Alterations in Genomic Organization and Gene Expression in Colorectal Carcinogenesis.

by

Sarah Elizabeth Anne Leigh

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ABSTRACT

This study has examined some of the features that characterize the changing pattern of genomic organization and gene expression in colorectal carcinogenesis. The transition from normal colonic mucosa, to adenoma, to carcinoma is accompanied by the progressive accumulation of genetic defects.

DNAs from a panel of premalignant adenomas, predominantly from familial adenomatous polyposis (FAP) patients, and carcinomas mostly from non-FAP patients, were screened for the presence of somatic mutations. RNA was extracted from a similar panel of samples for use in gene expression studies. Allele losses were detected in tumour DNA samples with polymorphic markers from chromosomes 1, 5q, 7 and 11p. Such loss of genetic material may indicate the presence of tumour suppressor genes at affected loci. As the adenomatous polyposis coli gene has been assigned to 5q21, allele loss in this region was expected in carcinomas, however loss of chromosome 5q markers had not previously been reported in premalignant adenomas. The absence of allele loss on chromosome 3p, suggests that the small cell lung cancer tumour suppressor gene, was not involved in colorectal carcinogenesis. Genetic instability was manifest in some carcinoma samples by the generation of novel alleles at various hypervariable loci. The retinoblastoma susceptibility gene was over-represented in two carcinomas and elevated levels of expression were detected in RNA from 90% of adenomas and 50% of carcinomas. Such uncharacteristic findings may indicate that unknown factors are interacting with this tumour suppressor gene in colorectal tumours. Expression of the p53 tumour suppressor gene declined from high levels in adenomas to low levels in carcinomas. Comparison of these findings with published data, suggests that an inverse relationship may exist between gene expression and mutation at this locus. The most striking and consistent change observed in this study, was the loss of carbonic anhydrase 1 gene expression associated with epithelial de-differentiation. Expression of the mucin genes
also declined with the progression of colorectal carcinogenesis. In mucinous tumours however, although mucin transcript levels were high in some cases, the pattern of mucin gene expression varied between individual samples.

Construction of a normal colonic mucosal cDNA library, allowed a cross hybridization strategy to be employed, in an attempt to isolate clones from human chromosome five that contained sequences expressed in the colon. Six clones were isolated and their preliminary characterization undertaken.
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ABBREVIATIONS.

APC: Adenomatous polyposis coli.
ATCC: American Type Culture Collection.
bp: base pair.
BSA: Bovine serum albumin.
cDNA: Complementary deoxyribonucleic acid.
cpm: counts per minute.
DNA: Deoxyribonucleic acid.
DMSO: Dimethylsulphoxide.
E.coli: Escherichia coli.
FAP: Familial adenomatous polyposis.
FPC: Familial polyposis coli.
GDP: Guanine diphosphate.
GTP: Guanine triphosphate.
HNPCC: Hereditary non-polyposis colorectal cancer.
Kb: kilobase pair.
Kd: kilodalton.
M: Mole.
Mb: megabase pair.
ml: millilitre.
μl: microlitre.
ng: nanogramme.
nm: nanometre.
p: short arm of a chromosome.
PCR: polymerase chain reaction.
q: long arm of a chromosome.
RFLP: restriction fragment length polymorphism.
RNA: ribonucleic acid.
SV40: simian virus 40.
VNTR: variable number tandem repeat.
YAC: yeast artificial chromosome.
CHAPTER 1

INTRODUCTION

1.1. Epidemiology of Colorectal Cancer.

The incidence of colorectal cancer varies throughout the world, between different countries, regions and ethnic groups. It is primarily a disease of the developed "Western world", whilst it is relatively uncommon in under-developed "third world" countries (Shottenfeld & Haas, 1978). In the United Kingdom 27,250 new cases were reported in 1990, representing 11% of all cancers. Nationally, therefore, colorectal cancer is the second most common malignancy. In 1984 the five year survival rate for colorectal cancer was approximately 37%, with mortalities accounting for 12% (19,460) of all cancer deaths in 1988 (CRC Fact Sheets 1988, 1989 & 1990).

It is apparent from the distribution of colorectal cancer throughout the world that environmental factors, especially diet, have a profound effect on the etiology of this disease (Muir & Staszewski, 1986). Studies on the incidence of colorectal cancer amongst immigrant populations, both to and from high risk regions, have shown that within one generation individuals take on the local colorectal cancer risk (Shottenfeld & Haas, 1978).

Despite the obviously strong influence of the environment on the etiology of colorectal cancer, ultimately, the cause of aberrant cell growth is genetic, resulting from mutations some of which may be inherited. Therefore, colorectal cancer develops as a result of a composite of environmental and genetic factors.

1.2. Etiology of Colorectal Carcinomas.

Colorectal carcinomas arise as a consequence of normal epithelial cell regeneration being disrupted. It is thought that all adenocarcinomas develop from previously benign outgrowths of the colorectal wall - adenomas (also known as polyps), in what is known as the adenoma - carcinoma sequence (Muto, et al, 1975, Hill, et al, 1978). Once a carcinoma has formed it has potential for growth, both into the lumen of
the gut and down into the intestinal wall. Given the appropriate conditions, metastatic disease, most commonly of the liver, may result from invasion of the cancer into the wall of the colorectum.


The colon and rectum represent the distal portion of the alimentary canal, from the ileocaecal valve to the anus. As shown in figure 1.1a the colorectum may be sub-divided into nine regions, although the composition of the colorectal wall remains uniform throughout. The colorectal wall is made up of essentially five layers, with the mucosa forming the luminal surface and the serosa the peritoneal surface (figure 1.1b). As indicated in the legend to figure 1.1b, the mucosa is further sub-divided into three distinct tissue types (epithelium, lamina propria mucosa & longitudinal muscularis mucosa). It is in the surface epithelium that 99% of colorectal carcinomas arise. The single layer of epithelial cells, comprising columnar, goblet and isolated enterochromaffin cells, is folded into crypts, known as the Crypts of Lieberkühn (figure 1.1c). Studies on the uptake of tritiated thymidine, together with microscopic observation, have illustrated that the base of the crypts represent the area of proliferation and regeneration (Deschner, 1980), with a single progenitor cell per crypt (Ponder, et al, 1985). Columnar cells migrate from the base of crypts up to the luminal surface, undergoing differentiation in the process. Mucin containing goblet cells are abundant in the upper two thirds of the crypts. Migration continues to the surface epithelium, where well differentiated columnar cells predominate. Such columnar cells are frequently polar, with a well balanced cytoplasmic architecture and a uniform coating of microvilli on the luminal surface. Regarded as the area of extrusion, cell death occurs in the surface epithelium and necrobiotic cells are normally observed (figure 1.1c) (Hermanek, et al, 1983).
Figure 1.1. Macro and Microscopic Anatomy of the Colorectum.

1.1.a. Subdivisions of the Colorectum.

1.1.b. Subdivisions of the Colorectal Wall.
M: mucosa consisting of epithelium, lamina propria mucosa & longitudinal muscularis mucosa, SM: submucosa consisting of connective tissue and adipose cells, MPC: muscularis propria consisting of a circular muscle layer, MPL: muscularis propria consisting of a longitudinal muscle layer, SS: subserosa consisting of loose connective tissue, S: serosa consisting of a mesothelial layer forming the visceral peritoneal cover of the intestinal wall.

1.1.c. Crypt of Lieberkühn.
SE, surface epithelium, CE, crypt epithelium, Ex, region of extrusion, D & M, region of division and migration, P & R, region of proliferation and regeneration, e, enterochromaffin cell, c, columnar cell, cd, columnar cell undergoing division, g, goblet cell, n, necrotic cell.
Figure 1.1.  
Macro & Microscopic Anatomy of the Human Colorectum.

a

HF SF TC AC DC SC C An

b

M SM MPC MPL SS

M

Ex g cd e

D & M

P & R

SE CE
1.22. Adenoma Formation.

The condition familial adenomatous polyposis (FAP) has provided a unique opportunity to study adenoma formation from the earliest stages. Characterized by the presence of hundreds to thousands of colorectal adenomas, histological samples from FAP patients reveal not only adenomas at all stages of development, but also areas of the epithelium where adenoma formation is likely to occur (FAP will be discussed in detail in section 1.32a). Adenomas in non-FAP patients are usually only detected when they become symptomatic, by which time they are well advanced (Bussey, 1975).

Adenomas develop from areas of epithelial hyperplasia, in which there is hyperchromatism, an increase in mitotic activity, stratification and reduced mucin production. It would appear from studies on the uptake of tritiated thymidine, that crypts with hyperplasia have a proliferative compartment which extends to the surface epithelium (Dreschner, 1980). Microscopic examination suggests that areas of hyperplasia spread from one crypt to adjoining crypts. As the adenomatous growth continues the surface of the epithelium protrudes from the surroundings and the adenoma becomes apparent.

Adenomas may be classified according to their macroscopic appearance, microscopic architecture, and degree of cellular atypia. Macroscopically, pedunculated or sessile adenomas occur, with either a smooth or papillary surface. The microscopic architecture may be: (a) tubular, consisting of branching tubules or glands, lined with epithelial cells, (b) villous, where the mucosa is in the form of finger like projections which reach down to the muscularis mucosa, or (c) tubulovillous, in which both morphological types are represented. Seventy five percent of adenomas are found to be tubular, villous and tubulovillous forms occur at 10% and 15% respectively (Muto, et al, 1975). The degree of cellular atypia and dysplasia may range from mild to severe. In mild dysplasia there is a slight increase in the number of mitotic figures, some cells have hyperchromatic nuclei and there is a general reduction in mucin secretion. However, there is minimal loss of cellular polarity, normal epithelial cells are still apparent and the regular architecture of the
tubules or villi is maintained. In contrast, severe dysplasia is typified by irregular tubules or villi, with budding and bridging between glands. Layers of epithelial cells, in which polarity has been lost, lie back to back, with the result that stromal tissue is reduced. The increase in mitotic figures, including abnormal forms, accompany the large polymorphic, hyperchromatic nuclei present in the cells, which now secrete little or no mucin (Bussey, 1975, Muto, et al, 1975, Hermanek, et al, 1983). The degree of dysplasia may not be uniform throughout an adenoma, with mild, moderate and severe forms being represented in a single polyp.

1.23. Adenoma - Carcinoma Sequence.

The only distinction between an adenoma with severe dysplasia and an adenocarcinoma, is invasion of the neoplastic growth through the muscularis mucosae. The malignant potential of an adenoma depends on the interaction of number of variables, including size, microscopic architecture, degree of cellular atypia and the macroscopic growth pattern (Hermanek, et al, 1983). Muto et al (1975) observed that villous adenomas are the most likely to progress to carcinomas, with an approximate malignancy rate of 40%. This was contrasted by the figures for tubular and tubulovillous, which were 5% and 22% respectively. Although a positive correlation exists between adenoma size and malignant potential, the morphology of an adenoma is of considerable significance, in that a small adenoma of the villous type has a greater malignant potential, than a larger adenoma of the tubular type. However, as severe atypia is uncommon in small adenomas (<5mm), large adenomas are more likely to progress to cancers (Muto, et al, 1975). Regions of mild dysplasia may persist in an adenocarcinoma, where the severely dysplastic cellular growth has yet to infiltrate. When large adenomas occur with severe atypia and dysplasia, but which have failed to breach the muscularis mucosae they are known as a carcinomas in situ or mucosal carcinomas.

Carcinomas may be of various morphological forms as shown in figure 1.2. Of these, the polypoid type, which represents 25% of cancers, is the least likely to result in metastases. However, the more frequently occurring ulcerated
carcinomas (55-60%) have a poorer prognosis, but it is the uncommon scirrhous cancers which have the greatest malignant potential (Hermanek, et al, 1983). Of the six histological types of colorectal cancer defined by the World Health Organization (Morson & Sobin, 1976), adenocarcinomas are the most common (representing approximately 85% of tumours). Mucinous adenocarcinomas, characterized by secretion of excessive quantities of mucin, account for a further 10%. The rarer signet-ring cell, squamous cell, adenosquamous and undifferentiated carcinomas represent the remaining tumour types.

Figure 1.2

Macroscopic Colorectal Cancer Types.

1.24. Dukes Staging of Colorectal Carcinomas.

In 1932 Cuthbert Dukes devised a scheme for the classification of colorectal cancers which is still in use to-day. A cancer which has extended to the muscularis propria is classified as Dukes stage A. Dukes stage B is a cancer which has breached the muscularis propria, but has not spread to the lymph nodes. Histological involvement of the lymph nodes determines Dukes stage C classification of the cancer (C1: local nodal involvement, C2: local and distant nodal involvement) (Dukes, 1932). As Dukes staging is based on the degree of tumour infiltration into the intestinal wall and local lymph node involvement, typing may only be made following surgical resection of the sample and adjoining
lymph nodes. A number of other regimes have been devised to classify colorectal tumours and provide some prognostic indication. Such a system was outlined by Payne (1989), in which depth of tumour infiltration, lymph node involvement, metastasis, and grade of tumour are all recorded.

1.25. Metastasis from Colorectal Carcinomas.

Malignant cells may be shed from the primary colorectal cancer to form local and distant metastasis. In general, the tissues in which secondary metastatic growth occurs appears to depend upon the site of the primary cancer. In the case of colorectal cancers, the most common sites are the local lymph nodes and the liver, and less commonly the lung. As long ago as 1889, Paget (Zetter, 1990) recorded the site specific nature of metastatic disease. His "seed and soil" hypothesis suggested that there were characteristics specific to both the "seed" (the malignant cell) and the "soil" (the tissue type in which the secondary tumour developed), which specifically favoured the growth of a metastasis. In 1928 Ewing (Zetter, 1990) attributed the specificity simply to the routes (vascular and lymphatic) which transported tumour cells away from the primary site (mechanical theory of metastasis). Thus, tumour cells would accumulate and grow in the first organ encountered. It seems probable that both theories have some foundation.

The mechanical theory of metastasis would appear to play a large part in colorectal metastatic disease, in that infiltration of the lymphatic system results in local lymph node tumours, and hematogenous spread results in hepatic tumours. However, findings that rat liver macrophages (Kupffer cells) will bind specifically to, and yet not kill rat colon carcinoma cells, suggests a validation of the seed and soil theory. In fact it would seem that rather than protecting the liver from invasion, Kupffer cells may actually be contributing to metastatic infiltration (Gjøen, et al, 1989). Cells are also shed from colorectal cancers into the lumen of the gut, as demonstrated by the detection of activated Kirsten-ras oncogene in faecal samples (Sidransky, et al, 1992). Metastases only form from this source if the integrity of the intestinal wall is
compromised, by for example a surgical wound (Hermanek, et al, 1983).

1.3. Causative Agents in Colorectal Carcinogenesis.

Environmental and genetic factors influence adenoma formation and the adenoma-carcinoma sequence in colorectal carcinogenesis. Hill et al (1978), put forward a hypothesis to suggest how such factors may interact in tumour formation. The hypothesis states that an environmental agent (A), causes adenoma development in susceptible individuals, another agent (B) causes the adenomas to grow and finally a carcinogen (C) causes a malignant change in a high proportion of large adenomas. From epidemiological evidence, it was likely that agents A & B were abundant in the Western world, where colorectal cancer is common. As small adenomas are evenly distributed along the length of the colorectum, it was possible that agent A was ingested in its active form. Agent B however, could be a digestion product, as large adenomas were more abundant in the proximal colorectum. The very high risk of malignant conversion associated with large adenomas, suggested that agent C was ubiquitous along the length of the gut and in all nations (Hill, et al, 1978).

A series of population based studies suggest that susceptibility to apparently sporadic or discrete adenomas, may be inherited as an autosomal dominant trait, present in up to 40% of the population. Variable penetrance of the gene, and the fact that many adenomas remain asymptomatic and therefore undetected, may explain why although familial aggregations of colorectal cancer have been recorded, the genetic process involved has remained unclear (Burt, et al, 1985, Cannon-Albright, et al, 1988). Recently however, Houlston et al (1992) have concluded from a complex segregation analysis, that the dominant genes responsible for colorectal cancer are not rare, and have a lifetime penetrance of 0.63. These findings are still consistent with the hypothesis put forward by Hill et al (1978), and reinforce the view that colorectal carcinogenesis is influenced by genetic and environmental factors.
1.31. Dietary and Other Environmental Risk Factors in Colorectal Carcinogenesis.

An extensive survey of cancer incidence in the Bantu tribe of the South African Transvaal, was the first to reveal low frequency of many cancers. Cancer of the large intestine was a tenth of that expected, by comparison with North American blacks and whites (Higginson & Ottlé, 1960). The one readily identifiable difference between the Bantu and the other groups studied, was that the diet of the African tribe was simple and contained large amounts of roughage. The low incidence of malignant and benign large bowel disease was observed in a number of rural African groups by Burkitt (1969, 1971). He correlated this finding with diet, noting that stool bulk, bacterial flora, transit time and intraluminal pressure could all be altered by the removal of dietary fibre. He hypothesized that the virtue of increasing stool bulk and reducing transit time was to dilute potential carcinogens and minimize their contact with the gut lumen (Burkitt, 1971). Subsequent epidemiological studies have served to reinforce and expand upon this hypothesis, in particular identifying potential risk and protective factors present in the varied human diet. In addition it has been possible to give some indication of the exposure time required for such agents to exert their effect.

Complex interactions occur between the various dietary components with each other and with the bacterial flora, and metabolic products present in the gut. It is therefore difficult to attribute colorectal cancer protection or risk to any factor in isolation. However, a number of clearly influential factors have been identified in addition to fibre, these are chiefly fat and meat, resulting in increased risk and vegetables, calcium and selenium resulting in reduced risk.

The positive correlation between colorectal cancer and fat in the diet, especially animal fat is now well established (Willet, 1989). High levels of dietary fat result in increased hepatic synthesis and excretion of cholesterol and bile acids into the gut (Reddy, 1981a). These are hydrolyzed to secondary bile acids by the action of Clostridium paraputrificum. Fatty acids and free bile acids
are irritating to the colonic epithelium, causing increased cellular loss and consequent increased proliferation to replace the lost cells (Reddy, 1981b, Newmark, et al, 1984). The role of the intestinal flora in this process was exemplified by the reduction of fat tumourigenicity in germ free animals (Reddy, et al, 1975). Furthermore, bile salts induce ornithine decarboxylase activity, this enzyme has been linked with tumour promotion (Tempero, 1986, Sun & Li, 1988).

It is thought that the protective effects of both fibre and calcium stem at least in part from their interaction with faecal bile acids. High levels of fibre and starch in the diet alter the composition and fermentation of the intestinal flora, in such a way as to affect bile acid metabolism and hence carcinogenicity (Reddy, 1981b, Cummings & Bingham, 1987). It has been proposed that the reduced pH which results from increased bacterial fermentation of fibre directly inhibits bile acid degradation (Thornton, 1981). In addition some forms of fibre partake in physiochemical binding with bile acids, resulting in their expulsion in the faeces and thus disruption of the enterohepatic circulation of these metabolites (Reddy, 1981a). Calcium also binds strongly with free bile acids and fatty acids, in this case to form insoluble calcium soaps, which are found in the faeces of individuals with sufficient calcium in their diet (Newmark, et al, 1984, Slattery, et al, 1988a). Phosphates, which are present in high levels in the Western diet, compete with free bile acids and fatty acids for calcium, to form calcium phosphate. Therefore, calcium is required in the diet to interact with both phosphates and fat metabolites in order to give effective protection.

The protective properties of in particular cruciferous vegetables (Brussel sprouts, cauliflower, cabbage, etc) result from the naturally occurring indoles in these vegetables (Graham, et al, 1978). Indoles induce microsomal mixed function oxidases (Wattenberg, 1975), which in turn inactivate polycyclic aromatic carcinogens that may be present in the gut (Wattenberg & Loub, 1978). A number of other dietary components have been implicated in colorectal carcinogenesis, selenium has been associated with a reduced risk, particularly of male rectal cancer (Stampfer, et al,
1989, Nomura, et al, 1987). Vitamin A and β-carotene seem also to have a negative association, although it is difficult to study their effects in isolation, for both substances are present in fruit and vegetables, which are already considered to be protective for other reasons (Modan, et al, 1981, Wald, et al, 1987). Although it is known that carcinogens are formed when food is broiled and fried at high temperatures, no positive correlation with this type of cooking and colorectal cancer has been observed (Lyon & Mahoney, 1988). The link between colorectal cancer and refined carbohydrates (Bristol, et al, 1985), may reside in the high energy/fibre ratio for foods such as refined flour and sugar, as at least two studies have revealed total calorific intake to be a definite risk factor (Graham et al, 1988, Lyon & Mahoney, 1988).

Non-dietary considerations associated with colorectal carcinogenesis include overall physical activity, occupation, sex and parity. Increased physical activity may reduce the cancer risk by stimulating peristalsis and hence reducing faecal transit time (Slattery, et al, 1988b). Occupational risks are associated with physical activity and exposure to hazardous chemicals. The correlation that exists between high socio-economic grouping and increased risk, may be due to a sedentary life style, coupled with a refined, high fat, calorific diet (Brownson, et al, 1989). Women run a greater risk of developing colorectal cancer than do men, being particularly susceptible to cancer of the colon (Wynder & Shigematsu, 1967). This sex dependent risk is reversed in post-menopausal women and reduced in women with one or more children, suggesting a definite hormonal effect (McMicheal & Potter, 1983, West, et al, 1989). However, in addition to hormonal considerations it has been shown that women have a longer transit time and a reduced stool bulk (irrespective of dietary intake) than men. The reduced colorectal cancer risk attributable to parenthood in both men and women, could possibly be brought about by the increased physical activity and reduced socio-economic grouping that is often associated with the arrival of children (Kune, et al, 1989).

Although the carcinogenic properties of a number of environmental factors has now been documented, much
information is still required, to establish exactly how such factors influence the molecular genetic changes associated with colorectal neoplasia. However, it is apparent that environmental considerations have a rapid effect on colorectal carcinogenesis, as migrants from low to high risk countries acquire the increased risk (if they integrate into the host community) within the first generation (McMicheal & Giles, 1988, Walker & Segal, 1989).

1.32. Inherited Conditions Associated with a Predisposition to Colorectal Neoplasia.

A number of inherited conditions exist which predispose affected individuals to develop colorectal cancer. Although such diseases are uncommon, they provide an interesting insight into the events which occur during colorectal carcinogenesis. Various diseases which result in intestinal polyposis fall into this category, first recognized as long ago as the eighteenth century, work continues into assessing the neoplastic potential and phenotypic classification of such conditions (Bussey, 1975).

1.32a Familial Adenomatous Polyposis.

Familial adenomatous polyposis (FAP), also known as familial polyposis coli (FPC) and adenomatous polyposis coli (APC), is an autosomal dominantly inherited condition, with an approximately 90% degree of penetrance (Bussey, 1975, Utsunomiya, 1990). FAP occurs at an average frequency of 1 in every 16,000 live births in Great Britain, Japan, and the United States of America, representing about 0.006% of the population (Utsunomiya, 1990).

Patients with FAP develop hundreds to thousands of colorectal adenomatous polyps, usually during their teens and early twenties. The adenomas are distributed throughout the length of the colorectum, although there is a tendency for the numbers to increase from the proximal to distal portions of the gut (figure 1.3). In some patients the adenomas are so densely packed that normal mucosa is no longer evident, even by microscopic examination (Bussey, 1975). Numerous adenomas in the colorectum presents a high risk of colorectal cancer, in that at least one will inevitably undergo malignant change and develop into an adenocarcinoma. The
adenocarcinomas which would normally arise during an FAP patients' late twenties to early thirties, would probably prove fatal by the fifth decade of life (Bussey, 1975, Bülow, 1987). It is therefore desirable to screen members of FAP families for the earliest manifestations of the disease, as prophylactic measures may be taken, which can greatly improve their life expectancy.

The establishment of polyposis registers has facilitated identification of "at risk" family members. The St.Marks Hospital Polyposis Registry was the first, set up by Lockhart-Mummery in 1925 and by 1975 it contained some 294 families (Bussey, 1975). A number of other polyposis registries now exist, both regionally and nationally (Burn, et al., 1991, Bülow, 1987, Utsunomiya, 1990).

Presymptomatic screening commonly takes the form of annual sigmoidoscopy or colonoscopy to detect colorectal adenomas. If adenomas are present, then treatment is usually complete removal of the colon, followed by joining of the ileum to the rectum (ileorectal anastomosis). This procedure considerably improves the survival rate of FAP patients, whilst maintaining a reasonable quality of life (Bülow, 1987). There are however, several disadvantages associated with this method of screening. These include patient resistance to annual endoscopic examinations, and the need to continue screening from puberty until 50 years of age, for although adenomas have usually developed by the third decade of life, there are reports of individuals in which they do not develop until middle age. Taking these limitations into account efforts have been made to base screening on one of the other manifestations of FAP.

Over the years the extracolonic features of FAP have been increasingly well characterized. In 1951 Gardner reported his observations of a large kindred in which there was a high prevalence of colorectal cancer. In addition to colorectal adenomas, affected members of the family had benign osteomas of the skull and jaw, and soft epidermoid cysts. Together these dominantly inherited features became known as Gardner's syndrome. However, it is increasingly thought that Gardner's syndrome represents a phenotypic variant of the FAP genetic defect. Other extracolonic
Figure 1.3. Ressected Colon of an FAP Patient.

This figure shows photographs taken of the formalin fixed colon removed from patient FAP139. AC, ascending colon, TC, transverse colon, DC, descending colon. Arrows point to a number of adenomas, it is apparent that there is a greater density of adenomas in the terminal regions of the descending colon than elsewhere in the colon.
Figure 1.3.

Resected Colon of an FAP Patient.

Colon

Adenomas

AC  TC  DC

Initially it was thought that occult osteomas of the jaw offered the potential as a screening tool, because they may be detected prior to the presentation of colorectal symptoms (Utsunomiya & Nakamura, 1975). This was however discounted by Woods et al, (1989), who in a survey only detected occult osteomas in 14% of patients, in whom other extracolonic manifestations of FAP were already apparent.

In 1935 a report was made in a Gardner's syndrome patient of several punctate pigmented areas of the fundus (McKittrick, et al, 1935). It was not until the 1980s that the potential of congenital hypertrophy of the retinal pigment epithelium (CHRPE), was examined as a screening tool (Blair & Trempe, 1980). Although one study only detected CHRPE in Gardner's syndrome patients and not in patients classified as having FAP (Traboulsi, et al, 1988), subsequent reports did find CHRPE in FAP patients (Chapman, et al, 1989). CHRPE do occur in the general population, however, in FAP patients the expression of CHRPE is usually multiple (>4), bilateral lesions which develop at an early age (Romania, et al, 1989). The value of the relevant ophthalmological examination in presymptomatic FAP screening has been assessed by a number of groups. The findings were that the procedure causes minimal trauma to the patient, may be performed at an early age, is independent of the other extracolonic features of FAP and is an accurate indicator of gene carrier status (Romania, et al, 1989, Baba, et al, 1990, Burn, et al, 1991). However, in a minority of FAP families, the number of CHRPEs is not significantly greater than that detected in the general population. In such instances CHRPEs may not be used for presymptomatic screening (Romania, et al, 1989).

Desmoid tumours would appear to be a complication which arises in FAP patients following abdominal surgery, although
in some cases they develop prior to colorectal symptoms. Characterized by uncapsulated fibrous growth, they usually develop in the abdominal cavity, where they may become very large. Although desmoids grow slowly and do not metastasize, they are associated with a poor prognosis. This is because their sheer size may result in damage to local organs (eg: pressure on urethras resulting in renal damage), furthermore, their infiltration into surrounding tissues, makes surgical removal impossible (MacAdam & Goligher, 1970).


1.32b. Peutz-Jeghers Polyposis.

Peutz-Jeghers syndrome is an autosomal dominantly inherited condition, characterized by polyposis of the small intestine and melanotic macules of the lips and buccal mucosa (Bussey, 1975, Correa & Haenszel, 1978). The hamartomatous Peutz-Jeghers polyps differ from adenomatous polyps, by having a branched architecture of muscularis mucosa, surrounded by a layer of well differentiated epithelial cells (Hermanek, et al, 1983). In Peutz-Jeghers syndrome the polyps may become very large, and can cause intestinal obstruction and intussusception (Erbe, 1976). Giardiello, et al (1987) reported that Peutz-Jeghers patients were eighteen
times more likely to develop cancer, than members of the general population. However, only 27% of the neoplasias were gastrointestinal, it is therefore unclear whether such cancers develop from the hamartomatous polyps, or from one of the isolated adenomatous polyps, which have been observed in some Peutz-Jeghers patients (Giardiello, et al, 1987).

1.32c Juvenile Polyposis.

Juvenile polyposis is an autosomal dominantly inherited condition with complete penetrance, which is not genetically linked to FAP (Erbe, 1976, Petersen, et al, 1990). Patients with this disease develop polyps throughout the length of the gastrointestinal tract, during the first decade of life. Although the polyps are hamartomatous, they have a polypoid appearance and differ from Peutz-Jeghers polyps, by having large amounts of lamina propria, in which mucin filled cysts may develop (Hermanek, et al, 1983). It is thought that together with other congenital abnormalities, juvenile polyposis is associated with an increased risk of colorectal cancer. However, it remains unclear what role the polyps may play in this predisposition (Bussey, 1975, Erbe, 1976).

1.32d. Turcot's Syndrome.

Turcot's syndrome is characterized by colorectal polyposis and malignant tumours of the central nervous system (CNS) (Correa & Haenszel, 1978). In this syndrome, up to one hundred often large adenomas may develop, resulting in a strong predisposition to colorectal cancer. The CNS tumours which arise, are most commonly medulloblastomas, these usually prove fatal in the patients' teens or early twenties (Bussey, 1975, Itoh, et al, 1979). Controversy exists as to whether or not Turcot's syndrome is yet another manifestation of FAP, as FAP patients have been reported with tumours of the CNS (Bussey, 1975, Kropilak, et al, 1989). It would appear however, that unlike FAP, Turcot's syndrome is an autosomal recessive disorder. This conclusion was reached both from the presence of two affected sibs in the progeny of a consanguineous marriage of unaffected parents, and from the observation that affected individuals usually die before they become reproductively active (Itoh, et al, 1979). Furthermore, a recent report has excluded genetic linkage
between Turcot's syndrome and FAP-linked markers, thus supporting the view that Turcot's syndrome and FAP are two genetically distinct diseases (Tops, et al, 1992).

1.32e. Hereditary Non-Polyposis Colorectal Cancer.

Hereditary non-polyposis colorectal cancer (HNPCC) was first reported by Lynch et al, in 1966. Subsequently termed Lynch syndrome, further work on HNPCC led to the classification of two related disorders, Lynch syndromes I & II, which probably account for at least 5% of all colorectal cancers (Mecklin, 1987). Lynch syndrome I, is an autosomal dominantly inherited condition, in which patients have a predisposition to develop, at an early age, multiple primary adenocarcinomas of the proximal colon (Lynch, et al, 1990, Ushio, 1990). Lynch syndrome II, which is also known as the Cancer Family Syndrome, has all the features of Lynch syndrome I, but in addition, patients develop extracolonic adenocarcinomas. Women with Lynch syndrome II frequently develop tumours of the endometrium or ovary. Other tumours reported in this condition include gastric, small intestinal, pancreatic, hepatobiliary, renal and laryngeal (Lynch, et al, 1990). Due to the heterogeneity of the Lynch syndromes a set of criteria were drawn up to assist with diagnosis. To be classified as Lynch syndrome a family must have: (i) at least three family members affected by colorectal cancer, with one individual a first degree relative to the other two, (ii) at least two generations affected, (iii) early onset of colorectal cancer (<50 years of age) in at least one family member and (iv) FAP must have been excluded from the diagnosis (Lynch, et al, 1990).

1.32f. Other Conditions Associated a Predisposition to Colorectal Cancer.

The inflammatory condition ulcerative colitis (UC) is associated with an increased susceptibility to colorectal cancer. However, in UC, carcinomas develop directly from flat areas of dysplastic mucosa and not from precancerous adenomas (Hermanek, et al, 1983). The etiological differences are reflected in the observation that K-ras is activated less frequently in UC carcinomas (9%) than in
sporadic tumours (72%), thus suggesting that different genetic processes are involved (Bell, et al, 1991).


1.4. Molecular Genetics of Carcinogenesis.

Theories were formulated in the 1950s to explain why the incidence of cancer increased with age. Both Nordling (1953) and Armitage & Doll (1954) proposed that this was due to the accumulation of a succession of genetic mutations. Nordling recognized that such mutations were most likely to occur in naturally proliferating cells. Whilst Armitage & Doll suggested that the effect of a carcinogen, was dependent not only on the cell type exposed, but also upon the time of life at which the carcinogen was encountered. Ashley (1969) attempted to refine these multi-stage theories of carcinogenesis, and concluded that two major steps were involved: initiation and promotion. It seemed likely that a number of discrete structural and functional changes were involved in both initiation and promotion of tumour growth.

In 1971 Knudson put forward his two hit hypothesis for the development of inherited malignancies. An epidemiological study of the rare childhood malignancy retinoblastoma, revealed that tumours in both eyes were more frequent in patients with the dominantly inherited form of the disease. Knudson concluded that such individuals had inherited a germline mutation, the "first hit", which predisposed them to develop retinoblastoma. The "second hit", required for tumour development, was acquired somatically, in cells of the developing retina. In patients with no family history of the disease, both the first and second hits would occur in the retina. Statistically this hypothesis was in agreement with the observed frequency of bilateral tumours and early age of onset, in familial retinoblastoma.

Conclusive evidence that cancer has a genetic basis was therefore provided by the occurrence of inherited cancers, the observation of chromosomal damage in the cells of certain cancers and the association between mutagens and
carcinogenesis. As the precise nature of the genetic mechanisms responsible for the development of cancers were studied, it became apparent that two distinct classes of genes were involved. There were oncogenes, whose activation resulted in the promotion of carcinogenesis, and tumour suppressors or anti-oncogenes, whose inactivation allowed tumourigenesis to proceed (Land, et al, 1983a, Nowell, 1988). What also became apparent from experimental evidence, was that more than one genetic change was required for expression of the cancer phenotype, this was in agreement with the early theories put forward.

1.41. Oncogenes.

The existence of genes with the ability to promote tumourigenesis was alluded to from the study of tumourigenic retroviruses, and DNA transfection experiments. The first oncogene to be identified was v-src, isolated from the Rous sarcoma virus (Martin, 1970). This retrovirus, which can induce sarcomas in vivo and transform fibroblasts in vitro, was found to contain a gene (v-src) encoding a tyrosine kinase (pp60src)(Brugge & Erikson, 1977, Hunter & Sefton, 1980). Closer examination of the v-src gene, revealed that it was derived from a normal gene of the chicken, which is the host of the Rous sarcoma virus (Stehelin, et al, 1976). This therefore led to the conclusion that a normal cellular gene (proto-oncogene), had the potential to exhibit strong transforming properties if activated, in this case by transduction into a retrovirus. A number of oncogenes, and their cellular homologues have been identified from the study of retroviruses, including Harvey-ras (H-ras), Kirsten-ras (K-ras) and c-myc (Land, et al, 1983a).

Cellular oncogenes were also identified from transfection studies, in which DNA from cancer cells was used to transfect normal cells in culture. Foci of transformed cells were then examined, and dominantly acting oncogenes were detected (Shih, et al, 1979a). As with the retroviral studies, the genes identified also had cellular homologues. Although these assays were initially performed using DNA from chemically induced cancer cell lines, it soon became apparent that similar results were obtainable from tumours of various cell types and from different species (Shih, et al, 1981).
It has become increasingly evident that proto-oncogenes and their products constitute a heterogeneous group of genes and peptides. Products of different proto-oncogenes have been reported in the cytoplasm, associated with the nuclear and plasma membranes and in the nucleus. The biological functions of these gene products are frequently associated with cell cycle regulation, and include protein kinases, GTPases and transcription factors (Land, et al, 1983a, Bishop, 1985). However, it would appear that there is only a limited group of cellular genes with transforming potential, as the same oncogenes have been isolated both from different viruses, and from transfection studies (Eva, et al, 1982, Der, et al, 1982).

The transition from proto-oncogene to oncogene may occur either as a result of aberrant expression of the gene or by expression of a mutated product (Land, et al, 1983a, Bishop, 1985). Although a large number of genes with an oncogenic potential have now been identified, it would still appear to be the case that activation of a single proto-oncogene is insufficient for tumourigenesis (Land, et al, 1983b, Weinberg, 1989). Furthermore, it has been revealed that c-myc and K-ras, amongst others, can co-operate with each other to produce a transformed phenotype (Weinberg, 1985, Land, et al, 1986). Thus, demonstrating that different oncogenes may have distinct and complementary effects on the normal cellular phenotype.

1.42. Tumour Suppressor Genes (Anti-oncogenes).

In contrast to the tumour promoting properties of oncogenes, tumour suppressor genes appear to be involved in restraining tumourigenesis. The existence of such genes was suggested by cell hybrid experiments, cytogenetic observations and the occurrence of familial cancers (Marshall, 1991).

In the cell hybrid experiments, it was observed that fusions between normal cells and certain tumour cell lines, resulted in non-tumourigenic hybrids. Thus, the normal cells were able to suppress the tumour phenotype (Stanbridge, 1976). These experiments were extended to fusions between different tumour cell lines. It was found that whilst hybrids between carcinoma cell lines of epithelial origin
remained tumourigenic, complementation occurred in those between lines of differing cellular origin, resulting in a non-tumourigenic hybrid (Weissman & Stanbridge, 1983). Therefore, it was concluded that tumourigenicity was essentially recessive, or dependent on gene dosage, and that because complementation only occurred between lines of different cell type, a variety of genes existed with the ability to suppress neoplastic expression (Weissman & Stanbridge, 1983).

Chromosomal instability and non-random loss of specific regions was observed by karyotypic analysis in numerous tumours (Mitelman, 1985). The loss of chromosomal material was in direct agreement with the concept of genes acting as tumour suppressors, in that loss or inactivation of at least one copy of the gene allowed tumourigenesis to proceed. Furthermore, the occurrence of consistent chromosomal alterations in certain cancers, ultimately led to the identification and isolation of a number of tumour suppressor genes by positional cloning. The first tumour suppressor gene to be cloned was that associated with retinoblastoma. Following reports of cytogenetic deletions of chromosome 13q14 in retinoblastomas (Balaban, et al, 1982), Cavenee, et al (1983) pioneered the use of polymorphic DNA markers in the search for chromosomal deletions in tumour DNA. Using markers known to map into the correct region, allele losses were observed in the DNA of tumour samples, one of the markers used was esterase-D. A chromosomal walk from the esterase-D region ultimately led to isolation of the retinoblastoma susceptibility gene (Rb) (Lee, et al, 1987a). The tumour suppressor genes involved in the familial cancer syndromes Wilms' tumour (WT), neurofibromatosis type 1 (NF1) and familial adenomatous polyposis (FAP) have now all been cloned (Rose, et al, 1990, Wallace, et al, 1990, Viskochill, et al, 1990, Joslyn, et al, 1991, Kinzler, et al, 1991b). In addition, the genes deleted in colorectal cancer (DCC) and mutated in colorectal cancer (MCC), have also been cloned (Fearon, et al, 1990, Kinzler, et al, 1991a). Whilst the chromosomal regions associated with Wilms' tumour, FAP, MCC and DCC were identified by cytogenetics (Francke, et al, 1979, Herrera, et al, 1986, Reichmann, et al, 1981), NF1 was
localized to 17q11.2 by linkage analysis in affected families (Goldgar, et al., 1989). However, reports of chromosomal translocations in two NF1 patients hastened identification of the NF1 gene. The specific chromosomal regions associated with a number of other malignancies have now been identified (Green, 1988), including 3p21 for small cell carcinoma of the lung (SCLC) (Wang-Peng, et al., 1982) and 11q13 for multiple endocrine neoplasia type 1 (MEN1) (Larson, et al., 1988, Thakker, et al., 1989). But the genes responsible have yet to be cloned.

The tumour suppressor gene whose involvement has been reported in the largest number of different human malignancies is p53 (Levine, et al., 1991). Although p53 was first identified through its ability to form an oligometric complex with the large T-antigen of simian virus 40 (SV40) (Lane & Crawford, 1979). And despite reports of cooperation between the ras oncogene and p53 in neoplastic transformation (Parada, et al. 1984, Eliyahu, et al., 1984), subsequent studies have shown p53 to be a genuine tumour suppressor gene (Finlay, et al., 1988). The p53 gene encodes a 393 amino acid nuclear phosphoprotein (Lane & Crawford, 1979, Lamb & Crawford, 1986), which appears to bind to specific DNA sequences and act as a transcriptional activator, presumably on genes involved in negative regulation of cell growth (Raycroft, et al., 1990, Kern, et al., 1992). This normal function is disrupted in many human malignancies, both by mutations within conserved regions of the p53 gene, and by deletions encompassing the gene (Nigro, et al., 1989, Hollstein, et al., 1991). In common with the tumour suppressor genes identified through their association with particular inherited malignancies, p53 germline mutations have been detected in the cancer-prone condition, Li-Fraumeni syndrome, and in other non-Li-Fraumeni cancer families (Malkin, et al., 1990, Srivastava, et al., 1990, Prosser, et al., 1992).

Although the biological properties of the tumour suppressor genes so far identified are generally diverse, the product of the retinoblastoma susceptibility gene (pRB) shares several features with p53. The pRB peptide is also a nuclear phosphoprotein, with DNA binding properties, and it
forms complexes with oncoproteins of DNA tumour viruses (Lee, et al, 1987b, Whyte, et al, 1988, Dyson, et al, 1989). In addition, deletions and mutations of the Rb gene have been reported in many cancers, other than those clinically associated with retinoblastoma, notably breast cancer (T'Ang, et al, 1988, Lee, et al, 1988) and small cell carcinoma of the lung (Harbour, et al, 1988). It seems likely that the Wilms' tumour gene is also involved in transcriptional regulation, as it encodes a zinc finger protein (Call, et al, 1990). The products of the NF1, MCC and APC genes are all cytoplasmic. The NF1 protein probably acts as a GTPase activating protein, in signal transduction (Martin, et al, 1990), whilst MCC and APC may well be involved in maintaining the normal architecture of the cell, as they both include domains capable of forming coiled coil proteins (Bourne, 1991, Groden, et al, 1991, Kinzler, et al, 1991b). Finally, it would appear that the product of the DCC gene may be involved in cell-cell interaction, as it shares homology with various cell surface glycoproteins (Fearon, et al, 1990).

Confirmation that candidate genes are truly tumour suppressors, may be provided by transfection assays or from the creation of transgenic animals. The normal Rb and p53 genes have been used in both types of assay. Soon after the Rb gene was cloned, constructs of the normal Rb gene were made and transfected into retinoblastoma and osteosarcoma cell lines. The neoplastic phenotype of the cells was suppressed in both cases (Huang, et al, 1988). Similar results were obtained when a normal p53 gene was introduced into a colorectal cancer cell line (SW837), from which one normal p53 gene had been deleted and the remaining allele was mutated (Baker, et al, 1990b). Transgenic mice homozygous for a null p53 gene, were found to be developmentally normal, but prone to a variety of spontaneous neoplasms (Donehower, et al, 1992). Homozygosity for null Rb mutations, resulted in non-viable embryos, apparently due to the failure of certain neuronal and haematopoietic cells to undergo terminal differentiation (Lee, et al, 1992, Jacks, et al, 1992). Interestingly, although some heterozygotes for the mutated Rb gene, were susceptible to pituitary tumours, retinoblastomas
were not detected in any of the animals examined (Jacks, et al, 1992, Lee, et al, 1992).

1.5. Molecular Genetics of Colorectal Carcinogenesis.

The molecular genetic events involved in the development of colorectal cancer have been studied extensively. In accordance with the etiology of the condition, a series of molecular changes appear to be responsible for progression from normal mucosa, to adenoma, to carcinoma (Vogelstein, et al, 1988). As with many other malignancies, the first clue to the location of colorectal specific genetic changes were given by cytogenetic observations of tumour specimens.

1.5.1. Cytogenetics of Colorectal Carcinogenesis.

Numerical gains and losses of every human chromosome have been reported in colorectal carcinomas (Reichmann, et al, 1981). However, surveys of large numbers of samples have revealed non-random chromosomal alterations. The chromosomes most frequently lost from colorectal tumours are 17p and 18 (Muleris, et al, 1987, 1988, 1990). Muleris, et al (1988) observed that cancers which had lost both 17p and 18, tended, in decreasing order to also have lost, 1p, 4, 14 and 5q, and gained 20, 8q and 13. Whilst in cancers that had lost either 17p or 18, the most frequent additional changes were trisomy 7, 8q and 13. Overall, trisomy 7 was the most commonly reported chromosomal gain, followed by trisomy 8q and 13 (Reichmann, et al, 1981, Ochi, et al, 1983, Ferti-Passantonopoulou, et al, 1986, Muleris, et al, 1990). Structural rearrangements were also reported, these occurred most frequently in chromosomes 1 and 5 (Kovacs, 1978, Reichmann, et al, 1981, 1984, Ferti-Passantonopoulou, et al, 1986, Griffin, et al, 1990). Although the general cytogenetic trends associated with colorectal tumours have been reported here, it is important to remember that individual karyotypes may be very different (Kovacs, 1978, Lothe, et al, 1987).

most recently reported case seems to have resulted from a complex rearrangement, involving a direct insertion, followed by a microdeletion (Cross, et al, 1992). It is interesting to note that an interstitial deletion in the same region (5q12-22), was reported in a sporadic adenocarcinoma (Ferti-Passantonopoulou, et al, 1986). Thus, suggesting a link between the genetic events involved in the etiology of FAP and sporadic colorectal tumours.

1.52 Allelic Loss in Colorectal Carcinogenesis.

Polymorphic DNA markers have been used in linkage analysis of genetic diseases, and in the identification of chromosomal deletions associated with inactivation of tumour suppressor genes. Based on the reports of interstitial deletions in the long arm of chromosome 5 (Herrera, et al, 1986, Ferti-Passantonopoulou, et al, 1986), Bodmer, et al, (1987) performed linkage analysis on thirteen FAP families, with polymorphic DNA markers from chromosome 5. A maximum LOD score of 3.26 at zero recombination, was obtained with the marker C11P11, which maps to 5q21 (Bodmer, et al, 1987). Although this result was confirmed in a further five pedigrees (Leppert, et al, 1987), recombinants were soon reported between FAP and C11P11 (Aldred, et al, 1988). Comparison of the genotype of paired tumour and normal colorectal samples, revealed allele loss of highly polymorphic markers on chromosome 5, in 20% of cases (Solomon, et al, 1987). Many similar studies followed, one of the most extensive was that performed by Vogelstein et al (1989), in which 56 tumour/normal DNA pairs were examined with a total of 54 markers representing all the autosomes. Loss of heterozygosity appeared to be a common event, in that allele loss was detected with every probe used. However, the most significant finding was that markers from 17p and 18q were lost in more than 75% of tumours. This appeared to be a consistent observation, and was reported in both sporadic and FAP carcinomas (Vogelstein, et al, 1988, Law, et al, 1988, Fey, et al, 1989, Sasaki, et al, 1989, Miyaki, et al, 1990). Although loss of 22q markers were the most frequently detected change in one series of carcinomas, the results were slightly biased by the use of five markers from 22q, compared with only one relatively uninformative marker from 17p.
Closer examination of events in the long arm of chromosome 5, suggested that interstitial deletions surrounding the FAP locus occurred more frequently than mitotic recombination or partial arm loss (Ashton-Richardt, et al, 1989). Hence, with a higher density of markers in the FAP region, loss of heterozygosity was reported in up to 53% of cases (Ashton-Richardt, et al, 1989, Okamoto, et al, 1990).


It was implicit from the agreement between the cytogenetic and the molecular genetic evidence, that the tumour suppressor genes involved in colorectal carcinogenesis were located on 5q, 17p, 18q, and 22q. Although the candidate genes on 5q, 17p, and 18q have been identified, the gene or genes on 22q have yet to be isolated.

1.53. Tumour Suppressor Genes in Colorectal Carcinogenesis.
1.53a. Adenomatous Polyposis Coli Gene (APC) and Mutated in Colorectal Cancer Gene (MCC), 5q21.

Once the gene responsible for FAP had been localized to 5q, considerable effort was focused on identifying more markers from the region, with the ultimate goal of cloning the gene. Nakamura et al (1988) isolated numerous clones from a 5q specific cosmid library. The location of the new clones with respect to FAP and existing markers was assessed by linkage analysis. In addition to demonstrating that there was no genetic heterogeneity between FAP and Gardners' syndrome, this study identified the marker YN5.48 (D5S81), which was tightly linked to FAP (LOD score of 8.25 at zero

The deletions in sporadic colorectal tumours which led to isolation of the MCC gene, were centred around the cosmid 5.71. Using an exon-connection strategy (Fearon, et al, 1990), exons of the MCC gene were identified, and a brain cDNA library was screened (Kinzler, et al, 1991a). The resultant 2511bp open reading frame of MCC had not previously been reported. However, there was a 19 amino acid region of similarity shared between MCC and the human and porcine G protein-coupled m3 muscarinic acetylcholine receptor (mAChR) (Kinzler, et al, 1991a). MCC was identified as a candidate gene responsible for FAP, however, the observation that it did not map within FAP specific deletions and the absence of germline mutations in more than 90 FAP patients excluded this possibility (Nishisho, et al, 1991). The role of the MCC gene in tumour suppression is still under review. So far mutations and deletions have been detected in a number of colorectal and other tumours (Kinzler, et al, 1991a, Nishisho, et al, 1991, Boynton, et al, 1992, D'Amico, et al, 1992).

Isolation of the APC gene was reported simultaneously by two groups (Joslyn, et al, 1991, Kinzler, et al, 1991b). Joslyn, et al, (1991) identified germline deletions in two FAP patients, with the cosmid L5.71 using pulsed field gel electrophoresis (PFGE). Subclones from the yeast artificial chromosome (YAC) contig spanning the deletions, were then used to screen cDNA libraries. Three transcripts were identified, one of which was considered to be the APC gene. Kinzler, et al, (1991b) screened for coding sequences in each of three YAC contigs from the region, spanning a total of 5.5Mb of DNA. Of the six genes identified, including MCC,
only one was implicated in FAP. As with MCC, the APC gene sequence was novel, encoding a predicted 2844 amino acid peptide (Joslyn, et al, 1991, Kinzler, et al, 1991b).

The authenticity of the APC gene was initially suggested by identification of nine non-conservative germline mutations in APC patients (Groden, et al, 1991, Nishisho, et al, 1991). Analysis of PCR products of the APC gene by denaturing gradient gel electrophoresis (DGGE), ribonuclease (RNase) protection and single strand conformational polymorphism (SSCP), has revealed germline mutations in a large number of unrelated FAP patients (Fodde, et al, 1992, Miyoshi, et al, 1992a, Cottrell, et al, 1992). The majority of APC germline mutations occur in exons 7-11 and 15 of the gene (Fodde, et al, 1992), and result in truncation of the APC transcript. The somatically acquired mutations in sporadic colorectal tumours, tend to be clustered between codons 1286 and 1513 within exon 15, but also predominantly result in transcriptional termination (Miyoshi, et al, 1992b). As suggested by earlier observations (Rees, et al, 1989), mutation or loss of genes on 5q represents an early event in colorectal carcinogenesis. A study on a series of adenomas from one FAP patient, has shown the normal APC allele to be deleted in ten of fifteen adenomas, including five of a small size (≤3mm)(Ichii, et al, 1992). Similar findings have also been reported in sporadic adenomas, with somatic mutations detectable in 63% of samples (Powell, et al, 1992). These results, whilst highlighting the significance of APC gene inactivation in carcinogenesis, demonstrate that loss of both APC alleles was not necessarily required for adenoma formation.

The biological functions of both the MCC and APC gene products remain unclear. From the predicted peptide sequences it would appear that both the MCC and APC gene products are cytosolic, and share the ability to form coiled coil complexes (Groden, et al, 1991, Kinzler, et al, 1991a). It has therefore been suggested, that both peptides could participate in the formation of either homo- or hetero-peptidê oligomers (Groden, et al, 1991). No significant homology was shared between the MCC and APC genes, however, the region of local similarity between MCC and G protein-
coupled m3 muscarinic acetylcholine receptor (mAChR), was also evident in the APC peptide. This suggests that both MCC and APC may be involved in the regulation of ras oncogene activity.

In common with the retinoblastoma susceptibility gene, it appears that the APC and MCC genes may be involved in malignancies other than those of the colorectum. So far frequent allele loss has been reported with PCR based APC and MCC markers, in 80% of lung and 70% of oesophageal cancers (D'Amico, et al, 1992, Boynton, et al, 1992). Furthermore, somatic mutations of the APC gene have been identified in pancreatic cancers and gastric adenomas and carcinomas amongst Japanese patients (Horii, et al, 1992, Nakatsura, et al, 1992, 1993).

1.53b. Deleted in Colorectal Cancer Gene (DCC), 18q21-ter.

The frequent loss of markers on 18q in colorectal tumours (Muleris, et al, 1987, Vogelstein, et al, 1988, 1989), le Fearon, et al, (1990) to search for a candidate tumour suppressor gene in this region. Centered around the markers most commonly deleted, a 370kb phage contig was constructed. Using a combination of cross-species hybridization and PCR based exon-connection, expressed sequences were isolated from the contig, and the deleted in colorectal cancer (DCC) gene was identified (Fearon, et al, 1990). Mutations of the DCC gene were detected in tumour samples and included point mutations, homozygous deletions and insertions. In addition, the level of DCC expression in tumours was depressed, in comparison with the surrounding normal mucosa (Fearon, et al, 1990, Kikuchi-Yanoshita, et al, 1992a).

Although only 2854 bases of the potentially 10kb DCC transcript have been isolated, a 725 amino acid peptide has been predicted. The DCC peptide shares regions of homology with neural cell adhesion molecules and other related cell surface glycoproteins (Fearon, et al, 1990). This finding is interesting in light of the observation that inactivation of the DCC gene was associated with tumour invasiveness and metastasis. It is possible therefore, that the DCC peptide may be involved in maintenance of contact inhibition and normal cell-surface interactions (Fearon, et al, 1990).
As with other tumour suppressor genes, reversal of tumourigenicity has been demonstrated with DCC. The long arm of chromosome 18 was introduced by microcell hybridization, into a colon carcinoma cell line. The effect was that hybrids developed a flattened morphology and were no longer tumourigenic in nude mice (Tanaka, et al, 1991).

Previous reports of linkage between the Kidd blood group (18q11.1-q21.1) and hereditary non-polyposis colorectal cancer (HNPCC) (Lynch, et al, 1985, Geitvik, et al, 1987), prompted speculation that the DCC gene may be responsible for susceptibility to this condition. This possibility was excluded, following linkage analysis in seven HNPCC families with markers from 18q, including three from within the DCC gene (Peltomäki, et al, 1991). The APC locus on chromosome 5q21 has also been excluded on the basis of linkage analysis (Peltomäki, et al, 1992).

1.5.3c. p53 Tumour Suppressor Gene, 17p13.

Complete or partial loss of 17p is a frequent event in colorectal carcinogenesis (Muleris, et al, 1987, Vogelstein, et al, 1988, 1989). Characterization of the region consistently deleted in different colorectal tumour samples, revealed that it encompassed the p53 gene (Baker, et al, 1989). It was found that the remaining allele was commonly mutated within conserved regions of the gene (Baker, et al, 1989, 1990a, Nigro, et al, 1989, Rodrigues, et al, 1990), the effect of such mutations was to greatly extend the biological half life of the p53 peptide, from 6 to 30 minutes (Finlay, et al, 1988). Previous studies had suggested that loss of markers on 17p was a relatively late event in colorectal carcinogenesis, this premise was supported by the observation that p53 genes were seldom mutated in either sporadic or familial adenomas (Baker, et al, 1990a).

The specific role of p53 in suppression of colorectal neoplasia was demonstrated in transfection assays. Wild-type and mutant forms of p53 were introduced into colorectal carcinoma cell lines. The effect of the wild-type construct was to inhibit progress through the cell cycle, whilst cells carrying the mutant gene expressed elevated levels of p53 mRNA and formed colonies readily (Baker, et al, 1990b).
1.54. Oncogenes in Colorectal Carcinogenesis.


1.54a. K-ras Oncogene in Colorectal Carcinogenesis.

Activation of Kirsten-ras oncogene in colorectal cancer by somatic point mutation was reported as long ago as 1983 (Capon, et al, 1983). Subsequent studies have shown such activation to be not only a frequent event, but one which occurs early in the tumourigenic pathway (Bos, et al, 1987, Forrester, et al, 1987).

K-ras is a member of a highly conserved family of genes, which also includes Harvey (H) ras and neuroblastoma associated (N) ras. The ras genes all encode 21kd peptides (p21)(Shih, et al, 1979b), which are bound to the inner surface of the plasma membrane (Willingham, et al, 1980). The p21 peptides have weak intrinsic GTPase activity (McGrath, et al, 1984), bind guanine nucleotides (Scolnick, et al, 1979) and are probably involved in intercellular signal transduction. The proposed model for the role of p21 in signal transduction suggests that in the inactive state GDP is bound to p21. Stimulation from, for example a receptor, results in exchange of the GDP for GTP, with a consequent conformational change of p21 to an active state. The active p21 interacts with an effector and is immediately deactivated by its intrinsic GTPase activity, back to the inactive GDP bound form (Barbacid, 1987).

Carcinogenic activating point mutations of the ras genes are confined to codons 12, 13 and 61, and apparently result in peptides with reduced GTPase activity (McGrath, et al, 1984). Hence, the half life of the GTP bound-active form of p21 would be prolonged (Barbacid, 1987). Hypomethylation of H and K-ras genes has been reported in colorectal adenocarcinomas, suggesting that the genes may be
overexpressed in tumours, compared with adjacent normal mucosa (Fienberg & Vogelstein, 1983b).


Any correlation between K-ras activation and morphology, pathological staging and clinical prognosis remains controversial. Although Kerr, et al (1986) detected no correlation between these factors, Michelassi, et al (1988) reported the highest levels of p21 in archival samples of metastatic colorectal tumours, which ultimately had the worst prognosis. These observations were countered by those of Gallick, et al (1985), who found low levels of p21 in metastases. They proposed that as a tumour progresses and becomes more autonomous, it no longer requires ras activation. Morphological comparisons revealed that villous adenomas and the carcinomas which develop from them, were more likely to contain activated K-ras than their tubular counterparts (Forrester, et al, 1987, Michelassi, et al, 1987). In addition, Forrester et al (1987) observed that mutations at the second base of codon 12 were frequently associated with a more invasive phenotype, than those at the first base. Although Vogelstein, et al (1988) failed to correlate ras mutation with the location of the tumour, Bell, et al (1991) found rectal carcinomas to contain K-ras codon 12 mutations more frequently than colon carcinomas.

Overall therefore, activation of K-ras would appear to play an active role in colorectal carcinogenesis. Although ras activation occurs at an early stage in carcinogenesis, it is clear that it does not represent an initiating event, as only 9% of small adenomas (<1cm) contain activated ras genes (Vogelstein, et al, 1988). Involvement of ras in a large proportion of premalignant lesions allows presymptomatic
screening of individuals to be attempted. An immunocytochemical assay was proposed for examining colorectal mucosal brush smears (Czerniak, et al., 1987). However, a less invasive method, based on PCR analysis of faecal samples has recently been put forward (Sidransky, et al., 1992).

1.54b. c-myc Oncogene in Colorectal Carcinogenesis.

The human homologue (c-myc) of the avian myelocytomatosis virus (M29) oncogene, was found to encode a 64kd nuclear protein (p64c-myc), with DNA binding properties (Dalla-Favera, et al., 1982, Donner, et al., 1982). Elevated expression of c-myc was reported in approximately 70% of colorectal adenocarcinomas, and 60% of adenomas (Erisman, et al., 1985, 1988, Rothberg, et al., 1985, Sikora, et al., 1987, Sugio, et al., 1988, Finlay, et al., 1989b). In view of the fact that c-myc is a cell cycle dependent gene, elevated levels of expression would be expected in populations of proliferating cells, such as those in tumours (Calabretta, et al., 1985). Taking this into account, genuine transcriptional deregulation of c-myc was estimated to occur in 56% of primary colorectal carcinomas (Viel, et al., 1990). Although in the colon carcinoma cell line, COLO320, the c-myc gene was amplified in the formation of double minute chromosomes and homogeneously staining regions (Alitalo, et al., 1982), increased expression of c-myc in other colorectal samples, was generally not associated with genomic amplification or rearrangement (Erisman, et al., 1985, Sikora, et al., 1987, Dolcetti, et al., 1988).

There does not appear to be a strong correlation between clinical outcome and c-myc expression in colorectal neoplasia (Rothberg, et al., 1985, Erisman, et al., 1988). However, it has been suggested that tumours expressing raised levels of c-myc tend to be located in the left colon (Rothberg, et al., 1985) and that in these tumours raised c-myc levels are associated with chromosome 5 allele loss (Erisman, et al., 1989). Furthermore, a recent study has demonstrated that deregulated c-myc expression in colon carcinoma cell lines may be suppressed, by introduction of a normal chromosome 5 by microcell fusion (Rodriguez-Alfageme, et al., 1992). These observations have led to speculation that the APC tumour suppressor gene on 5q21, may act as a trans-acting regulator

1.6. Aims of This Study

1.6.1. Analysis of Genomic Sequences.

The initial aim of this project was to identify chromosomes whose loss, gain or structural rearrangement, represented significant events in colorectal carcinogenesis. Based on previous cytogenetic and molecular evidence chromosomes 1, 3 and 7 were chosen for study. Polymorphic DNA markers from loci on these chromosomes were used to analyse matched tumour and normal colorectal DNA samples, by Southern blot hybridization (Southern, 1975).

Abnormalities of chromosome 1 have been cited in many types of malignancy (Mitelman, et al, 1985). In colorectal tumours, involvement of chromosome 1 was reported in up to 39% of cases (Reichmann, et al, 1984), with trisomies and structural rearrangements, including isochromosome formation, representing common events (Reichmann, et al, 1981, Paraskeva, et al, 1984).

The short arm of chromosome 3 was examined to establish whether the putative small cell lung cancer (SCLC) tumour suppressor gene at 3p21 (Wang-Peng, et al, 1982, Naylor, et al, 1986), was also involved in colorectal neoplasia. Preliminary data had suggested that allele loss was occurring at this locus, in the DNA of at least one colorectal tumour (Rider, 1986).


Various other loci were examined for genomic rearrangements, including the trk oncogene, which was originally identified in a colon carcinoma (Pulciani, et al, 1982). The retinoblastoma susceptibility gene was also examined, in light of observations that it was rearranged in malignancies other than those associated with retinoblastoma.
(T'Ang, et al, 1988, Harbour, et al, 1988). And the hypervariable loci of two mucin genes were studied, as the mucin status of colorectal carcinomas may have prognostic significance (Symonds & Vickery, 1976, Umpleby, et al, 1985).

1.62. Analysis of Expressed Sequences.

The expression patterns of a variety of genes were studied in a panel of RNA samples, from FAP adenomas and sporadic carcinomas. Genes whose involvement had already been implicated in colorectal carcinogenesis, or whose chromosomal location suggested that expression may be altered by genomic rearrangement, were examined. Thus, the proto-oncogenes trk, c-myc and met were studied, the retinoblastoma susceptibility gene, together with p53 and MCC tumour suppressor genes were also investigated. The status of carbonic anhydrase 1 expression was examined, and finally the expression level of three different mucoproteins was established.

1.63. Isolation and Analysis of Chromosome Five Specific Clones.

In an attempt to isolate genes from chromosome 5 which may be involved in colorectal carcinogenesis, a cross hybridization screening strategy was employed. Wong et al, (1989) had developed a method to isolate genes involved in retinal disorders which were known to map to the X chromosome. The method relied upon identification of clones which cross hybridized between a cDNA library from a given tissue, and a chromosome specific genomic library. Two libraries were therefore required to apply this strategy to the isolation of genes from chromosome 5 that may be involved in colorectal carcinogenesis. A chromosome 5 specific genomic DNA library was purchased commercially. However, all the commercially available colon cDNA libraries were derived from RNA of mucosal tissue adjoining neoplastic lesions, it was therefore considered preferable to construct a cDNA library from the RNA of stripped colonic mucosa which was known to be normal.
CHAPTER 2

MATERIALS & METHODS.

2.0. Tissues Samples & Cell Lines.

The tissues and patients used in this study are given in tables 2.1, 2.2 & 2.3. Surgical specimens were kindly provided by the Polyposis Registry, St Marks Hospital, London, Mr A. Gunn, Northumberland Area Health Authority and Cmdr A.R. Mugridge, The Royal Naval Hospital, Plymouth. The samples were obtained during elective surgery, either for colectomy in FAP patients or for resection of carcinomas in non-FAP patients. In most cases material was obtained from the tumour or adenoma and from normal mucosal tissue in the same region of the colorectum. Ideally surgical samples were flash frozen in liquid nitrogen shortly after surgery, in which case they could be used for either DNA or RNA analysis. However, this was not always possible or desirable (if cells from the specimens were to be cultured), in which case the tissues were stored at 4°C in phosphate buffered saline (appendix 1.3) and then flash frozen when convenient, such samples were used only in DNA analysis.

A number of colorectal cancer derived cell lines were also used as a source of DNA and RNA (table 2.4), these were obtained from various sources.

In addition to all the colorectal samples of neoplastic origin, a sample of stripped colonic mucosa was obtained from a patient (LS) with no colorectal pathology. The patient had suffered from endometriosis, which had resulted in adhesion of the uterus to part of the colon, thus necessitating removal of a section of normal colon during the hysterectomy. This sample was kindly provided by Mr A. Gunn.
Table 2.1.

Sporadic Colorectal Cancers from Which DNA was Isolated.

<table>
<thead>
<tr>
<th>Patient No</th>
<th>Age</th>
<th>Sex</th>
<th>Site</th>
<th>Diagnosis</th>
<th>Dukes Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>142</td>
<td>40</td>
<td>M</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>C1</td>
</tr>
<tr>
<td>149</td>
<td>48</td>
<td>M</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>C1</td>
</tr>
<tr>
<td>150</td>
<td>58</td>
<td>F</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>C1</td>
</tr>
<tr>
<td>151</td>
<td>81</td>
<td>F</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>B</td>
</tr>
<tr>
<td>177</td>
<td>52</td>
<td>M</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>C1</td>
</tr>
<tr>
<td>183</td>
<td>56</td>
<td>M</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>C2</td>
</tr>
<tr>
<td>184</td>
<td>45</td>
<td>M</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>C1</td>
</tr>
<tr>
<td>185</td>
<td>48</td>
<td>M</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>C1</td>
</tr>
<tr>
<td>192</td>
<td>55</td>
<td>M</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>B</td>
</tr>
<tr>
<td>193</td>
<td>59</td>
<td>M</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>C1</td>
</tr>
<tr>
<td>195</td>
<td>59</td>
<td>M</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>C1</td>
</tr>
<tr>
<td>200</td>
<td>57</td>
<td>F</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>B</td>
</tr>
<tr>
<td>202</td>
<td>45</td>
<td>M</td>
<td>Des. Colon</td>
<td>Adenocarcinoma</td>
<td>B</td>
</tr>
<tr>
<td>214</td>
<td>37</td>
<td>F</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Recto-Sigmoid</td>
<td>Adenocarcinoma</td>
<td>A</td>
</tr>
<tr>
<td>219</td>
<td>49</td>
<td>M</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>C1</td>
</tr>
<tr>
<td>225</td>
<td>58</td>
<td>M</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>B</td>
</tr>
<tr>
<td>244</td>
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<td>M</td>
<td>Rectum</td>
<td>Undifferentiated</td>
<td>C1</td>
</tr>
<tr>
<td>254</td>
<td>37</td>
<td>M</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>B</td>
</tr>
<tr>
<td>260</td>
<td>59</td>
<td>M</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>A</td>
</tr>
<tr>
<td>311</td>
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<td>F</td>
<td>Asc. Colon</td>
<td>Adenocarcinoma</td>
<td>B</td>
</tr>
<tr>
<td>315</td>
<td>74</td>
<td>F</td>
<td>Sig. Colon</td>
<td>Adenocarcinoma</td>
<td>C2</td>
</tr>
<tr>
<td>823</td>
<td>76</td>
<td>F</td>
<td>Caecum</td>
<td>Adenocarcinoma</td>
<td>C</td>
</tr>
</tbody>
</table>

Table 2.2. Familial Adenomatous Polyposis Samples from which DNA was Isolated.

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Age</th>
<th>Sex</th>
<th>Adenoma Size</th>
<th>Adenoma Site and Morphology (if known)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAP29</td>
<td>34</td>
<td>M</td>
<td>4mm, 4mm, 4mm</td>
<td>Ascending Transverse Transverse Transverse</td>
</tr>
<tr>
<td>FAP30</td>
<td>48</td>
<td>M</td>
<td>5mm, 6mm, 6mm</td>
<td>Ascending Transverse Ascending Transverse</td>
</tr>
<tr>
<td>FAP46</td>
<td>16</td>
<td>F</td>
<td>5mm, 5mm, 5mm</td>
<td>Ascending Transverse Transverse Transverse</td>
</tr>
<tr>
<td>FAP57</td>
<td>15</td>
<td>M</td>
<td>NK, NK, NK</td>
<td>Ascending Ascending Transverse Descending</td>
</tr>
<tr>
<td>FAP70</td>
<td>52</td>
<td>F</td>
<td>NK, NK, NK, 20mm x 10mm</td>
<td>Ascending Ascending Transverse Descending Descending Sessile Villous</td>
</tr>
<tr>
<td>FAP83</td>
<td>40</td>
<td>M</td>
<td>7mm, 3mm, 5mm</td>
<td>Ascending Transverse 3 adenomas pooled Asc., Trans., Des.</td>
</tr>
<tr>
<td>FAP84</td>
<td>22</td>
<td>F</td>
<td>3mm, 3mm, 5mm</td>
<td>Ascending Ascending Transverse Descending Descending Asc., Trans.</td>
</tr>
<tr>
<td>FAP86 (Son of FAP 83)</td>
<td>M</td>
<td></td>
<td>4mm, 4mm, 4mm</td>
<td>Ascending Transverse Descending Asc., Trans., Des.</td>
</tr>
<tr>
<td>FAP87</td>
<td>45</td>
<td>M*</td>
<td>NK, NK, NK</td>
<td>Ascending Transverse Descending Asc., Trans., Des.</td>
</tr>
<tr>
<td>FAP99</td>
<td>49</td>
<td>M</td>
<td>15mm, 35mm, Normal Mucosa</td>
<td>Polyp Suspected cancer</td>
</tr>
<tr>
<td>FAP112</td>
<td>29</td>
<td>M</td>
<td>60mm x 80mm, Normal Mucosa</td>
<td>Sig. Colon adenocarc Dukes B</td>
</tr>
<tr>
<td>FAP119</td>
<td>40</td>
<td>F</td>
<td>NK, Normal Mucosa</td>
<td>Adenocarcinoma Dukes C</td>
</tr>
<tr>
<td>176 not an FAP patient</td>
<td>NK</td>
<td>M</td>
<td>10mm, 3mm, Normal</td>
<td>Ascending Hep. Flex. Asc., Hep. Flex.</td>
</tr>
</tbody>
</table>

Table 2.2. NK: not known, Sig. Colon: sigmoid colon, Hep. Flex.: hepatic flexure, Patient 176 was a non-polyposis patient with approximately 100 polyps distributed throughout his colorectum.
Table 2.3a.

Sporadic Colorectal Cancers from Which RNA was Isolated.

<table>
<thead>
<tr>
<th>Patient No</th>
<th>Age</th>
<th>Sex</th>
<th>Site</th>
<th>Diagnosis</th>
<th>Dukes Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>323</td>
<td>73</td>
<td>F</td>
<td>Rectum</td>
<td>Muc. Adenocarcinoma</td>
<td>C1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Adenoma</td>
<td></td>
</tr>
<tr>
<td>390</td>
<td>73</td>
<td>F</td>
<td>Sig. Colon</td>
<td>Adenocarcinoma</td>
<td>C1</td>
</tr>
<tr>
<td>428</td>
<td>64</td>
<td>M</td>
<td>Sig. Colon</td>
<td>Adenocarcinoma</td>
<td>C1</td>
</tr>
<tr>
<td>500</td>
<td>83</td>
<td>F</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>C</td>
</tr>
<tr>
<td>517</td>
<td>78</td>
<td>M</td>
<td>Caecum</td>
<td>Muc. Adenocarcinoma</td>
<td>B</td>
</tr>
<tr>
<td>522</td>
<td>64</td>
<td>M</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>A</td>
</tr>
<tr>
<td>556</td>
<td>73</td>
<td>M</td>
<td>Splenic Flex.</td>
<td>Muc. Adenocarcinoma</td>
<td>C1</td>
</tr>
<tr>
<td>567</td>
<td>69</td>
<td>M</td>
<td>Sig. Colon</td>
<td>Adenocarcinoma</td>
<td>C1</td>
</tr>
<tr>
<td>570</td>
<td>74</td>
<td>F</td>
<td>Sig. Colon</td>
<td>Muc. Adenocarcinoma</td>
<td>C1</td>
</tr>
<tr>
<td>575</td>
<td>65</td>
<td>F</td>
<td>Sig. Colon</td>
<td>Adenocarcinoma</td>
<td>A</td>
</tr>
<tr>
<td>587</td>
<td>67</td>
<td>F</td>
<td>Sig. Colon</td>
<td>Adenocarcinoma</td>
<td>B</td>
</tr>
<tr>
<td>596</td>
<td>59</td>
<td>M</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>C1</td>
</tr>
<tr>
<td>598</td>
<td>62</td>
<td>M</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>B</td>
</tr>
<tr>
<td>602</td>
<td>65</td>
<td>M</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>C1</td>
</tr>
<tr>
<td>610</td>
<td>70</td>
<td>M</td>
<td>Sig. Colon</td>
<td>Tub.Vill. Adenocarcinoma</td>
<td>NK</td>
</tr>
<tr>
<td>616</td>
<td>40</td>
<td>M</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>C1</td>
</tr>
<tr>
<td>621</td>
<td>73</td>
<td>M</td>
<td>Sig. Colon</td>
<td>Adenocarcinoma</td>
<td>B</td>
</tr>
<tr>
<td>638</td>
<td>NK</td>
<td>M</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>B</td>
</tr>
<tr>
<td>643</td>
<td>78</td>
<td>F</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>B</td>
</tr>
<tr>
<td>667</td>
<td>75</td>
<td>M</td>
<td>Sig. Colon</td>
<td>Adenocarcinoma</td>
<td>A</td>
</tr>
<tr>
<td>668</td>
<td>66</td>
<td>M</td>
<td>Sig. Colon</td>
<td>Adenocarcinoma</td>
<td>C2</td>
</tr>
<tr>
<td>681</td>
<td>58</td>
<td>F</td>
<td>Sig. Colon</td>
<td>Adenocarcinoma</td>
<td>B</td>
</tr>
<tr>
<td>693</td>
<td>61</td>
<td>M</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>B</td>
</tr>
<tr>
<td>708</td>
<td>80</td>
<td>M</td>
<td>Sig. Colon</td>
<td>Adenocarcinoma</td>
<td>B</td>
</tr>
<tr>
<td>717</td>
<td>54</td>
<td>M</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>B</td>
</tr>
<tr>
<td>757</td>
<td>75</td>
<td>F</td>
<td>Sig. Colon</td>
<td>Adenocarcinoma</td>
<td>B</td>
</tr>
<tr>
<td>762</td>
<td>64</td>
<td>F</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>B</td>
</tr>
<tr>
<td>769</td>
<td>52</td>
<td>M</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>B</td>
</tr>
<tr>
<td>776</td>
<td>68</td>
<td>F</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>B</td>
</tr>
<tr>
<td>777</td>
<td>84</td>
<td>F</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>B</td>
</tr>
<tr>
<td>784</td>
<td>61</td>
<td>M</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>C2</td>
</tr>
<tr>
<td>800</td>
<td>60</td>
<td>M</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>B</td>
</tr>
<tr>
<td>808</td>
<td>70</td>
<td>F</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>C1</td>
</tr>
<tr>
<td>854</td>
<td>80</td>
<td>M</td>
<td>Sig. Colon</td>
<td>Adenocarcinoma</td>
<td>B</td>
</tr>
<tr>
<td>860</td>
<td>26</td>
<td>F</td>
<td>NK</td>
<td>NK</td>
<td>NK</td>
</tr>
<tr>
<td>904</td>
<td>80</td>
<td>M</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>B</td>
</tr>
<tr>
<td>927</td>
<td>78</td>
<td>M</td>
<td>Sig. Colon</td>
<td>Muc. Adenocarcinoma</td>
<td>C</td>
</tr>
<tr>
<td>945</td>
<td>75</td>
<td>M</td>
<td>Rectum</td>
<td>Adenocarcinoma</td>
<td>B</td>
</tr>
<tr>
<td>988</td>
<td>52</td>
<td>F</td>
<td>Recto-Sigmoid</td>
<td>Adenocarcinoma</td>
<td>C1</td>
</tr>
<tr>
<td>1001</td>
<td>65</td>
<td>M</td>
<td>Sig. Colon</td>
<td>Muc. Adenocarcinoma</td>
<td>C1</td>
</tr>
<tr>
<td>1011</td>
<td>86</td>
<td>M</td>
<td>Sig. Colon</td>
<td>Adenocarcinoma + UC</td>
<td>C2</td>
</tr>
<tr>
<td>1015</td>
<td>71</td>
<td>M</td>
<td>Sig. Colon</td>
<td>Adenocarcinoma</td>
<td>A</td>
</tr>
</tbody>
</table>

Table 2.3a. Muc.: mucinous, Sig. colon: sigmoid colon, Splenic Flex.: splenic flexure, Tub.Vill.: tubulo villous, UC.: ulcerative colitis, NK: not known.
Table 2.3b. Familial Adenomatous Polyposis Samples from Which RNA was Isolated.

<table>
<thead>
<tr>
<th>Patient No</th>
<th>Age</th>
<th>Sex</th>
<th>Adenoma number</th>
<th>Adenoma size</th>
<th>Adenoma site</th>
<th>Histopathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAP112</td>
<td>29</td>
<td>M</td>
<td>Cancer</td>
<td>6 x 8 cm</td>
<td>Sigmoid colon</td>
<td>Dukes' B</td>
</tr>
<tr>
<td>FAP133</td>
<td>P1</td>
<td>NK</td>
<td>NK</td>
<td>NK</td>
<td>NK</td>
<td></td>
</tr>
<tr>
<td>FAP136</td>
<td>P1</td>
<td>8mm</td>
<td>Transverse colon</td>
<td>Mild</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAP139</td>
<td>P1</td>
<td>10mm</td>
<td>Descending colon</td>
<td>Tubular, mild</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAP145</td>
<td>P1</td>
<td>NK</td>
<td>NK</td>
<td>NK</td>
<td>NK</td>
<td></td>
</tr>
<tr>
<td>FAP146</td>
<td>P1</td>
<td>NK</td>
<td>NK</td>
<td>NK</td>
<td>NK</td>
<td></td>
</tr>
<tr>
<td>FAP147</td>
<td>P1</td>
<td>NK</td>
<td>NK</td>
<td>NK</td>
<td>NK</td>
<td></td>
</tr>
<tr>
<td>FAP156</td>
<td>P1</td>
<td>2mm</td>
<td>NK</td>
<td>Mild</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAP164</td>
<td>P1</td>
<td>10mm</td>
<td>NK</td>
<td>Mild</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mild/ Moderate/ Severe: degree of dysplasia. Tubular/ Villous: adenoma architecture, NK: not known.
Table 2.4.

**Cell Lines from which DNA and RNA were Isolated.**

<table>
<thead>
<tr>
<th>Line Name</th>
<th>Origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCA-7</td>
<td>Colorectal adenocarcinoma</td>
<td>Kirkland, 1985.</td>
</tr>
<tr>
<td>COLO320</td>
<td>Neuroendocrine tumour derived from colon cancer</td>
<td>American Type Culture Collection.</td>
</tr>
<tr>
<td>KMS-4</td>
<td>Lymph node metastases of FAP patient</td>
<td>Namba, et al, 1983. (RNA only)</td>
</tr>
<tr>
<td>JWNF</td>
<td>Normal fibroblasts from above patient</td>
<td>J.Delhanty (personal communication)</td>
</tr>
</tbody>
</table>

2.1. Analysis of Genomic Sequences.

A panel of DNA samples prepared from tumour and normal tissues and carcinoma cell lines (tables 2.1, 2.2 & 2.4), were screened with a series of DNA probes for genomic rearrangements, including loss of heterozygosity. The genomic DNA was Southern blotted (Southern, 1975) and hybridized to radiolabelled probes (listed in section 2.3, tables 2.5a & c). For probes which detect restriction fragment length polymorphisms (RFLPs), the genotype of the constitutional DNA was determined. If this was heterozygous, the tumour DNA was analysed and compared with the normal. When highly polymorphic DNA markers, such as the minisatellite probes were used, it was assumed that the constitutional DNA would be heterozygous, and hence tumour and normal DNAs were compared in the first instance. In addition to the loss of genetic material detected by RFLPs, rearrangements at non-polymorphic loci were also detected in these samples by Southern blotting.
2.11. Isolation of Genomic DNA from Frozen Tissue.

Frozen tissue was transferred directly from liquid nitrogen storage into lysis buffer (10mM Tris.HCl pH 8, 150mM NaCl, 10mM EDTA, 0.5% SDS (w:v), 250µg/ml proteinase K) and chopped finely with scalpels. Lysis was allowed to proceed for at least 16 hours at 37°C, when the mixture was gently extracted twice with phenol and once with chloroform. The DNA was precipitated by the addition of two volumes of ice cold ethanol and incubation overnight at -20°C, the resulting DNA was hooked from the liquid with a pipette tip and dissolved in TE pH 7.4. A second precipitation was performed by the addition of an equal volume 7.5M ammonium acetate and twice the final volume of ice cold ethanol, incubation at -20°C was usually not required. The DNA was washed in 80% ethanol (20% H₂O (v:v)), allowed to air dry and finally resuspended in TE pH 7.4. Once the yield of DNA had been calculated spectrophotometricly (appendix 2.1), the volume of the sample was adjusted to give a DNA concentration of 250µg/ml.

2.12. Isolation of Genomic DNA from Cultured Cells.

Human fibroblasts or colorectal cancer cells were grown to confluence in the appropriate medium at 37°C (cell culture was kindly performed by J. Delhanty). The cells were removed from the flasks by treatment with 0.4% trypsin (w:v), and were washed twice in 0.9% saline (w:v) by successive centrifugation at 1000rpm and resuspension. The resulting cell pellet was resuspended in lysis buffer and the DNA was isolated as above.

2.13. Digestion, Electrophoresis and Blotting Genomic DNA.

Five microgrammes of genomic DNA was digested to completion with 20 units of the required restriction endonuclease, in the presence of the manufacturers' reaction buffer and 10mM spermidine. After digestion for at least 2 hours at the designated temperature, the digestions were heat inactivated at 65°C for 10 minutes where appropriate. The samples were then size fractionated by gel electrophoresis through agarose in TAE buffer (appendix 2.2a) and blotted onto either Gene Screen Plus hybridization membrane (NEN
DuPont) or Hybond-N+ membrane (Amersham International Plc) as described in appendix 2.4a.


Membranes with DNA immobilised on them were processed according to the manufacturers recommendations (NEN DuPont & Amersham International plc) as given in appendix 2.4b.

2.2. Analysis of Expressed Sequences.

A panel of RNA samples prepared from tumour and normal tissues and carcinoma cell lines (tables 2.3a & b, & table 2.4) were screened with a series of DNA probes for the presence and relative abundance of a variety of expressed sequences.

RNA is very sensitive to nuclease degradation, therefore special precautions were taken during isolation and subsequent manipulations of RNA to protect it initially from endogenous and then exogenous ribonucleases. Hence, disposable gloves were worn throughout all procedures, glassware was baked at 180°C for 2 hours and non-Tris containing solutions were treated with the potent ribonuclease inhibitor diethylpyrocarbonate (dpc)(Ehrenberg, et al, 1974), thus inactivating any contaminating nucleases. DPC was added to solutions after their pH had been adjusted, to give a final concentration of 0.1% (v:v), the solutions were mixed thoroughly and left overnight at room temperature. The dpc was then removed by standard autoclaving.

2.21. Isolation of Total Cellular RNA From Tissue.

Total cellular RNA was isolated from tissue by the method of Chomczynski & Sacchi (1987) this procedure, like that of Chirgwin et al (1979), relies upon guanidinium thiocyanate denaturation of protein, including endogenous ribonucleases, but avoids the necessity for cesium chloride density centrifugation by direct extraction of the homogenate with organic solvents.

Preweighed frozen tissue was transferred directly from liquid nitrogen to 2ml solution D (4M guanidinium thiocyanate, 25mM sodium citrate pH 7 (dpc), 0,5% sarcosyl (w:v), 0.1M 2-mercaptoethanol) in a small polystyrene petri
dish. The tissue was minced with scalpels, and the homogenate was transferred to a 7ml polypropylene stoppered centrifuge tube. Two hundred microlitres 2M sodium acetate pH 4 (dpc), 2ml water saturated phenol and 400μl chloroform were added sequentially to the homogenate, with thorough mixing between each addition. Finally the suspension was shaken vigorously for 10 seconds and incubated on ice for 15 minutes. The two phases were separated by centrifugation at 10000g for 20 minutes at 4°C. The aqueous phase containing the RNA was removed, taking care not to disturb the DNA containing interface and organic phase. The RNA was precipitated by the addition of 2ml propan-2-ol and incubation at -20°C for at least an hour, it was then recovered by centrifugation at 10000g for 10 minutes at 4°C, and resuspended in 600μl solution D. Following transfer to a 1.5ml microcentrifuge tube the RNA was reprecipitated by addition of an equal volume of propan-2-ol and incubation overnight at -20°C. The resulting precipitate was recovered by centrifugation at 13000g for 20 minutes at 4°C, washed in 80% ethanol 20% H2O (dpc), dried under vacuum and resuspended in either H2O (dpc) or 10mM HEPES pH 7.5 (dpc) and stored at -70°C.

2.22. Isolation of Total Cellular RNA from Cultured Cells.

Total cellular RNA was isolated from cultured cells by an adaptation of the method of Strohman et al (1977). A culture of the cells of interest were grown to subconfluence (cell culture was kindly performed by J. Delhanty), in the appropriate medium in 10 medium sized (260ml) tissue culture flasks. The cells were washed twice with 10ml 0.9% saline (w:v) (dpc). Three millilitres 6M guanidinium hydrochloride, 200mM sodium acetate pH 5.6 (dpc) were added to each flask and cell lysis was assisted by incubation for 5 minutes on a rocking platform. The flasks were then stood upright and the viscous lysate was allowed to settle. The lysates from each flask were pooled into a 50ml polypropylene centrifuge tube, and were drawn several times though a 21 gauge hypodermic needle attached to a 20ml syringe, thus considerably reducing the viscosity of the lysate. The resulting suspension was divided equally between two 50ml polypropylene centrifuge tubes and a half volume ethanol was added to each whilst
being shaken vigorously (to reduce local precipitation of DNA). Following incubation overnight at -20°C, the nucleic acid precipitate was collected by centrifugation in 30ml glass Corex centrifuge tubes (Corning Ltd, Staffs) at 10000g for 15 minutes at 4°C. The pellet was resuspended in 10ml urea solution (7M urea, 350mM NaCl (dpc), 50mM Tris.HCl pH 7.5, 1mM EDTA (dpc), 0.2% SDS (w:v:)) and was extracted by shaking vigorously with an equal volume phenol:chloroform. The resulting emulsion was centrifuged at 5000g for 5 minutes at 20°C, and the nucleic acid was precipitated from the aqueous phase by addition of two volumes of ethanol and incubation overnight at -20°C. Again the precipitate was collected by centrifugation at 10000g for 10 minutes at 4°C, the pellet was washed in 80% ethanol (20% 10mM HEPES pH 7.5 (dpc)) and dried under vacuum. The pellet was dissolved in 1ml 10mM HEPES pH 7.5 (dpc) and the RNA was selectively precipitated by the addition of solid NaCl to 3M and incubation overnight at -20°C (Houghton et al, 1980). The RNA was collected by centrifugation as before and was washed twice with 3M sodium acetate pH 7 (dpc) and once with 80% ethanol (20% 10mM HEPES pH 7.5 (dpc)), it was dried under vacuum, finally to be resuspended in either H2O (dpc) or 10mM HEPES pH 7.5 (dpc) and stored at -70°C. It was possible to recover genomic DNA from the supernatant of the NaCl precipitation by addition of 2.5 volumes of ethanol.

2.23. Oligo (dT)-Cellulose Affinity Chromatography.

The basic principle of enrichment for polyadenylated (poly-A) messenger RNA (mRNA) from total cellular RNA was that of Aviv & Leder (1972). In this study however, a spin column procedure was employed.

Approximately 0.5g oligo(dT) cellulose (Gibco, BRL) was suspended in 10mM HEPES pH 7.5 (dpc) in the barrel of a 1ml disposable syringe which had been plugged with siliconized glass wool. The syringe/column was placed in a sterile 10ml centrifuge tube and was spun at 1500g for 2 seconds at room temperature (these were the conditions for all subsequent centrifugations). The 10mM HEPES collected in the centrifuge tube was discarded, the column was then washed twice with 0.1M KOH (spinning between each wash) to remove any residual nucleic acids present on the cellulose or the syringe.
Contaminating nucleases were then removed by two washes with 
H2O to which dpc was added freshly (0.05% (v:v)). The column 
was then equilibrated with 0.5M KCl, 10mM HEPES pH 7.5 (dpc) 
by successive washes until the pH of the eluant had risen to 
7.5. The sample was then loaded onto the column following 
addition to the sample of an equal volume of 1M KCl, 10mM 
HEPES pH 7.5 (dpc) resulting in a final KCl concentration in 
the sample of 0.5M. The column was spun and the eluant of 
unbound RNA was reapplied to the column, this was repeated 
four times. All remaining unbound RNA was removed from the 
column with successive 0.5M KCl, 10mM HEPES pH 7.5 (dpc) 
washes, until the A260 spectrophotometric reading of the 
eluant was negligible. Short length adenylated RNA sequences 
were removed from the column by two washes with 0.1M KCl, 
10mM HEPES pH 7.5 (dpc). Finally the poly-A mRNA was 
released from the oligo (dT) cellulose by washing with 10mM 
HEPES pH 7.5 (dpc). The resulting fractions were pooled and 
precipitated with a tenth volume 3M sodium acetate pH 7 
(dpc), 2.5 volumes ethanol and incubation overnight at -20°C. 
The mRNA precipitate was retrieved as before, resuspended in 
a minimal volume H2O (dpc), and stored at -70°C.

2.24. Agarose Gel Electrophoresis of RNA.

2.24a. For Ethidium Bromide Visualization.

RNA was examined by agarose gel electrophoresis both 
before and after oligo (dT) affinity chromatography. Such 
analysis allowed some assessment of the RNA quality to be 
made, it also revealed the presence of any contaminating DNA 
in the samples.

Agarose (1.2% (w:v)) was melted in 1X RE buffer (36mM 
Tris.HCl pH 7.8, 30mM NaH2PO4.2H2O, 1mM EDTA, 0.01% SDS(w:v)), 
allowed to cool to 60°C and was poured into a minigel mould. 
The RNA samples (1-5 μg) were denatured at 80°C for 5 minutes 
in a total volume of 30μl containing 1X RE, 10% glycerol 
(v:v), 2% Ficoll type 400 (w:v), 0.025% bromophenol blue 
(w:v). The samples were quenched on ice prior to loading. 
The gel running buffer was 1X RE. Electrophoresis was allowed 
to proceed at 50 volts until the bromophenol blue had 
migrated to three quarters of the length of the gel. The gel 
was stained with ethidium bromide (1μg/ml) for 15 minutes and 
was then viewed on a UV transilluminator.
2.24b. For Northern Blotting.

RNA was size fractionated by agarose gel electrophoresis, transferred to Nylon hybridization membrane and hybridized to radiolabelled DNA probes according to the principles of Thomas (1980).

A 1% agarose gel was prepared by melting the agarose in water, after cooling to 60°C, a 1/10 volume of 10X MOPS buffer (0.2M MOPS pH 7, 50mM sodium acetate, 10mM EDTA) was added followed by formaldehyde solution to give a final concentration of 6% (v:v) (Lehrach, et al, 1977). The gel was then mixed well, cast and allowed to set.

The RNA samples (1-10μg) and radiolabelled DNA molecular weight markers (appendix 2.2c) were denatured at 60°C for 15 minutes in the presence of 50% deionized formamide (v:v), 6% formaldehyde (v:v) and 10% 10X MOPS buffer (v:v). The samples were quenched on ice and loading buffer was added to give a final concentration of 5% glycerol (v:v), 1mM EDTA, 0.04% bromophenol blue (v:v) and 0.04% xylene cyanol FF (v:v). The samples were then loaded onto the gel and electrophoresed at 50 volts, with 1X MOPS buffer as the running buffer, until the bromophenol blue had migrated three quarter of the length of the gel. The gel was rinsed in H₂O and the RNA was transferred to Gene Screen Plus hybridization membrane (NEN DuPont, Southampton, Hants.), by capillary blotting with 10X SSC as the transfer buffer, according to the manufacturers specifications. Alternatively, the gels were rinsed in 0.4N NaOH and capillary blotted onto Hybond N+ nylon hybridization membrane (Amersham International Plc), with 0.4N NaOH as the transfer buffer, as indicated in the manufacturers specifications.

2.25 Dot Blot Analysis of RNA.

Large numbers of total cellular RNA samples were hybridized to a variety of DNA probes using the dot blot procedure, this allowed quantitative estimates of expression levels to be made, and thus direct comparisons could be made between samples derived from tumour and normal colonic tissue.

The 96 well Hybri Dot apparatus (Gibco BRL, Paisley, Scotland.) was used with Gene Screen Plus hybridization membrane (NEN DuPont). Total cellular RNA (5μg) was denatured
at 50°C for an hour in 50% deionized formamide (v:v), 6% formaldehyde (v:v), in a total volume of 30μl. It was then quenched on ice and loaded onto the dot blot apparatus, which was assembled as recommended by the manufacturers (NEN DuPont, Gibco BRL). The samples were left in contact with the membrane for 30 minutes before a vacuum was applied. The membrane was removed from the dot blot assembly and baked at 80°C for 2 hours. On occasion a slot blot manifold was used (S&S Minifold II, Schleicher & Scheull, Germany).


Filters with RNA immobilised on them were treated according to the manufacturers recommendations as outlined (appendix 2.4b).

2.3. Preparation of DNA Probes.

The DNA probes used in this study are shown in tables 2.5.a,b & c, they were obtained from many sources.

The clones were supplied in one of three forms: as freeze dried bacterial cell pellets, as bacterial colonies in or on agar, or as plasmid DNA. In the latter case the DNA was transformed into E.coli host cells to establish a permanent culture of the clone. For long term storage glycerol stocks of each clone were prepared. One millilitre of stationary phase culture was mixed with 150μl glycerol and stored at -70°C.

2.31. Transformation of Competent Cells with Plasmid DNA.

Preparation of competent bacterial cells and their transformation with plasmid DNA was achieved by CaCl₂ treatment and a heat shock, essentially as outlined by Cohen, et al (1972) but including the adaptations of Dagert & Ehrlich (1979). E.coli DH1 was the strain of bacteria which was used regularly in transformations, however, if the E.coli lacZ gene was to be selected for (eg: with the pUCseries of plasmids (Vieira & Messing, 1982, Yanish-Perron, et al, 1985)) then the plasmids were transformed into E.coli JM109 which has a defective lac operon. Subsequently Sure™ (Stratagene Ltd, Cambs) became available and were used for
<table>
<thead>
<tr>
<th>Probe name</th>
<th>Locus Symbol</th>
<th>Map Location</th>
<th>Gene if any</th>
<th>Enzyme</th>
<th>Allele Size Kb</th>
<th>Frequency</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>p1-79</td>
<td>D1Z2</td>
<td>1p36.3</td>
<td></td>
<td>TagI</td>
<td>VNTR 32bp</td>
<td>NK</td>
<td>Buroker, et al, 1987</td>
</tr>
<tr>
<td>pPGDH1</td>
<td>PGD</td>
<td>1p36.3-p36.13</td>
<td>Phospho-gluco-dehydrogenase</td>
<td>BamHI</td>
<td>11.0, 9.5, 6.0</td>
<td>.75, .25</td>
<td>Kleyn, et al, 1989</td>
</tr>
<tr>
<td>AF3</td>
<td>FUCA1</td>
<td>1p35-p34</td>
<td>α-fucosidase</td>
<td>PvuII</td>
<td>7.0, 6.0</td>
<td>.75, .25</td>
<td>Darby, et al, 1986</td>
</tr>
<tr>
<td>pMUC10</td>
<td>MUC1</td>
<td>1q21-q23</td>
<td>Polymorphic epithelial mucin</td>
<td>HinfI</td>
<td>VNTR 60bp</td>
<td>.9</td>
<td>Swallow, et al, 1987b</td>
</tr>
<tr>
<td>pHeA2</td>
<td>THR8 (ERBA2)</td>
<td>3p24-p22</td>
<td>c-erbA2 proto-oncogene</td>
<td>BamHI</td>
<td>2.5, 5</td>
<td>.56, .44</td>
<td>Middleton, et al, 1986</td>
</tr>
<tr>
<td>H3H2</td>
<td>D3F15S2</td>
<td>3p21</td>
<td></td>
<td>HindIII</td>
<td>2.3, 2.0</td>
<td>.46, .54</td>
<td>Carritt, et al, 1986</td>
</tr>
<tr>
<td>pmetH</td>
<td>MET</td>
<td>7q31</td>
<td>met proto-oncogene</td>
<td>TaqI</td>
<td>7.5, 4.0</td>
<td>.56, .44</td>
<td>Dean, et al, 1985</td>
</tr>
<tr>
<td>SMUC41</td>
<td>MUC2</td>
<td>11p15</td>
<td>Intestinal mucin</td>
<td>HinfI</td>
<td>VNTR 69bp</td>
<td>&gt;.50</td>
<td>Gum, et al, 1989</td>
</tr>
</tbody>
</table>
## Table 2.5b.

**Hypervariable Minisatellite Probes Used in the Analysis of DNA.**

<table>
<thead>
<tr>
<th>Probe</th>
<th>Locus</th>
<th>Map location</th>
<th>Enzyme</th>
<th>Frequency</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>λMS1</td>
<td>D1S7</td>
<td>1p35-p33</td>
<td>HinfI</td>
<td>.98</td>
<td>Wong, et al, 1987</td>
</tr>
<tr>
<td>λMS32</td>
<td>D1S8</td>
<td>1q42-q43</td>
<td>AluI</td>
<td>.97</td>
<td>Wong, et al, 1987</td>
</tr>
<tr>
<td>λMS8</td>
<td>D5S43</td>
<td>5q35-pter</td>
<td>HinfI</td>
<td>.85</td>
<td>Wong, et al, 1987</td>
</tr>
<tr>
<td>pλg3</td>
<td>D7S22</td>
<td>7q36-pter</td>
<td>HinfI</td>
<td>.97</td>
<td>Wong, et al, 1986</td>
</tr>
</tbody>
</table>

## Table 2.5c.

**DNA Probes Used in the Analysis of Expressed Sequences.**

<table>
<thead>
<tr>
<th>Probe</th>
<th>Map location</th>
<th>Gene</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCC100</td>
<td>5q21-q22</td>
<td>mutated in colorectal cancer gene</td>
<td>Kinzler, et al, 1991a</td>
</tr>
<tr>
<td>pmethH</td>
<td>7q31</td>
<td>met proto-oncogene</td>
<td>Dean, et al, 1985</td>
</tr>
<tr>
<td>CA1</td>
<td>8q13-q22</td>
<td>carbonic anhydrase 1</td>
<td>Barlow, et al, 1987</td>
</tr>
<tr>
<td>pUCCDIA</td>
<td>8q24</td>
<td>c-myc proto-oncogene</td>
<td>Rabbits, et al, 1984</td>
</tr>
<tr>
<td>PG3.8M</td>
<td>13q14</td>
<td>retinoblastoma susceptibility gene</td>
<td>Fung, et al, 1987</td>
</tr>
<tr>
<td>β-actin</td>
<td>7pter-q22</td>
<td>3' UTR muscle β-actin</td>
<td>Gillespie, et al, 1984</td>
</tr>
<tr>
<td>Mucins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pMUC10</td>
<td>1q21-q23</td>
<td>tumour associated epithelial mucin</td>
<td>Swallow, et al, 1987b</td>
</tr>
<tr>
<td>SMUC41</td>
<td>11p15</td>
<td>intestinal mucin</td>
<td>Gum, et al, 1989</td>
</tr>
<tr>
<td>SIB124</td>
<td>7q22</td>
<td>intestinal mucin</td>
<td>Gum, et al, 1990</td>
</tr>
</tbody>
</table>

*Tables 2.5a, b & c.* Enzyme: restriction endonuclease used to detect polymorphism. Frequency: frequency with which alleles appear in the general population (Human Genome Mapping 10).
all transformations, as they were versatile and completely restriction and modification negative.

2.31a Preparation of Competent Cells.

A 5ml overnight culture was used to inoculate 250ml L-broth (appendix 1.2a) in a 1L conical flask and was grown to early-log phase at 37°C with good aeration. The cells were incubated on ice for 15 minutes and were kept chilled for all subsequent manipulations. The cells were harvested at 5000g for 10 minutes at 4°C, the pellet was resuspended in 500ml 100mM CaCl₂ (cold) and stood on ice for 30 minutes, with occasional swirling. The cells were sedimented as before and resuspended in 40ml 100mM CaCl₂, 15% glycerol (v:v). The suspension was aliquoted into 1.5ml microcentrifuge tubes and stored overnight on ice, prior to flash freezing in liquid nitrogen and long term storage at -70°C.

2.31b Transformation of Competent Cells.

The competent cells (200µl) were thawed on ice and transformed by the addition of approximately 100ng plasmid DNA and incubation on ice for 30 minutes, followed by a heat shock at 42°C for 2 minutes and a further 5 minutes on ice. The cells were allowed to grow for 1 hour without any selection pressure by the addition of 800µl L-broth and incubation at 37°C with agitation. An appropriate dilution of the culture was then spread onto agar plates containing the relevant antibiotic (appendix 1.2b) and incubation overnight at 37°C. Negative control transformations were also performed, in the absence of plasmid DNA. Invariably no colonies were evident on the control plates. The colonies from the transformation were used to inoculate cultures for glycerol stocks and plasmid DNA preparations.

2.32. Isolation of Plasmid DNA.

Plasmid DNA was isolated from bacterial cells by the alkali lysis method of Birnboim & Doly (1979).

Ten millilitres of L-broth containing the appropriate antibiotic (appendix 1.2b) was inoculated with either a single colony from an agar plate or with 10µl of glycerol stock of the E.coli harbouring the plasmid of interest. This was grown overnight at 37°C with constant agitation. Five
millilitres of the overnight culture was used to inoculate 250ml fresh L-broth/antibiotics in a 1 litre conical flask, this was grown to stationary phase at 37°C with agitation for at least 20 hours. The bacteria were harvested by centrifugation at 6000g for 10 minutes at 4°C. The cell pellet was resuspended in 10ml freshly prepared solution I (25mM Tris.HCl pH 7.4, 10mM EDTA, 50mM glucose, 2mg/ml lysozyme), 40 and 30ml respectively of solutions II (0.1N NaOH, 1% SDS (w:v)) and III (5M sodium acetate pH 4.8) were then added at 5 minute intervals, after which the suspension was allowed to stand for a further 5 minutes prior to centrifugation at 3000g for 10 minutes. The nucleic acid was precipitated from the resulting supernatant by addition of 40ml propan-2-ol and incubation at room temperature for 10 minutes. The precipitate was recovered by centrifugation at 3000g for 20 minutes, the pellet was resuspended in 2ml TEN (10mM Tris.HCl pH 7, 1mM EDTA, 0.3M sodium acetate) and was extracted twice with phenol and once with chloroform. The nucleic acid was then reprecipitated by the addition of 2.5 volumes ethanol and incubation at -20°C for 30 minutes.

The precipitate was recovered as before and was resuspended in 1ml TE pH 7.4. An equal volume of freshly boiled ribonuclease-A (1mg/ml) was added and contaminating RNA was digested at 37°C for 30 minutes, the reaction was stopped by extractions with phenol, phenol:chloroform and chloroform. The DNA was precipitated with 1/10 volume 4M NaCl, 2.5 volumes ethanol and incubation at -20°C overnight. The plasmid DNA was recovered as before and finally resuspended in 100μl TE pH 7.5.

It was possible to perform a scaled down version of this method on 1.5ml of an overnight culture, in which case the ribonuclease-A digestion was usually omitted. If contaminating RNA obscured the presence of DNA on agarose gels, the RNA was removed by incubating the gels in 1μg/ml ribonuclease-A at 37°C for 30 minutes.
2.4 Construction of a Normal Colonic Mucosal cDNA Library.

2.41. Preparation of Double Stranded cDNA.

Double stranded cDNA was prepared by an adaptation of the method of Gübler & Hoffman (1983), using the Böehringer Mannheim cDNA synthesis kit (Böehringer Mannheim, Sussex.) precisely as outlined by the manufacturers.

First and second strand synthetic reactions were set up in duplicate, such that the first strand could be labelled with 5'\([\alpha-32P]\) dCTP in one reaction and the second strand in the other. Thus a total of 5µg LS poly-A mRNA was oligo(dT) primed and reverse transcribed to single stranded cDNA with 80 units AMV reverse transcriptase in two reactions of 20µl each. A 1µl sample of the labelled reaction was removed and diluted to 5µl with H₂O for analysis by TCA precipitation and agarose gel electrophoresis.

Components for the second strand reaction were added directly to the first strand mixture. The mRNA was digested with ribonuclease-H and the second cDNA strand synthesized by the action of E.coli DNA polymerase I. A 1µl sample was again removed and diluted from each sample for analysis as above.

The ends of the cDNA were "polished" with T4 polymerase to allow efficient ligation of EcoRI adaptors onto the cDNA termini.

2.41a. Estimation of cDNA Yield by TCA Precipitation.

The yield of cDNA synthesized was estimated by comparing the amount of TCA insoluble radionucleotide in the final reaction mix (FC) with the total amount of radionucleotide present (TC).

A microlitre of the diluted sample was pipetted onto a small piece of glass fibre filter paper (GF/C) and allowed to air dry. The Cerenkov radiation was measured directly using the ³H channel on the liquid scintillation counter (LKB Minibeta), to give the TC. The DNA was then precipitated by incubation in 5% TCA (w:v), 20mM sodium pyrophosphate on ice for 30 minutes, the filter was then dehydrated by a brief wash in methanol and allowed to air dry before being counted.
The following equation was used to calculate the yield of cDNA (ng):

\[
\frac{\text{FC} \cdot 4 \cdot 330 \cdot 20}{\text{TC}}
\]

Where FC and TC are the counts determined above, 4 represents the four nucleotides (dATP, dCTP, dGTP & dTTP), 330 is the average molecular weight of a nucleotide monophosphate (Daltons), 20 represents the number of nanomoles dCTP added to the reaction.

2.41b. Analysis of cDNA by Agarose Gel Electrophoresis.

Samples of the radiolabelled products of the synthetic reactions were visualised by autoradiography following agarose gel electrophoresis. Both neutral (non-denaturing) TAE and alkali (denaturing) gels were used, thus enabling an assessment of the quality of both duplex (neutral gels) and single cDNA strands (alkali gels) to be made.

Neutral TAE agarose (1.2% (w:v)) gel electrophoresis was as described (appendix 2.2a). For alkali gels 1.2% agarose (w:v) was melted in water and allowed to cool to approximately 55°C, a tenth volume 10X ALE (0.3M NaOH, 10mM EDTA) (MacDonell, et al, 1977) was added and the gel was cast. The samples were loaded onto the gel in 1X ALE, 0.8% Ficoll type 400(w:v), 0.01% bromocresol green (w:v). The electrophoresis running buffer was 1X ALE.

Following electrophoresis, the agarose gels were blotted dry onto DEAE ion exchange paper (DE81, Whatman) prior to analysis by autoradiography. The gel was placed onto Saran Wrap and a piece of DEAE paper was positioned on top of it. Blotting was facilitated with a stack of paper towels weighed down by a heavy weight. The blot was left for at least 3 hours, the towels were removed and the gel together with the DEAE paper were wrapped in Saran Wrap and autoradiographed.

2.42. Cloning cDNA into λgt10.

The Amersham cDNA cloning system was used to clone the cDNA into the EcoRI site of λgt10, and to in vitro package and plate recombinant phages onto the relevant bacterial strains (E.coli L87 & NM514). Various control DNAs were supplied with the kit to allow the success of each step in
the procedure to be assessed. The first control was HaeIII digested bacteriophage M13mp8 DNA, these blunt ended fragments were end labelled with \( [\alpha^{-32}\text{P}] \text{dCTP} \) as given in the manufacturers specifications and were processed alongside the cDNA throughout all subsequent manipulations.

2.42a. Addition of Oligonucleotide Adaptors.

EcoRI adaptors were ligated onto the blunt ends of the cDNA molecules overnight at 15°C with T4 DNA ligase. Adaptors were used in preference to EcoRI linkers, for they do not require EcoRI digestion and thus there was no necessity to protect the EcoRI sites in the cDNA with EcoRI methylase. In addition, the adaptors provided with the kit encoded the recognition site for BamHI, KpnI and NcoI restriction endonucleases, as shown below:

\[
\begin{array}{c}
\text{EcoRI} \\
5' \text{AATTCGAGGATCCGGTGACCATGG} 3' \\
3' \text{GCTCCTAGGCCCATGGTACC} 5' \\
\text{BamHI} \\
\text{KpnI} \\
\text{NcoI}
\end{array}
\]


Excess adaptors were separated from the cDNA by gel filtration through columns supplied with the kit, in addition to removing unligated adaptors, this step also size fractionated the cDNA. The sample - now in a volume of 20\( \mu \text{l} \) - was allowed to pass into the matrix of the column under gravity, and was eluted with TE pH 8. Fractions of 3 drops (\( \approx 120\mu \text{l} \)) were collected, and the level of radioactivity in each (cpm Cerenkov) was measured directly. Five microlitre samples were removed from successive fractions containing the highest levels of radioactivity, these were electrophoresed in TAE (appendix 2.2a) and blotted onto DEAE paper for autoradiography as before. This allowed the size distribution of the cDNA in each of these fractions to be assessed. The five fractions containing the largest cDNA molecules were pooled and processed further. For the control DNA five fractions - those containing the 2.53, 1.6, and 0.8kb fragments - were used.
2.42c. Kinasing Adapted cDNA.

The cohesive ends of the adaptor molecules were unphosphorylated to prevent self-ligation, therefore to allow ligation of the adapted cDNA to the dephosphorylated vector arms, phosphate groups were added to the 5' termini of the cDNA, by the action of T4 polynucleotide kinase. The reaction mix was stopped by extraction with a series of organic solvents, and the DNA was precipitated with sodium acetate and ethanol.

2.42d. Ligation of cDNA to λgt10 Vector Arms & In Vitro Packaging.

At this stage three additional controls were introduced into the ligation and subsequent packaging reactions (i) whole λgt10, to test overall packaging efficiency and host selection, (ii) phosphatased λgt10 arms ligated to one another, to determine the background of non-recombinant phage on each strain and (iii) λgt10 vector arms ligated to EcoRI ended control DNA, to check ligation and number of recombinants attainable. All ligations were performed in 10μl reaction volume, at 15°C overnight as specified in table 2.6.

Table 2.6.

<table>
<thead>
<tr>
<th>Sample No</th>
<th>Insert DNA</th>
<th>λgt10</th>
<th>λgt10 arms</th>
<th>L/K buffer</th>
<th>T4 DNA Ligase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>5μl(5μg)</td>
<td>-</td>
<td>1μl</td>
<td>2.5 units</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>2μl(1μg)</td>
<td>1μl</td>
<td>2.5 units</td>
</tr>
<tr>
<td>3</td>
<td>100ng EcoRI ended control</td>
<td>-</td>
<td>2μl(1μg)</td>
<td>1μl</td>
<td>2.5 units</td>
</tr>
<tr>
<td>4</td>
<td>50ng adapted blunt ended control</td>
<td>-</td>
<td>2μl(1μg)</td>
<td>1μl</td>
<td>2.5 units</td>
</tr>
<tr>
<td>5</td>
<td>25ng adapted cDNA</td>
<td>-</td>
<td>2μl(1μg)</td>
<td>1μl</td>
<td>2.5 units</td>
</tr>
<tr>
<td>6</td>
<td>50ng adapted cDNA</td>
<td>-</td>
<td>2μl(1μg)</td>
<td>1μl</td>
<td>2.5 units</td>
</tr>
</tbody>
</table>

Table 2.6. L/K: ligation/kinase buffer supplied with cloning kit.
After ligation the reactions were in vitro packaged with the aliquots of sonicated extract and freeze thawed lysate provided with the kit as specified. The phage suspensions were then infected at the suggested dilutions into the MgSO₄ treated host strains E.coli L87 and NM514 and were plated for incubation overnight at 37°C.

2.43. Amplification and Storage of λgt10 cDNA library.

All of the library was plated at a high titre onto E.coli NM514, as above. A confluent lawn of lysis resulted and each 90mm petri dish was overlayed with 5ml SM (appendix 1.2a). Phage particles were allowed to diffuse into the SM at 4°C on a rocking platform for at least 4 hours. The phage suspension was carefully collected and pooled. A cleared lysate was formed by the addition of several drops of chloroform and centrifugation at 4000g for 5 minutes at room temperature. The amplified library was divided into 1ml aliquots, a number of which were flash frozen in liquid nitrogen, following the addition of 70μl dimethylsulphoxide (DMSO), for long term storage at -70°C. Whilst a drop of chloroform was added to the rest for storage at 4°C. Dilutions of the amplified library were plated onto E.coli NM514 to establish the titre of viable phages.

2.5. Screening Bacteriophage Libraries.

Two bacteriophage libraries were used in this study: the LS normal colonic mucosal library (LSNCM) described above and a chromosome 5 specific genomic library. The chromosome 5 library (LAO5NS01) was purchased from the American Type Culture Collection (ATCC). This library was prepared from the complete EcoRI digestion of a human/hamster hybrid cell line (640-12), cloned into Charon 21A, with an average insert size of 4kb.

2.5.1. Plating a Bacteriophage Library onto L-Agar.

The cDNA library LSNCM was plated onto E.coli. NM514 cells as before, whilst the genomic library was plated onto E.coli. LE392, which were prepared as follows: 10ml L-broth (appendix 1.2a), 0.2% maltose (w:v) was inoculated with E.coli. LE392 and grown overnight at 37°C,
the cells were harvested and resuspended in 4ml cold 10mM MgSO₄.

For each library two 24.5cm square tissue culture tray dishes (Nunc. BRL) containing L-agar (appendix 1.2a) were used, ≈100,000 plaques could be plated onto each plate. An appropriate dilution of the phage suspension was incubated with 800µl plating cells and incubated at 37°C for 15 minutes, this was then mixed with 50ml molten top-agar (appendix 1.2a), and poured onto the plate, it was then inverted and incubated overnight at 37°C.

2.52. Transfer of Plaques to Nylon Hybridization Membrane.

The plates were cooled at 4°C for at least an hour prior to transfer to the nylon membranes (Hybond-N, Amersham International Plc). A piece of Hybond-N was placed carefully on top of the agar for 1 minute, during which time the membrane was pierced with a hypodermic needle for subsequent realignment, the Hybond-n was removed and placed plaque side up onto a piece of Whatman 3mm paper soaked in 1.5M NaCl, 0.5M NaOH. The plaque denaturation was allowed to proceed for 3 minutes, when the membrane was neutralized for 7 minutes by transfer to a piece of 3mm paper soaked in 0.5M Tris.HCl pH 7.2, 1.5M NaCl, 1mM EDTA. The membrane was then rinsed in 2xSSPE (appendix 1.3) and allowed to air dry. Finally the membrane was baked at 80°C for 2 hours and the DNA was cross linked by ultraviolet light for 3 minutes on a transilluminator. A second replica membrane of the plate was routinely taken as above, except that it was left in contact with the plaques for 90 seconds and the alignment marks of the first membrane were copied onto the second.


The membranes with plaques immobilised on them were processed as described in appendix 2.4b.

The chromosome 5 specific genomic library was probed with an aliquot of the cDNA library LSNCM which had been radiolabelled using the polymerase chain reaction (PCR). The reaction was as described in section 2.55c, except that 1µl of the amplified library (5x10¹¹ pfu/ml) was the template and 50µCi 5'[α-³²P]dCTP were added to the reaction during the
elongation phase of the twelfth cycle. After a total of 30 cycles the reaction was extracted with chloroform and applied to a G50 Sephadex spin column as described in appendix 2.4d.

2.54. Second and Third Round Screening.

After the first round of library screening had been performed, successive rounds were required in order to identify single positively hybridizing plaques. Thus, a hybridizing region present on the first round of screening, was physically removed as a 1cm diameter plug of agar from the bioassay tray. This plug was placed into 1ml SM (appendix 1.2a), to which a drop of chloroform had been added and the phage particles were allowed to diffuse from the agar for at least an hour at 4°C. The phage suspension was titrated to result in a dilution which gave approximately 300 plaques per 90mm petri dish. Sections 5.52 & 3 were then repeated and any positively hybridizing plaques were removed as plugs with the tip of a glass Pasteur pipette. These plugs were again suspended in 1ml SM buffer, plus chloroform, and the whole procedure was repeated once more, until individually hybridizing plaques could be reliably identified.

2.55. Analysis of Plaque Purified Bacteriophage Clones.
2.55a. Small Scale Isolation of Bacteriophage DNA.

Small scale DNA preparations were performed on phage suspensions prepared from plate lysates. A 90mm petri dish was plated with a dilution of a single plaque suspension to give a confluent lawn of plaques on the relevant plating cells. After incubation overnight at 37°C, the plate was overlayed with 4ml SM buffer as described in section 2.43 above. DNA was isolated from the resulting phage suspension precisely as outlined in the λgt10 cloning system booklet (Amersham International plc), using polyethyleneglycol-6000 (PEG)/NaCl precipitation and DEAE cellulose DE52 (Whatman). The final product of between 20 and 50µg was resuspended in 50µl TE pH 7.4 buffer.

2.55b. Medium Scale Isolation of Bacteriophage DNA.

A medium scale preparation was used to obtain a higher yield of a purer product. A 24.5cm tissue culture tray was plated to confluence with a dilution of a single plaque
suspension. After incubation overnight this was overlayed with 30 ml SM buffer to obtain a phage suspension as above. Chloroform lysed bacterial debris was removed from the suspension by centrifugation at 4000g for 10 minutes at 4°C. The phage particles were precipitated from the supernatant by the addition of 2.8 g PEG6000 and 1.2 g NaCl, and incubation at 4°C for at least 6 hours. The phage were then sedimented at 3000g for 10 minutes and the resulting pellet was resuspended in 10 ml TM (100 mM Tris·HCl pH 7.4, 50 mM MgCl₂). 7.5 g CsCl were added and the suspension was subjected to ultracentrifugation at 35000 rpm for 16 hours at 15°C. This resulted in opalescent band of phage particles, which was removed by puncturing the side of the centrifuge tube with a 21 gauge hypodermic needle attached to a syringe. Ultracentrifugation was repeated, following dilution of the phage suspension with TM, CsCl (0.75 g ml⁻¹). The opalescent band was again removed and transferred to dialysis tubing, for dialysis against 10 mM Tris·HCl pH 7.4, 10 mM MgCl₂ overnight at 4°C. The phage particles were lysed by the addition of EDTA to 10 mM and SDS to 0.1% (w:v) and the DNA was extracted twice with phenol and once with chloroform. Finally the DNA was removed as a precipitate following the addition of 2.5 volumes ice cold ethanol.

2.55c. Polymerase Chain Reaction Amplification of λgt10 Insert.

DNA cloned into the EcoRI site of λgt10 could be amplified directly from phage suspensions, with primers flanking the cloning site as shown below. Thus providing a pure source of insert DNA.

Forward: 5'-TGAGCAAGTTCAGCCTGGTTAAGTC-3'
Reverse: 5'-GGTGGCTTATGAGTATTTCTTCCAG-3'

A single plaque was picked from an agar plate into 1 ml SM with the tip of a glass Pasteur pipette. A drop of chloroform was added and the phage were allowed to diffuse for at least an hour at room temperature. 1 μl of the suspension was added to 26 μl H₂O in a 0.5 ml microcentrifuge tube and frozen at -70°C for 10 minutes, when the tube was plunged into a boiling water bath and boiled for 3 minutes.
The standard PCR mix (appendix 2.7) and 50pM of each of the above primers were added to the still hot phage suspension to bring the total volume of the reaction up to 100μl. The DNA was amplified after a 5 minute incubation at 95°C and addition of 1 unit Taq DNA polymerase (Promega) through 30 cycles as follows: 90°C for 30 seconds, 50°C for 30 seconds and 70°C for 2 minutes. The products of the reaction were analysed on 1.2% agarose (w:v) TAE gel (appendix 2.2a).

2.55d. Subcloning Bacteriophage Derived Inserts into Plasmid Vectors.

For ease of handling, inserts derived from phage clones were routinely subcloned into one of the pUC series of plasmid vectors (Vieira & Messing, 1982, Yanish-Perron, et al, 1985). Five nanogrammes of EcoRI digested pUC vector DNA were ligated to approximately 1μg EcoRI digested phage DNA in the presence of 50mM Tris.HCl pH 7.8, 10mM MgCl₂, 1mM ATP, 20mM dithiothreitol, 50μg/ml bovine serum albumin with 1 unit T4 DNA ligase (Boehringer Mannheim, Sussex) in a total volume of 10μl. The reaction was allowed to proceed overnight at 11°C followed by a 30 minute incubation at 37°C and heat inactivation at 68°C for 10 minutes. The whole ligation reaction was added to the competent cells for transformation as described in section 2.31b.

Recombinant colonies were identified by plating the transformation mix onto L-agar plates which had been overlayed with 3ml L-agar containing; 20μl isopropylthiogalactoside (IPTG)(1M), 40μl 5-bromo-4-chloro-3-indolyl-β-glucopyranoside (X-gal)(20mg/ml) and 3μl ampicillin (200mg/ml). Non-recombinants appeared as blue colonies, whilst recombinants were white. Small scale plasmid DNA preparations (section 2.32) were performed on a number of white colonies to check that they contained the insert of interest, once this had been established a large scale plasmid DNA preparation was under taken.

2.6. Analysis of Clones Isolated From the Chromosome Five Genomic Library.

2.61. Screening Genomic Clones for Hamster Sequences.

It was estimated that approximately 25% of the chromosome five library (LA05NS01) represented hamster
sequences, as it had been constructed from a human / hamster somatic hybrid cell line (ATCC product information). It was therefore important to screen any clones isolated from it for the presence of hamster sequences. This was done by digesting phage DNA with EcoRI and subjecting it to Southern blotting (section 2.13) and hybridization (section 2.14) with oligolabelled (appendix 2.4d) hamster total genomic DNA.

2.62. Restriction Mapping Genomic Clones.

Approximately 1µg of insert DNA isolated from the plasmid subclones (appendix 2.3) was digested with a variety of restriction endonucleases, in a total reaction volume of 40µl according to the manufacturers specifications. Single and double digests were performed and the products were electrophoresed through 1% agarose TAE gels (appendix 2.2a), in the presence of DNA molecular weight markers as described previously (section 2.13). After electrophoresis a permanent photographic record was made of the gel.

2.63. Identification of Single Copy Fragments in the Genomic Clones.

The gels obtained from restriction mapping (section 2.62) were alkali blotted onto Hybond-n+ nylon hybridization membrane (Amersham International Plc), according to manufacturers specifications. A total of three membrane replicas could be made of each gel, by blotting for 10, 20 and 60 minutes successively. One of the replicas was hybridized to oligolabelled total human genomic DNA (appendix 2.4d) as specified (appendix 2.4b). Single copy fragments were identified as those which did not hybridize to the total human genomic DNA. Once such fragments had been identified, restriction digests were set up, and the inserts were purified as in appendix 2.3.

2.64. Hybridization of Single Copy Fragments to Human/Rodent Somatic Cell Hybrid DNA.

A panel of well characterised human/rodent somatic cell hybrid DNAs (kindly provided by Dr Sue Povey), were screened with the single copy fragments isolated from the genomic clones, to check that these clones were in fact derived from human chromosome 5.
Ten microgrammes hybrid DNA was digested to completion with EcoRI as previously described and was fractionated by electrophoresis through a 1% agarose TAE gel (appendix 2.2a). EcoRI digested rodent and human DNAs were included as controls. The resulting gels were alkali blotted onto Hybond-N+ hybridization membrane (appendix 2.4a) and probed with oligolabelled single copy fragments as specified (appendix 2.4b).
CHAPTER 3

RESULTS.

3.1. Analysis of Genomic Sequences.

Genomic DNA isolated from tumour and normal colonic mucosal samples was screened for loss of heterozygosity, with restriction fragment length polymorphism (RFLP) detecting probes, from chromosomes 1, 3 and 7. The locus specific minisatellite DNA probes mapping to chromosomes 1, 5 and 7, available at the time were also used to detect allele loss and other genomic alterations. In addition, possible rearrangements at the RB-1 and \( trk \) loci, and allele loss and mutations at the MUC1 and MUC2 loci were investigated. In some instances the intensity of hybridization of an allele in a tumour sample appeared to be reduced, rather than lost completely. Such apparently normal hybridization may have been due to the presence of residual stromal tissue or infiltrating lymphocytes in the tumour. It is possible either to remove such normal tissue by dissection, or to assess the percentage of normal tissue microscopically and make the appropriate allowances. However, neither option was available in this study. Throughout the text and in tables and figures that follow, allele 1 will refer to the large allele and allele 2 to the small allele for a given polymorphism.

3.11. Alterations on Chromosome 1.

3.11a. \( p1-79, 1p36 \).

The probe \( p1-79 \) was the most distal tested on the short arm of chromosome 1. It gave a complex banding pattern on DNA digested with \( TaqI \) and also with \( PstI \) and \( PvuII \). Of all the samples tested (tables 3.1 & 3.2), allele loss was evident in the tumours of only three patients, these were the cancer cell line JW2 and sporadic cancers 260 and 823 (figure 3.1). In these cases a maximum of 2 bands were lost or showed reduced intensity of hybridization, from a total of at least 30. In an attempt to establish whether the loss of hybridization seen represented partial or complete loss of
Table 3.1

Genomic Alterations in the DNA of Sporadic Colorectal Tumours Detected with Indicated Probes.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Dukes Stage</th>
<th>p1-79 Reduced Int.</th>
<th>p36 Reduced Int.</th>
<th>p6C1-10 Reduced Int.</th>
<th>p24-22 Reduced Int.</th>
</tr>
</thead>
<tbody>
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<td>149</td>
<td>C1</td>
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<td>1.1</td>
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<tr>
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<td>1.1</td>
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<tr>
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</tbody>
</table>

Table 3.1. 1,2: heterozygote, 1,1 or 2,2: homozygote of indicated allele, 1 or 2: reduced intensity of indicated allele, 1 or 2: allele loss (indicated allele remaining), 2 or 4: presence of a new allele. NC: no change of hybridization pattern, red int: reduced intensity of some bands obtained with p1-79, 2 lost: 2 bands lost from the many obtained with p1-79, NT: not tested.
<table>
<thead>
<tr>
<th>Patient No</th>
<th>pl-79</th>
<th>pPGDH1</th>
<th>AF3</th>
<th>pMUC10</th>
<th>pHeA2</th>
<th>H3H2</th>
<th>pmetH</th>
<th>SMUC41</th>
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<td>NT</td>
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<td>1,2</td>
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</tr>
</tbody>
</table>

1,2: heterozygote, 1,1 or 2,2: homozygote of indicated allele, 1 or 2: reduced intensity of indicated allele, 1,1 or 2,2: homozygote of indicated allele, 1 or 2: reduced intensity of indicated allele, NC: no change of hybridization pattern, 2 lost: 2 bands lost from the many obtained with pl-79, NT: not tested.
Figure 3.1
Genomic Alterations Detected with p1-79.

Figure 3.1. p1-79 hybridized to Southern blots of tumour (T) and normal (N) genomic DNA (digested as indicated). Patient numbers are given. Somatic hybrids cell lines MOG2E5 and MOG2C2 were also used together with the mouse cell line RAG (see text). Allele losses indicated with arrows.
one p1-79 allele, two human/mouse somatic cell hybrid DNAs were tested (provided by Dr Sue Povey). The hybrid MOG2C2 contained two different copies of human chromosome 1, whilst MOG2E5, a derivative of MOG2C2, had lost one chromosome 1 and was thus homozygous for all loci on this chromosome. The DNA from the mouse parent cell line (RAG) was also tested to check for cross species hybridization. As with the cancer samples there were only 2 bands absent in MOG2E5 compared with the MOG2C2 DNA (no hybridization was evident to the mouse DNA) (figure 3.1). It seems therefore, that in the samples tested the difference between two haplotypes is just two bands, with the remaining bands being shared. Thus, in the samples where allele loss was apparent, it is likely that one entire p1.79 allele was lost. In addition to the TaqI digest, PstI and PvuII digested JW DNA was also hybridized to p1.79, two bands were missing from the cancer cell line in both digests. A PstI digest of 823 DNA revealed the absence of a single band in the cancer sample (figure 3.1).

Having found that the FAP derived cancer cell line JW2 had lost one p1.79 locus, four other FAP cancers were tested (DNA prepared by M. Rees). But no allele loss was evident in any of these samples on either TaqI or PstI digests.

3.11b. pPGDH1, 1p36.3-p36.13.

The cDNA for phosphoglucone dehydrogenase (PGD) detected a simple two allele RFLP in BamHI digested DNA. However, interpretation of results was slightly complicated by the comigration of allele one with a constant band at 11kb (figure 3.2a). Of the samples tested, 9 sporadic cancer patients and 4 FAP patients were heterozygous and therefore informative at this locus, but no allele loss or other genomic rearrangement was apparent in any of these samples (tables 3.1 & 3.2).

3.11c. AF3, 1p35-p34.

The PvuII RFLP detected by the 3' HincII fragment of the L-α-fucosidase cDNA (figure 3.2b), was heterozygous in 6 sporadic colorectal cancer patients and 5 FAP patients (tables 3.1 & 3.2). No loss of heterozygosity or genomic
alteration was detected with this probe in any of the samples.

Figure 3.2.

Restriction Fragment Length Polymorphisms Detected with pPGDH1 and AF3.

![Southern blots showing typical RFLP hybridization patterns obtained with the probes pPGDH1 & AF3](image)

- **a**: pPGDH1 hybridized to BamHI digested human genomic DNA, allele 1 (A1) 11kb, allele 2 (A2) 9.5kb.
- **b**: AF3 hybridized to PvuII digested human genomic DNA, allele 1 (A1) 7kb, allele 2 (A2) 6kb.

3.11d. pMUC10, 1q21-q24.

The mucin glycoproteins which have been examined so far, are characterized by the presence of a large domain composed of multiple repeats of a specific amino acid motif. In turn the DNA encoding these mucins also contain tandemly repeated elements, which in the case of MUC1 are 60bp long. The number of repeats present in different members of a population has been shown to be hypervariable with a heterozygososity frequency of 90% for MUC1 (Swallow, et al, 1987b). Thus, these probes are useful in allele loss studies, where they map to regions of interest. In addition, mucins have direct involvement in certain types of cancer including colorectal.

Of 20 sporadic colorectal cancer patients, 17 were constitutionally heterozygous at the MUC1 locus. In the equivalent cancer samples, alterations were apparent in 6 individuals (table 3.1). In patient 214 there was a reduction in the intensity of hybridization of the smaller allele in the Dukes' stage B cancer from the rectum, but the
alleles appeared normal in DNA from the stage A cancer which was from the recto-sigmoid colon (figure 3.3). This result was confirmed by densitomeric examination of the autoradiographs, with the ratio of the two alleles being 4.4 (allele 1 : allele 2) in the Dukes stage B cancer, compared with 2.2 in the normal mucosa and Dukes stage A cancer. Cancers 184, 185 and 260 remained heterozygous, but mutations occurred which resulted in an alteration to the allele sizes. In 184 the larger allele increased in size, whilst in 185 both the normal alleles were altered, with the large allele becoming smaller and the small allele becoming larger. In patient 260 not only was the large allele smaller, but the small allele was less intense in the cancer, with the ratio between the densitomeric measurements being 15.9 (allele 1 : allele 2) in the cancer, as opposed to 3.7 in the normal sample (figure 3.3). It is patient 193 however, where the most striking change occurred, with the appearance of a completely new band in addition to the normal alleles (figure 3.3). This finding was confirmed by hybridization of the pMUC10 probe to an EcoRI digestion of the 193 cancer DNA, which like the Hinfl digest revealed 3 bands (figure 3.3) (the allele losses and rearrangements could not be confirmed by EcoRI digestion of the other samples, as no more DNA was available for analysis). Finally the smaller allele was lost completely from the cancer of patient 315 (figure 3.3).

Nine of the FAP patients were heterozygous (table 3.2), but it was only in the cancer cell line JW2 where alteration at the MUC1 locus was apparent, with the intensity of the large allele being reduced in the cancer cell line compared to the normal fibroblast DNA (figure 3.3). Detection of reduced allele intensity rather than total allele loss was unexpected, as there is no contamination of the JW2 cell line with normal cells, and as such any alteration should be uniform. However, the observation was confirmed by densitometry, with the ratio of the readings changing from 0.74 (allele 2 : allele 1) in the normal cells to 2.1 in JW2.
Figure 3.3.
Genomic Alterations Detected with pMUC10.

Figure 3.3. pMUC10 hybridized to Southern blots of tumour (T) and normal (N) genomic DNA digested with HinfI (except where indicated otherwise). Patient numbers are as given. New alleles, altered size alleles and allele loss indicated with arrows. Molecular weights (kb) indicated.


Tables 3.1 and 3.2 shows the results obtained with the probe pHeA2. Four sporadic colorectal cancer patients and 5 FAP patients were informative at the erbA2 locus, but no loss or gain of alleles had occurred. Figure 3.4a shows the appearance of the alleles.

Figure 3.4

Restriction Fragment Length Polymorphisms Detected with pHeA2, H3H2 & pMetH.

<table>
<thead>
<tr>
<th>a</th>
<th>b</th>
<th>c</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHeA2</td>
<td>H3H2</td>
<td>pMetH</td>
</tr>
<tr>
<td>A1 A1 A2</td>
<td>A1 A1 A2</td>
<td>A1 A1 A2</td>
</tr>
<tr>
<td>A1 A2 A2</td>
<td>A2 A1 A2</td>
<td>A1 A2 A2</td>
</tr>
</tbody>
</table>

Figure 3.4. Southern blots showing typical RFLP hybridization patterns obtained with the probes pHeA2, H3H2 & pMetH. a: pHeA2 hybridized to BamHI digested human genomic DNA, allele 1 (A1) 5kb, allele 2 (A2) 2.5kb. b: H3H2 hybridized to HindIII digested human genomic DNA, allele 1 (A1) 2.3kb, allele 2 (A2) 2kb. c: pMetH hybridized to TaqI digested human genomic DNA, allele 1 (A1) 7.5kb, allele 2 (A2) 4kb.


Ten sporadic colorectal cancer patients were tested at this locus, of which 4 were constitutionally heterozygous. Six of the 11 FAP patients tested were heterozygous (tables 3.1 & 3.2). No allele loss or other rearrangement was evident in any of the samples, typical alleles are shown in figure 3.4b.

3.13a. pmetH, 7q31.

Nine sporadic colorectal cancer patients were tested with the met probe, of which 4 were informative, 6 FAP patients were heterozygous but no allele loss had occurred in any of the samples tested (tables 3.1 & 3.2). Figure 3.4c shows the appearance of alleles A1 and A2.

3.13b. ÀMS31, 7p22-pter & pÀg3, 7q36-qter.

Results obtained with these two hypervariable probes which map to chromosome 7 are given in section 3.15d & 3.15e respectively.


3.14a. trk, 1q32-q41.

When this project was initiated the location of the trk oncogene was unknown. Subsequently however, it was mapped to the long arm of human chromosome 1, using somatic cell hybrids and in situ hybridization (Miozzo, et al, 1990). The trk proto-oncogene was first described as a rearrangement in a colon carcinoma, which resulted in a novel 7kb band of hybridization in BamHI digested DNA (Martin-Zanca, et al, 1986). In all the samples tested here, namely: 21 sporadic colorectal cancers, 24 adenomas from 10 FAP patients and 7 colorectal carcinoma cell lines, only the normal BamHI hybridizing pattern was evident (figure 3.5a).


The normal restriction pattern detected by the PG3.8M EcoRI subclone of the retinoblastoma cDNA is illustrated in figure 3.5b. This pattern was not altered in the DNA of 21 sporadic colorectal cancers, 23 adenomas from 10 FAP patients, 2 FAP colorectal cancers and 7 colorectal carcinoma cell lines. However, in the sporadic colorectal cancer of patient 823, where karyotypic examination had revealed trisomy of chromosome 13q (J.D.A. Delhanty, personal communication), densitomeric comparison of the hybridization pattern for the tumour and normal DNA samples, revealed an overall increase in the intensity of hybridization in the tumour DNA by a factor of 1.6, this roughly equates the expected ratio for 2
Figure 3.5

Restriction Fragments Detected with trk and RB-1 (PG3.8M).

a.

150  151  177  183
T  N  T  N  T  N  T  N

Figure 3.5. Southern blots showing typical hybridization patterns obtained with the probes TRK and RB-1 (PG3.8M). a: trk hybridized to BamHI digested genomic DNA, normal sized fragments 14kb and 2.7kb. b: PG3.8M hybridized to HindIII digested genomic DNA, normal sized fragments 9.8, 7.5, 6.2, 5.3. Cell line and patient numbers are as given. The right hand panel of patients 823 & 149 demonstrates equal loading of tumour and normal DNA in the ethidium bromide stained gel tracks.
copies of chromosome 13 in the normal sample and 3 in the tumour (figure 3.5b). A similar increase in hybridization intensity would also appear to have occurred in the tumour DNA of patient 149 (figure 3.5b).

3.14c. SMUC41 (MUC2), 11p15.5.

In common with the probe pMUClO, the cDNA SMUC41, which encodes a mucin of the small intestine, detects a VNTR of 69bp in human DNA (Gum, et al, 1989). Although this probe has been mapped to chromosome 11p15.5 (locus MUC2)(Griffiths, et al, 1990), its significance in this study lies more in the association of the mucins with pre- and cancerous conditions, than with its chromosomal localization, as this region of chromosome 11 does not appear to undergo significant rearrangement in colorectal cancer (Vogelstein, et al, 1989).

Figure 3.6.

Genomic Alterations Detected in Sporadic Colorectal Cancers with SMUC41.

The frequency of heterozygosity at the MUC2 locus is 50% (D. Swallow, personal communication), in agreement with this 10 of the 20 sporadic colorectal cancer patients tested here were heterozygous (table 3.1). Of those which were informative two (195 and 315) had lost an allele in the cancer DNA (figure 3.6). Seven FAP patients were informative but in a total of 27 adenomas no allele loss was observed, this was true also for the two informative FAP patients with colorectal cancer (table 3.2).
3.15. Rearrangements Detected with Locus Specific Minisatellite Probes.

3.15a λMS1, 1p33-p35.

All the sporadic colorectal cancer patients who were constitutionally heterozygous at the λMS1 locus (21/22), retained both alleles in the tumour DNA (table 3.3). No allele loss was apparent in the adenomas from any of the FAP patients (table 3.4). The only allele loss which was detected with this probe was in the FAP cancer cell line JW2, where the larger of the two alleles present in the normal fibroblast DNA, was absent in the tumour cell line (table 3.4, figure 3.7a).

3.15b λMS32, 1q42-q43.

Table 3.3 shows that 19 of the sporadic colorectal cancer patients were informative with λMS32. Rearrangements were apparent in two patients. In patient 185, who was constitutionally homozygous, two bands were detected in the cancer specimen, neither of which was the same size as the normal allele (table 3.3, figure 3.7b). A size alteration was also evident in the DNA of patient 195, where the larger of the two alleles detected in the normal sample, was reduced in size in the tumour DNA. The alterations observed in AluI digested DNA from patients 185 & 195, were also evident when λMS32 was hybridized to TagI digests of the same DNA (figure 3.7c).

The λMS32 locus was apparently duplicated in the sporadic colorectal cancer 823 and the HNPCC derived cancer cell line LIM1215 (table 3.3, figure 3.7b). In both cases the intensity of hybridization of one allele, measured by densitometry, was approximately twice that of the unaffected allele. These results were in agreement with cytogenetic observations for these samples. A short term culture of the carcinoma from patient 823, revealed that an isochromosome 1q, was one of various karyotypic abnormalities present (J.D.A. Delhanty, personal communication). In the cell line LIM1215, the duplication and subsequent translocation of 1q32-qter to 13p, was demonstrated by fluorescent in situ hybridization (Griffin et al, 1990), this was thought to be the sole karyotypic alteration in the cell line LIM1215. All
Table 3.3. Alterations in Sporadic Colorectal Cancer Detected with Locus Specific Minisatellite Probes.

<table>
<thead>
<tr>
<th>Patient No</th>
<th>λMS1 1p33-p35</th>
<th>λMS2 1q42-q43</th>
<th>λMS8 5q35-qter</th>
<th>λMS31 7p22-pter</th>
<th>pλg3 7q36-qter</th>
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<tbody>
<tr>
<td>142</td>
<td>1,2</td>
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<tr>
<td>150</td>
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<td>151</td>
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<td>1,1</td>
<td>1,2</td>
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<tr>
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<td>1,2</td>
<td>1,1</td>
<td>1,2</td>
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<td>1,1</td>
<td>1,2</td>
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<td>200</td>
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<tr>
<td>214</td>
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<tr>
<td>219</td>
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<td>2</td>
<td>1,2</td>
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<tr>
<td>225</td>
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<tr>
<td>254</td>
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<td>1,2</td>
<td>1,2</td>
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<tr>
<td>260</td>
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<tr>
<td>311</td>
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<td>1,2</td>
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<tr>
<td>315</td>
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<td>1,2</td>
<td>1,2</td>
<td>1,2</td>
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</tr>
<tr>
<td>823</td>
<td>1,2</td>
<td>1,2</td>
<td>2</td>
<td>1,1</td>
<td>1,2</td>
</tr>
</tbody>
</table>

Table 3.3. 1,2: constitutional heterozygote; 1 or 2: reduced intensity of indicated allele; 1 or 2: allele loss (indicated allele remaining); 1,1: increased intensity of allele, even though the normal was homozygous; 3, 3 or 6: presence of a new allele.
Table 3.4.

Alterations in Familial Adenomatous Polyposis Adenomas and Cancers Detected with Locus Specific Minisatellite Probes.

<table>
<thead>
<tr>
<th>Patient No</th>
<th>( \lambda M S 1 ) lp33-p35</th>
<th>( \lambda M S 2 ) lg42-q43</th>
<th>( \lambda M S 8 ) 5q35-gter</th>
<th>( \lambda M S 1 ) 7p22-pter</th>
<th>p( \lambda g 3 ) 7q36-gter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenomas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAP29</td>
<td>1,1</td>
<td>1,2</td>
<td>1,2</td>
<td>1,1</td>
<td>1,2</td>
</tr>
<tr>
<td>FAP30</td>
<td>1,2</td>
<td>1,2</td>
<td>1,1</td>
<td>1,2</td>
<td>1,2</td>
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<tr>
<td>FAP46</td>
<td>1,2</td>
<td>1,2</td>
<td>1,2</td>
<td>1,2</td>
<td>1,2</td>
</tr>
<tr>
<td>FAP57</td>
<td>1,2</td>
<td>1,2</td>
<td>1,2,(1)(2)</td>
<td>1,2</td>
<td>1,2</td>
</tr>
<tr>
<td>FAP70</td>
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<td>1,2,(3)</td>
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<td>1,2</td>
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<td>FAP83</td>
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<td>1,2</td>
<td>1,1</td>
<td>1,2</td>
<td>1,2</td>
</tr>
<tr>
<td>FAP84</td>
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<tr>
<td>FAP99</td>
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<tr>
<td>Cancers</td>
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</tr>
<tr>
<td>Sporadic Adenoma</td>
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<td></td>
<td></td>
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<td>1,2</td>
<td>1,2</td>
<td>1,1</td>
<td>1,2</td>
</tr>
</tbody>
</table>

Table 3.4. 1,2: constitutional heterozygote; 1 or 2: reduced intensity of indicated allele, 1 or 2: allele loss, indicated allele remaining; 3 or 6: presence of a new allele; ( ) indicates alteration in one of a number of adenomas.
Figure 3.7.

Genomic Alterations Detected with λMS1 & λMS32.

Figure 3.7. Southern blots of tumour (T) and normal (N) genomic DNA. Patient numbers are as given. For FAP70, T1 denotes the sessile vilous adenoma, whilst T2 denotes one of the remaining four adenomas tested. New alleles, altered size alleles and allele loss indicated with arrows. 

a: λMS1 hybridized to HindIII digestions, 
b: λMS32 hybridized to AluI digestions, 
c: λMS32 hybridized to TaqI digestions.
of the FAP patients tested with \( \lambda \)MS32 were heterozygous, in two of these alterations had occurred. In FAP70 five adenomas were tested, all of which remained heterozygous. However, there was a size change in the DNA derived from a sessile villous adenoma, resulting in enlargement of the larger allele (table 3.4, figure 3.7b & c). The other alteration was a complete allele loss in the cancer cell line JW2 (table 3.4, figure 3.7b).

3.15c. \( \lambda \)MS8, 5q35-qter.

The genetic alterations detected with \( \lambda \)MS8 were simple allele losses no other rearrangements were evident. Thus, allele loss was found in the tumours of 4 out of the 16 informative sporadic colorectal cancers (200, 219, 315, 823) (table 3.3, figure 3.8), and in 2 adenomas from 1 of the 10 informative FAP patients. In FAP57 results were obtained from three adenomas, it was in the two adenomas from the descending colon that allele loss had occurred. Complete loss was evident in one adenoma and partial loss in the other (table 3.4, figure 3.8) (studies on \( \lambda \)MS8 were performed in collaboration with M.Rees).

![Figure 3.8. Genomic Alterations Detected with \( \lambda \)MS8.](image)

*Figure 3.8. \( \lambda \)MS8 hybridized to Southern blots of tumour (T) and normal (N) genomic DNA digested with Hinfl. Patient numbers are as given. Allele loss, reduced and increased allele intensity are indicated with arrows.*
3.15d λMS31, 7p22-pter.

Alterations at the locus detected by λMS31 were present in four sporadic colorectal cancer samples (tables 3.3 & 3.4). In sample 151, although homozygous, the intensity of hybridization was greater in the tumour specimen than in the normal (figure 3.9a). Equal loading of the two samples was confirmed by densitomeric examination of a photograph of the ethidium bromide stained agarose gel. A single extra band was present in the cancer DNA of patient 311, which was larger than the two normal alleles (figure 3.9a). In cancer 260 two new bands had appeared, which were smaller than those present in the normal DNA (figure 3.9a). Furthermore, the intensity of the largest allele in 260 tumour DNA was reduced, it is therefore possible, that the two smaller bands were restriction fragments of the largest normal allele, as their combined mass equals that of this allele. It was only in the tumour DNA of patient 200 where any allele loss at the λMS31 locus was evident, here the intensity of the large allele was considerably reduced (figure 3.9a). The λMS31 locus was not altered in the DNA of the 13 FAP patients tested (table 3.4).

3.15e. pλg3, 7q36-pter.

Of all the patients tested, three demonstrated alterations in the DNA of their tumour samples at the pλg3 locus (tables 3.3 & 3.4). In two cases (sporadic cancers 200 & 823) the intensity of one allele was reduced, in 200 it was the larger allele and in 823 it was the smaller (figure 3.9b). Conversely, the intensity of hybridization to pλg3 was increased in the cancer DNA of patient 151. Equal loading of tumour and normal DNA was confirmed by densitometry, in the same way as described in section 3.15d for this same patient. No alterations were detected in the DNA of the 13 FAP patients tested at this locus (table 3.4).
Figure 3.9. Southern blots of tumour (T) and normal (N) genomic DNA digested with \textit{Hinfl}. Patient numbers are as given. New alleles, altered size alleles, allele loss and reduced allele intensity indicated with arrows. The right hand panels for patient 151, demonstrate equal loading of tumour and normal DNA in the ethidium bromide stained gel tracks. \textbf{a}: Southern blots hybridized to \textit{\lambda MS31}, \textbf{b}: Southern blots hybridized to p\textit{Ag3}.

Tables 3.5 and 3.6 summarize the results obtained with the various probes used to detect genomic rearrangements in the colorectal DNA samples listed. With the exception of the retinoblastoma probe, genomic alterations were only detected with the hypervariable probes. As can be seen in tables 3.1 and 3.2 these probes were significantly more informative than the standard RFLP detecting probes, hence, increasing the chances of detecting any alterations present in the tumour samples. By their nature the hypervariable probes have a relatively high rate of germline and somatic mutation (Jeffreys, et al, 1988), with somatic mutations being more prevalent in gastrointestinal neoplasms than in other cancers (Thein, et al, 1987). Such mutations result in the appearance of novel hybridizing bands, and may arise as a result of a variety of mechanisms during mitosis (Jeffreys, et al, 1988). In this study, mutations resulting in the presence of novel bands (n=9), were less frequent than those resulting in the loss of genetic material (n=20). New alleles were detected with the three probes pMUC10, \( \lambda MS32 \) and \( \lambda MS31 \), whilst partial or complete allele losses were apparent at the p1.79, \( pMUC10, \lambda MS1, \lambda MS32, \lambda MS8, \lambda MS31, p\lambda g3 \) and SMUC41 loci. A high frequency of germline mutation has been reported at the MUC1 locus, in DNA from the CEPH families (Centre d'Etude du Polymorphisme Humaine) (D.M. Swallow, personal communication). It would appear from the present study that somatic mutations also occur frequently at this locus. It was surprising that novel alleles were not detected with \( \lambda MS1 \) in this study, as Armour et al (1989) reported a higher incidence of such somatic mutations with this probe in gastrointestinal cancers, than with \( \lambda MS32 \). Conversely, they failed to detect somatic mutations with \( \lambda MS31 \), whilst two of the four alterations detected using \( \lambda MS31 \) in this study revealed new bands. It would appear therefore, that such results are very sample dependent.

The accumulation of genomic changes on a particular chromosome can be seen in table 3.6. For example all the informative probes on chromosome 1 have shown partial or complete allele loss in the FAP cancer cell line JW2. Similarly, both minisatellite probes on chromosome 7 show a
Table 3.5. Changes indicated represent the total number of alterations at a given locus irrespective of the type of change detected, (): that the change occurred in one or more of the adenomas tested, but not in all of them, *: duplication of chromosomes 1 or 13, first identified karyotypically and subsequently confirmed by Southern blot analysis.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sporadic colorectal cancers</th>
<th>FAP adenomas</th>
<th>FAP cancers</th>
<th>Carcinoma cell lines</th>
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<tr>
<td>Chromosome 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p1.79 (1p36.3)</td>
<td>2/22 9%</td>
<td>0/10</td>
<td>1/3 33%</td>
<td>-</td>
</tr>
<tr>
<td>λMS1 (1p35-p33)</td>
<td>0/21</td>
<td>0/8</td>
<td>1/3 33%</td>
<td>-</td>
</tr>
<tr>
<td>pPGD (1p36)</td>
<td>0/10</td>
<td>0/3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AF3 (1p35-p34)</td>
<td>0/6</td>
<td>0/5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pMUC10 (1q21-q23)</td>
<td>6/19 32%</td>
<td>0/7</td>
<td>1/1 100%</td>
<td>-</td>
</tr>
<tr>
<td>TRK (1q23-31)</td>
<td>0/21</td>
<td>0/10</td>
<td>0/2</td>
<td>0/6</td>
</tr>
<tr>
<td>λMS32 (1q42-q43)</td>
<td>3/20 15%</td>
<td>(1)/10 10%</td>
<td>1/3 33%</td>
<td>1*/6 17%</td>
</tr>
<tr>
<td>Chromosome 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pHeA2 (3p22-p24)</td>
<td>0/5</td>
<td>0/4</td>
<td>0/1</td>
<td>-</td>
</tr>
<tr>
<td>H3H2 (3p21)</td>
<td>0/4</td>
<td>0/6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chromosome 5</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>λMS8 (5q35-qter)</td>
<td>4/16 31%</td>
<td>(1)/7 14%</td>
<td>0/2</td>
<td>-</td>
</tr>
<tr>
<td>Chromosome 7</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>λMS31 (7pter-p22)</td>
<td>4/18 22%</td>
<td>0/9</td>
<td>0/3</td>
<td>-</td>
</tr>
<tr>
<td>pmetH (7q31)</td>
<td>0/4</td>
<td>0/5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pλg3 (7q36-qter)</td>
<td>3/20 15%</td>
<td>0/9</td>
<td>0/2</td>
<td>-</td>
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<tr>
<td>Chromosome 11</td>
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<td></td>
</tr>
<tr>
<td>SMUC41 (11p15)</td>
<td>2/11 18%</td>
<td>0/6</td>
<td>0/2</td>
<td>-</td>
</tr>
<tr>
<td>Chromosome 13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PG3.8M (13q14)</td>
<td>2*/21 9.5%</td>
<td>0/23</td>
<td>0/2</td>
<td>0/6</td>
</tr>
</tbody>
</table>
Table 3.6.

**Summary of Rearrangements in Samples With One or More Alteration.**

<table>
<thead>
<tr>
<th>Patient No</th>
<th>Dukes Stage</th>
<th>Probes Detecting Alterations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sporadic cancers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>149</td>
<td>C1</td>
<td>PG3.8M(13q) increased copy number at both loci</td>
</tr>
<tr>
<td>151</td>
<td>B</td>
<td>λMS31(7p), pλg3(7q) increased copy number at both loci</td>
</tr>
<tr>
<td>184</td>
<td>C1</td>
<td>pMUC10(1q) novel allele</td>
</tr>
<tr>
<td>185</td>
<td>C1</td>
<td>pMUC10(1q), λMS32(1q) novel alleles at both loci</td>
</tr>
<tr>
<td>193</td>
<td>C1</td>
<td>pMUC10(1q) novel allele</td>
</tr>
<tr>
<td>195</td>
<td>C1</td>
<td>λMS32(1q) novel alleles, SMUC41(11p) allele loss</td>
</tr>
<tr>
<td>200</td>
<td>B</td>
<td>λMS8(5q), λMS31(7p), pλg3(7q) allele loss at each locus</td>
</tr>
<tr>
<td>214</td>
<td>B</td>
<td>pMUC10(1q) allele loss</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>No alteration</td>
</tr>
<tr>
<td>219</td>
<td>C1</td>
<td>λMS8(5q) allele loss</td>
</tr>
<tr>
<td>260</td>
<td>A</td>
<td>p1.79(1p) allele loss, pMUC10(1q) novel allele, λMS31(7p) allele loss, plus novel alleles</td>
</tr>
<tr>
<td>311</td>
<td>B</td>
<td>λMS31(7p) novel allele</td>
</tr>
<tr>
<td>315</td>
<td>C2</td>
<td>pMUC10(1q), λMS8(5q), SMUC41(11p) allele loss at each locus</td>
</tr>
<tr>
<td>823</td>
<td>C</td>
<td>p1.79(1p), λMS8(5q), pλg3(7q) allele loss at each locus. λMS32(1q), PG3.8M(13q) increased copy number at both loci</td>
</tr>
</tbody>
</table>

FAP Adenomas

**FAP57**

λMS8(5q) in 2/5 adenomas allele loss

**FAP70**

λMS32(1q) in 1/5 adenomas novel allele

FAP Cancer

**JW2**

p1.79(1p), λMS1(1p), pMUC10(1q), λMS32(1q) allele loss at each locus

Cancer Cell Line

**LIM1215**

λMS32(1q) increased copy number

*Table 3.6.* Allele loss refers both to reduced intensity of hybridization and total allele loss.
reduced copy number in the tumour of patient 200 and an increased copy number in the tumour of patient 151. Furthermore, the cancer from patient 185 revealed the presence of novel alleles with both of the probes from chromosome 1q. Few alterations were detected in the FAP adenoma samples, with only a loss of λMS8 alleles in two adenomas from patient FAP57, and a mutation at the λMS32 locus in a single polyp of FAP70 being observed. The allele loss on chromosome 5q was of note because it has occurred independently in two samples from the same patient, whilst the novel allele at λMS32 was consistent with the reported mutations at this locus in gastrointestinal epithelia (Armour, et al, 1989).

The progressive nature of colorectal carcinogenesis was epitomized by the observation that 61% of the tumours in which genomic alterations were detected, were classified as Dukes' stage C (table 3.6).

3.2. Analysis of Expressed Sequences.

Total cellular RNA was isolated from a panel of tumour and normal mucosal samples, derived from the rectum and sigmoid colon of patients with sporadic colorectal cancer, and from a series of adenomas and carcinomas from nine FAP patients. Dot and slot blots of the RNAs were prepared, and probed with a variety of DNA probes, to compare the levels of expression in tumour and normal RNA samples. This approach allowed the rapid and systematic analysis of large numbers of samples with the probes of interest.


Wherever possible samples were loaded onto dot blots in duplicate. After initial hybridization of the filters to the probe of interest, residual probe was removed and the filters were hybridized to a β-actin probe. This ubiquitously expressed gene allows an assessment of the amount of RNA present to be made. The intensity of the hybridization signals on autoradiographs from each probing were measured using scanning densitometry, and the following equations were used to determine whether expression of the test probe was elevated or reduced in the tumour samples compared with the normals.
\[ AN = AF \]
\[ AT \]
\[ PT \times AF = PTA \]
\[ PTA \times 100 = \text{Expression (\%)} \]
\[ PN \]

Where: \( AN \): actin reading of normal sample, \( AT \): actin reading of tumour sample, \( AF \): adjustment factor, \( PT \): probe reading of tumour sample, \( PTA \): probe reading of tumour sample adjusted, \( PN \): probe reading of normal sample. \text{Expression}: percentage expression of probed sequence in tumour sample compared with normal (expression in normal regarded as 100%).

The expression of a given probe in the normal mucosal sample was regarded as 100\% in the tables which follow. The levels of probe expression in tumour samples above 110\% were recorded as positive (+), whilst levels of expression below 90\% were recorded as negative (-). Representative histograms have been constructed for each probe with each series of samples. In the histograms the level of expression in the normal samples has been adjusted to a base line of zero, such that levels of probe expression in the tumour samples above normal have positive values and those below normal are negative.

In some instances a normal sample was not available from a given individual, in which case the tumour signal was compared with the normal signal from an equivalent patient. The densitometer was very sensitive and any blemish on the autoradiograph was faithfully recorded, consequently some genuine readings were obscured, in such instances an estimation was made by eye of any changes in expression levels (as indicated on tables 3.7 - 3.11). On occasion probe removal from dot and slot blots, followed by rehybridization to \( \beta \)-actin gave unsatisfactory results. In such cases new blots were prepared of the relevant samples and they were hybridized directly to \( \beta \)-actin.

3.21a. \( trk \) Proto-Oncogene (pDM17).

Expression of the \( trk \) proto-oncogene is normally restricted to certain ganglia of the peripheral nervous system (Martin-Zanca, \textit{et al}, 1990). Tables 3.7 & 3.8, and figure 3.10 show the results obtained with the \( trk \) cDNA probe. It is likely that there is very little or no expression of \( trk \) in these samples, unfortunately no neuronal
RNA was available for use as a positive control. The weak hybridization signals obtained may therefore be due to non-specific background hybridization. From the densitomeric measurements three rectal cancer samples (616, 717 & 777) appeared to be expressing greatly elevated levels of trk, examination of figure 3.10 and comparison with the actin hybridization would perhaps suggest a slight increase in the expression level, but not to the extent indicated by densitometry. As the hybridization signals were relatively weak, the sensitivity of the densitometer had to be increased, so amplifying any slight differences between samples. In addition, overlying background hybridization appears to have intensified the signal from the RNA samples. Both explanations could have contributed to the discrepancy in the results. The expression of trk in the adenomas of patients FAP136 and 139 was reduced compared with the normal, but in common with the sporadic samples trk expression in the normal mucosa was only detectable after a long exposure of the autoradiograph.

3.21b. met Proto-Oncogene (pmeth).

The met proto-oncogene was originally isolated from an osteosarcoma-derived cell line (HOS), treated with the chemical carcinogen MNNG (N-methyl-N'-nitro-N-nitrosoguanidine) (Cooper, et al, 1984). Although expression of met has not been characterised in colorectal mucosa, it was detected in the gastric tumour cell line GTL-16 (Giordano, et al, 1989). From the dot blot experiments it would appear that met was expressed in colorectal mucosa. Table 3.7 shows that the majority of colorectal cancers tested here revealed elevated levels of met expression in comparison with equivalent normal mucosal RNA (12/22). Cancers 616, 643, 570, 575 and 757 demonstrate this increase most clearly (figure 3.11). Five cancers showed a slight reduction in the level of expression, whilst the cancer of patient 800 showed a marked drop (figure 3.11). Met was expressed at a level greater than the equivalent normal mucosa in all the adenomas of patients FAP136 and 139, with adenomas 1, 4, 8 and 9 of FAP136 and adenomas 4 and 6 of FAP139 showing the highest levels of met expression (table 3.8, figure 3.11).
Table 3.7.

Expression of Various Genes in Sporadic Colorectal Cancers.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Site</th>
<th>Dukes Stage</th>
<th>trk 1q32-g41</th>
<th>met 7q31</th>
<th>c-myc 8q24</th>
<th>RB 13q14</th>
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<tr>
<td>596</td>
<td>R</td>
<td>C1</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
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<td>NT</td>
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<td>C2</td>
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</tr>
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<td>++</td>
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<td>=</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td>757</td>
<td>SC</td>
<td>B</td>
<td>+ (E)</td>
<td>+++</td>
<td>+</td>
<td>=</td>
</tr>
<tr>
<td>854</td>
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<td>NK</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>NT</td>
</tr>
</tbody>
</table>

Table 3.7. NT = not tested, +, ++, +++ & ++++ represent expression elevated between 11-50%, 51-100%, 101-150% & 151% and over respectively above normal, = represents expression within 10% above and below normal, - & -- represent expression 11-50% & 51-100% and lower respectively below normal, (E) indicates reading estimated.
Table 3.8.

Expression of Various Genes in Familial Adenomatous Polyposis Samples.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Dysplasia</th>
<th>trk 1q32-q41</th>
<th>met 7q31</th>
<th>c-myc 8q24</th>
<th>RB 13q14</th>
<th>MUC1 1q21</th>
<th>MUC2 11p15</th>
</tr>
</thead>
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<tr>
<td>FAP136</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>adenoma 1</td>
<td>Mild</td>
<td>-</td>
<td>+++</td>
<td>= (E)</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>2</td>
<td>Moderate</td>
<td>= (E)</td>
<td>+</td>
<td>=</td>
<td>+++</td>
<td>=</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Mild</td>
<td>-</td>
<td>++</td>
<td>=</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>4</td>
<td>Mild/Mod.</td>
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<td>+++</td>
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<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Moderate</td>
<td>=</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Moderate</td>
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<td>=</td>
<td>+++</td>
<td>+++</td>
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<td>=</td>
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<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>Mild</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
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<td>++</td>
<td>= (E)</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
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<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
</table>

FAP139

| adenoma 1   | Mild      | = (E)        | +      | -        | ++      | +++     | +       |
| 2           | Mild      | =            | +      | +        | +       | -       | -       |
| 3           | Mild      | -            | +      | ++       | ++      | =       | +       |
| 4           | Mild      | -            | ++     | +        | +++     | +++     | -       |
| 5           | Mild      | --           | +      | =        | ++      | +       | -       |
| 6           | Mild      | -            | +++    | =        | +++     | +++     | -       |
| 7           | Mild      | -            | ++     | = (E)    | ++      | +       | -       |
| 8           | Mild/Mod. | =            | +      | =        | (E)    | =       | -       |
| 9           | Mild      | -            | ++     | +        | +       | ++      | -       |
| 10          | Mild/Mod. | -            | ++     | +        | +       | +++     | -       |
| 11          | Mild      | = (E)        | ++     | +        | +       | +++     | +       |

Table 3.8. NT = not tested, +, ++, +++ & +++ represent expression elevated between 11-50%, 51-100%, 101-150% & 151% and over respectively above normal, = represents expression within 10% above and below normal, - & -- represent expression 11-50% & 51-100% and lower respectively below normal, (E) indicates reading estimated.
Expression of trk Proto-oncogene in Colorectal RNA.

**Figure 3.10.**

*a:* Histograms showing levels of trk expression in sporadic cancers and FAP adenomas (patient and sample numbers indicated).  
*b:* dot blots hybridized to trk, left panel sporadic cancers, right panel FAP adenomas.  
*c:* dot blots shown in b, hybridized to β-actin.  
*d:* key to sample loading on dot blots.
Expression of *met* Proto-oncogene in Colorectal RNA.

Figure 3.11. a: Histograms showing levels of *met* expression in sporadic cancers and FAP adenomas (patient and sample numbers indicated). b: dot blots hybridized to *met*, left panel sporadic cancers, right panel FAP adenomas. c: dot blots shown in b, hybridized to \( \beta \)-actin. d: key to sample loading on dot blots.
3.21c. c-myc Proto-Oncogene (pUCCDIA).

Over expression of c-myc has been well characterised in both colorectal cancers and adenomas (Sikora, et al, 1987, Heerdt et al, 1991). In this study 9/17 cancers showed elevated c-myc expression, although the level of increased expression was not very high in any of these cases (table 3.7, figure 3.12). Five of the adenomas from FAP136 showed increased expression of c-myc to a greater or lesser extent, as did six of the 11 adenomas from FAP139. It may be of interest to note that of the five FAP136 adenomas showing signs of moderate cellular dysplasia, four revealed elevated c-myc expression, whilst only two of the five with mild dysplasia showed such elevated levels (table 3.8). One of the adenomas from FAP139 appeared to be expressing less c-myc than the normal mucosal sample (from FAP136) (table 3.8, figure 3.12). Reduced expression was also observed in four sporadic colorectal cancers (table 3.7, figure 3.12).

3.21d Retinoblastoma Susceptibility Gene (Rb) (pG3.8M).

Aberrant expression and rearrangements of the Rb susceptibility gene have been reported in tumours other than retinoblastoma and its associated cancers. These include breast, small cell lung, bladder and mesenchymal tumours (Lee, et al, 1988, Harbour, et al, 1988, Horowitz, et al, 1989, Friend, et al, 1987). In the colorectal cancers tested here, ten showed increased expression, seven reduced and three equal when compared with their equivalent normal RNA (table 3.7, figure 3.13). The majority of the samples in which Rb expression was elevated (7/10) were Dukes stage B tumours. All the adenomas from FAP136 showed significantly increased expression of the Rb gene, and nine of the eleven FAP139 adenomas also showed an increased signal (table 3.8, figure 3.13).
Figure 3.12.

Expression of *myc* Proto-oncogene in Colorectal RNA.

**Figure 3.12. a:** Histograms showing levels of c-*myc* expression in sporadic cancers and FAP adenomas (patient and sample numbers indicated). **b:** dot blots hybridized to c-*myc*, left panel sporadic cancers, right panel FAP adenomas. **c:** dot blots shown in **b**, hybridized to β-actin. **d:** key to sample loading on dot blots.
Figure 3.13.

Expression of Retinoblastoma Susceptibility Gene in Colorectal RNA.

(a) Histograms showing levels of RB-1 expression in sporadic cancers and FAP adenomas (patient and sample numbers indicated). (b) Dot blots hybridized to RB-1, left panel sporadic cancers, right panel FAP adenomas. (c) Dot blots shown in (b), hybridized to β-actin. (d) Key to sample loading on dot blots.
3.21e. MCC (Mutated in Colorectal Cancer) Gene (MCC100).

Somatically acquired point mutations and genomic rearrangements of the MCC tumour suppressor gene have been reported in sporadic colorectal carcinomas (Kinzler, et al, 1991a). In some instances such mutations may alter the level of MCC expression, and thereby influence colorectal carcinogenesis. However, in this study equivalent levels of tumour/normal MCC expression were detected in 85% (17/20) of the sporadic colorectal cancers tested, and those which did not conform to this pattern were only slightly elevated or reduced (table 3.9, figure 3.14). Similarly all the FAP adenomas and cancers tested expressed MCC at an equivalent level to the normal mucosa (table 3.10, figure 3.14).


Carbonic anhydrases are expressed throughout the length of the digestive tract, however CA1 is expressed predominantly in the large intestine (Lonnerholm, et al, 1985). It was thought that together with other intestinal specific genes, expression of CA1 may be dependent on the stage of cellular differentiation within the colonic mucosa. The CA1 gene has two promotors, which each transcribe a distinct message, one present in colonic epithelia and the other in erythroid cells (Brady, et al, 1991). Although the cDNA probe used here would not distinguish between the two transcripts, only the colon specific message should be present, because there was minimal contamination of the mucosal samples with blood. As can be seen in tables 3.9 & 3.10 and figure 3.15, the pattern of CA1 expression in colorectal neoplasia was dramatic. Expression was reduced in 100% of sporadic cancers and was effectively undetectable in 15/29 cases. This trend was also observed in FAP samples, with the majority of adenomas and carcinomas showing a profound reduction in CA1 expression. Although there was no apparent correlation between the Dukes' staging of the cancer samples and CA1 expression (table 3.9), there appeared to be some association between the degree of cellular dysplasia in the adenomas and CA1 expression (table 3.10). Equivalent levels of CA1 expression were, with one exception, only detected in adenomas with mild dysplasia, and not in those
with moderate or severe dysplasia. This may be seen in adenomas 1 and 3 of FAP139 and adenoma 6 of FAP147 (table 3.10, figure 3.15). The exception to this pattern was the carcinoma from FAP112, in which, unlike all the other cancers tested, CA1 expression was equivalent to the normal. It may be that this tumour sample was contaminated with a significant quantity of normal tissue, which would be expressing CA1, unfortunately it was not possible to establish whether or not this was the case.

CA1 expression was not detectable in the mRNA from any of the colon carcinoma cell lines tested, namely: HCA7, HT29, LIM1215, LIM1899, CACO2, COLO320, KMS4 and JW2 (data not shown). RNA from the cervical cancer cell line HeLa (kindly provided by Jane Sowden) was included in the hybridizations as a negative control (figure 3.15).
### Table 3.9
Expression of MCC, CA1 and p53 in Sporadic Colorectal Cancers.

<table>
<thead>
<tr>
<th>Patient No</th>
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<th>CA1</th>
<th>p53</th>
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Table 3.9. C: Caecum, SF: Splenic Flexure, R: Rectum, SC: Sigmoid Colon, NK: not known, NT: not tested. +, ++, +++ & ++++ represent expression elevated between 11-50%, 51-100%, 101-150% & 151% and over respectively above normal, = represents expression within 10% above and below normal, - & -- represent expression 11-50% & 51-100% and lower respectively below normal, * indicates no detectable expression, (E) indicates reading estimated.
Table 3.10

Expression of MCC, CA1 and p53 in Familial Adenomatous Polyposis Samples.

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<th>Patient No</th>
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<th>CA1</th>
<th>p53</th>
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<tr>
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<td>+++</td>
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Table 3.10. NT = not tested, +, ++, +++ & ++++ represent expression elevated between 11-50%, 51-100%, 101-150% & 151% and over respectively above normal, = represents expression within 10% above and below normal, - & -- represent expression 11-50% & 51-100% and lower respectively below normal, * indicates no detectable expression, (E) indicates reading estimated.
Figure 3.14

Expression of the MCC Gene in Colorectal RNA.

Figure 3.14. **a**: Histograms showing levels of MCC expression in sporadic cancers and FAP adenomas (patient and sample numbers indicated, for FAP164, sample 5 represents epidermoid cyst RNA). **b**: slot blots hybridized to MCC. **c**: key to sample loading on slot blots. Actin hybridizations taken from figure 3.22, or from previous dot blots.
Figure 3.15.

Expression of Carbonic Anhydrase 1 in Colorectal RNA.

Figure 3.15.  

a: Histograms showing levels of CA1 expression in sporadic cancers and FAP adenomas (patient and sample numbers indicated, for FAP145 sample 1 represents the cancer and 2 the adenoma, for FAP164 sample 5 represents the epidermoid cyst).  
b: Slot blots hybridized to CA1.  
c: Key to sample loading on slot blots. Actin hybridizations taken from figure 3.22, or from previous dot blots.
3.21g. p53 Tumour Suppressor Gene (pR4.2).

Allele loss at the p53 locus has been reported in many types of neoplasia, including colorectal (Mackay, et al, 1988, Baker, et al, 1989, 1990a, Fults, et al, 1989, Takahashi, et al, 1989, Mulligan, et al, 1990, Menon, et al, 1990). Concomitant mutation of the remaining allele and enhanced p53 peptide levels, would appear to be a common feature of allele loss at the p53 locus (Baker, et al, 1989, Nigro, et al, 1989, Iggo, et al, 1990, Thompson, et al, 1990). The levels of p53 transcript in colorectal neoplasia may also reflect the mutational status of the gene. In the samples tested here, elevated levels of the p53 message were detected in 19/38 sporadic cancers, 34/36 adenomas from six FAP patients and 1/2 FAP cancers (tables 3.9 & 3.10, figure 3.16). Although a strong correlation does not appear to exist between p53 expression and cellular dysplasia or Dukes' staging, it was apparent that elevated levels of p53 were detected in a greater proportion of Dukes' stage B cancers (9/16), than in stage C cancers (3/10) (only 2 Dukes' A cancers were tested) (table 3.9). Northern hybridizations were performed on those samples where sufficient RNA was available. The p53 cDNA probe hybridized strongly to the ribosomal RNA present in the samples, however, the normal 2.8kb p53 transcript was easily identifiable (figure 3.17). The results from the Northern hybridizations were broadly in agreement with the dot and slot blot findings (figure 3.17, tables 3.9 & 3.10), indeed, differences were clearly apparent between the hybridization patterns of the tumour and normal samples from patients 556, 717, 776 and 800 (figure 3.17). In addition to the samples tested previously, RNA from a number of cancer cell lines was also analysed. Previous studies had reported elevated levels of the p53 peptide, together with a G → A transition in codon 273 of the p53 gene, in the cell line HT29 (Rodrigues, et al, 1990). As may be seen in figure 3.17, the p53 transcript was evident in RNA from this and the other cell lines examined. The results presented here suggest that the levels of p53 RNA are elevated in the majority of neoplastic colorectal tissues, and reveal for the first time significant p53 expression in premalignant adenomas.
Figure 3.16.

Expression of p53 Tumour Suppressor Gene in Colorectal RNA.

(a) Graph showing p53 expression levels for different patient numbers.

(b) Table and images of p53 expression in various samples.

(c) Additional images and data points.
Figure 3.16. a: Histograms showing levels of p53 expression in sporadic cancers and FAP adenomas (patient and sample numbers indicated, sample 1 of FAP147 represents the cancer). b: dot & slot blots hybridized to p53. c: dot blots shown in b(i), hybridized to β-actin. d: key to sample loading on dot & slot blots. Actin hybridizations for slot blots taken from figure 3.22, or from previous dot blots.
Northern Hybridizations of p53 to Colorectal RNA.

Figure 3.17. Northern hybridizations of p53 to sporadic and familial colorectal RNA samples. Patient numbers as indicated, (T) or (ca) cancer, (N) or (n) normal, sample numbers for FAP patients refer to polyp numbers, (cy) epidermoid cyst, ribosomal RNA bands (28S & 18S) and p53 hybridizing bands indicated with arrows.
3.21h. Mucin Genes (MUC1, MUC2, MUC3).

Differences in the nature of colonic mucoprotein antigens in colorectal neoplasia have been recognised for some time (Gold & Miller, 1975 & 1978, Boland, et al, 1982, 1986). Probes specific for three mucin genes were used in this study, they were the mammary epithelial mucin pMUC10 (MUC1) (Gendler, et al, 1987), and the two intestinal mucins SMUC41 (MUC2) (Gum, et al, 1989) and SIB124 (MUC3) (Gum, et al, 1990). Further mucin genes have been identified, two encoding tracheo-bronchial mucins, designated MUC4 and MUC5 (Porchet, et al, 1991, Perini, et al, 1991), and more recently MUC6, which encodes a gastric mucin (Toribara, et al, 1993). However, probes for these genes were not available for study.

Table 3.11 and figures 3.18, 3.19, 3.20 and 3.21 show the results obtained with the three mucin probes hybridized to RNA from sporadic colorectal cancers, including five cancers which were classified as mucinous (cancers: 323, 517, 556, 570 & 927). MUC1 did not appear to be expressed in significant quantities in normal colorectal mucosa, this level of expression was reduced still further in the majority of non-mucinous colorectal cancers (13/22) (table 3.11, figures 3.18 & 3.21). The notable exception to this pattern was cancer 681, which appeared to express MUC1 at a level higher even than the two MUC1 expressing mucinous cancers, 517 and 570. The other mucinous cancers (323, 556 & 927) revealed low levels of MUC1, however, an adenoma from patient 323 showed a slight increase in expression compared with the normal sample. This was in agreement with the results obtained from the adenomas of patients FAP136 and 139, where MUC1 was expressed at a level greater than normal in 16/21 samples (table 3.8, figures 3.18 & 3.21).

Although MUC2 was clearly expressed in normal colonic mucosa, this expression was dramatically reduced in all of the sporadic cancers and in three of the five mucinous cancers (table 3.11, figures 3.19 & 3.21). It was therefore only in the two mucinous cancers 517 and 570, that significantly increased MUC2 expression was detected (table 3.11, figures 3.19 & 3.21). A consistent pattern of MUC2 expression was not apparent in the FAP adenomas, with the
The majority of FAP136 samples (8/10) expressing more MUC2 than the normal sample, and 8/11 adenomas from FAP139 expressing less (table 3.8, figures 3.19 & 3.21).

The third mucin gene (MUC3) was the only one to show significantly elevated levels of expression in a number of the sporadic colorectal cancers tested (7/24). However, the majority of the samples (14/24) revealed reduced MUC3 expression (table 3.11, figures 3.20 & 3.21). Unlike MUC1 and 2, expression of MUC3 was not elevated in any of the mucinous cancers (table 3.11, figures 3.20 & 3.21). The FAP adenomas were not tested with MUC3.

There would not appear to be any strong correlation between Dukes' staging and expression of MUC1 or 2. However, MUC3 expression was reduced in a greater proportion of Dukes' B tumours and it was increased most commonly in Dukes' C cancers (table 3.11), the strength of this observation was limited by the fact that the number of samples in each category was small. With a few exceptions mucin expression was generally reduced in colorectal carcinomas (figure 3.21a). It was of note that elevated levels of more than one mucin were only evident in two of the mucinous cancers and in a number of the FAP adenomas. The pattern of mucin expression differed between the two FAP patients, in the majority of FAP136 adenomas (7/10) where the level of MUC1 was increased, the same was also true for MUC2, but in FAP139 there was little correlation between the expression of MUC1 and 2 in the adenomas (table 3.8).
# Table 3.11

## Expression of Mucin Genes in Sporadic Colorectal Cancers.

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<th>MUC3</th>
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### Mucinous carcinomas

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<td>+</td>
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</tr>
<tr>
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<td>SC</td>
<td>NK</td>
<td>=</td>
<td>--</td>
<td>=</td>
</tr>
</tbody>
</table>

**Table 3.11.** SC: sigmoid colon, R: rectum, SF: splenic flexure, NK: not known, NT: not tested. +, ++, +++ & ++++ represent expression elevated between 11-50%, 51-100%, 101-150% & 151% and over respectively above normal, = represents expression within 10% above and below normal, - & -- represent expression 11-50% & 51-100% and lower respectively below normal, (E) indicates reading estimated.
Figure 3.18

Expression of MUC1 Mucoprotein in Colorectal RNA.

Figure 3.18. a: Histograms showing levels of MUC1 expression in sporadic and FAP adenomas (patient and sample numbers indicated). b: dot blots hybridized to MUC1, left panel sporadic cancers, right panel FAP adenomas. c: dot blots shown in b, hybridized to β-actin. d: key to sample loading on dot blots.
Figure 3.19

Expression of MUC2 Mucoprotein in Colorectal RNA.

Figure 3.19. **a:** Histograms showing levels of MUC2 expression in sporadic and FAP adenomas (patient and sample numbers indicated). **b:** dot blots hybridized to MUC2, left panel sporadic cancers, right panel FAP adenomas. **c:** dot blots shown in **b,** hybridized to β-actin. **d:** key to sample loading on dot blots.
Expression of MUC3 Mucoprotein in Colorectal RNA.

Figure 3.20. a; Histogram showing levels of MUC2 expression in sporadic cancers (patient and sample numbers indicated). b; dot blot hybridized to MUC2. d; key to sample loading on dot blots. Actin hybridizations taken from figure 3.22, or from previous dot blots.
Figure 3.21.

Expression of MUC1, MUC2 & MUC3 in Colorectal RNA.

**a**

![Histogram](image)

Patient No.

**b**

![Slot Blots](image)

**c**

<table>
<thead>
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</tbody>
</table>

**Figure 3.21.** **a:** Histogram showing levels of mucin expression in sporadic colorectal cancers (and adenoma, 323.2), black: MUC1, white: MUC2, shaded: MUC3. **b:** Slot blots hybridized to **i:** MUC1, **ii:** MUC2, **iii:** MUC3. **d:** Key to sample loading on slot blots. Actin hybridizations taken from figure 3.22, or from previous dot blots.
Figure 3.22.

Expression of β-Actin in Colorectal RNA Samples.

Figure 3.22. 

**a:** slot blots hybridized to β-actin. **b:** key to sample loading on slot blots.
3.3. Construction of Normal Colonic Mucosal cDNA Library.

The availability of flash frozen colonic mucosa from a patient with no apparent colorectal pathology, provided a valuable resource from which to make a cDNA library. Double stranded cDNA prepared from the mRNA was in the size range of 100-3000bp (figure 3.23a). EcoRI adaptor oligonucleotides were ligated onto the blunt ended cDNA and the resulting products were size fractionated by gel filtration (figure 3.23b). The five gel filtration fractions corresponding to the largest cDNAs were pooled (as indicated in figure 3.23b & c) and ligated into EcoRI cleaved λgt10 arms. The efficiency of the ligation and subsequent packaging reactions was estimated using control DNA samples supplied with the cloning kit (Amersham International plc) and by plating the reactions onto two different strains of E.coli (table 3.12).

The results obtained in sample 1 allowed the efficiency of the in vitro packaging reactions to be assessed, and also for the degree of biological selection provided by the two E.coli strains to be estimated. Although the actual number of plaques produced in L87 cells from sample 1 was 10 fold lower than the $3 \times 10^8$ pfu/μg recommended by Amersham, the ratio between the L87 and the NM514 results was 240, indicating that the biological selection was adequate (table 3.12). Non-recombinant phage DNA should not allow the formation of plaques in the high frequency of lysogeny (hfl) NM514 cells as the CI repressor protein would be present, allowing lysogenic growth. Thus the plaques present in sample 1 on this host represent an acceptable background.

In sample 2, EcoRI digested dephosphorylated λgt10 arms were ligated to one another. Under such conditions self-ligation occurs infrequently, resulting in a reduced titre. The titre obtained with reaction 2 on L87 was $2.6 \times 10^5$ pfu/μg, approximately 10 fold higher than that expected ($<5 \times 10^4$ pfu/μg according to the Amersham manual). Whereas that of $4.3 \times 10^3$ pfu/μg with NM514 cells was within the Amersham recommendations ($<2 \times 10^4$ pfu/μg) (table 3.12). The efficiency of the ligation reaction in the presence of appropriate insert.
Figure 3.23.

Analysis of cDNA Prepared from Normal Colorectal Mucosal mRNA.

a

PM2 1 2

5.4
2.3
1.05
0.475/0.45
0.275
0.11

b

Figure 3.23. a: Autoradiograph of an alkali agarose gel dried on to DEAE cellulose paper. PM2 phage DNA HindIII digested, sizes indicated (kb) Lane 1: product of 1st cDNA strand synthesis. Lane 2: product of 2nd cDNA strand synthesis (2nd strand radiolabelled). b: Graph showing cpm (Cerenkov) of cDNA gel filtration fractions, solid boxes represent fractions which were pooled and cloned into λgt10. c: Autoradiograph of TAE agarose gel dried on to DEAE cellulose paper. Lanes 10-23 represent fractions 10-23 of the cDNA gel filtration shown in figure 3.23b. Size markers in a & c were radiolabelled.
DNA was tested in reaction 3, where EcoRI ended control DNA was included. The titres for both strains of cells was within the recommended ranges (>3x10⁶pfu/µg & >2x10⁶pfu/µg for L87 & NM514 respectively), and were greatly increased over those for sample 2 (table 3.12). The ratio between the NM514 titres for samples 3 & 2 was 651, indicating that the presence of a suitable insert allowed a substantial increase in the number of phages growing lyticly, and hence producing plaques, compared with the background of plaques produced by vector arms alone.

The final control - sample 4, tested all the reactions in the cloning system (namely: ligation of adaptors, kinasing and ligation to the vector DNA). The results indicate that all reactions were working well, with titres over 1x10⁶ for both E.coli strains (table 3.12).

Table 3.12. The λgt10 whole DNA and arms were as provided in the Amersham cloning kit. pfu/µg = plaque forming units per µg vector DNA. Clear refers to the clear plaques obtained in lytic growth and turbid to the plaques obtained in lysogenic growth in L87 cells.

In reactions 5 and 6, 25 and 50ng of normal colonic mucosal cDNA was ligated to 1µg of λgt10 arms respectively. The number of recombinant phages present was calculated by subtracting the titre of reaction 2 for NM514 cells, from the titres obtained in reactions 5 and 6 (table 3.12). Therefore, 2.39x10⁶ recombinants were present in the 25ng
ligation, and 1.19x10^6 in the 50ng ligation, over 99% of the final titre in each case. The cloning efficiency was determined as follows:

\[
\text{Cloning efficiency (per } \mu\text{g cDNA)} = \frac{\text{number of recombinants}}{\text{ng cDNA used}} \times 1000
\]

For reaction 5 the cloning efficiency was 9.6x10^7 pfu/\mu g cDNA, and for reaction 6 it was 2.4x10^7, both these results were at least 10 fold above the recommendations made by Amersham.

The remainder of the packaging reactions 5 and 6 were plated onto NM514 cells, and were amplified to give libraries with titres of 4x10^11 and 6.25x10^11 pfu/ml (respectively). The libraries were known as LSNCM5 & LSNCM6 respectively.

3.4. Isolation and Analysis of Chromosome Five Specific Genomic Clones.

In an attempt to isolate genes expressed in normal colonic mucosa, which were located on human chromosome 5, a chromosome 5 specific genomic library was screened with a radiolabelled aliquot of the LSNCM cDNA library, according to the principles of Wong et al (1989). The human chromosome five genomic library (LA05NS01 from the ATCC), was prepared from a hamster/human somatic cell hybrid line (640-12), which contains chromosome 5 as the only human component.

3.4.1. Screening Chromosome Five Genomic Library with Normal Colonic Mucosal cDNA Library.

The cDNA library probe was prepared by including \( ^{32}\text{P} \left[ \alpha - \text{dCTP} \right] \) into a PCR, in which the inserts from an aliquot of LSNCM cDNA library (4x10^8 phage particles) were amplified with primers flanking the vector (\( \lambda gt10 \)) EcoRI cloning site. Figure 3.24 shows the typical size distribution of products.

Approximately 6x10^4 clones of the library LA05NS01 were screened, to provide twice full coverage of the human component of the library (according to the specifications provided by ATCC). After low stringency washing (2xSSPE), thirteen positively hybridizing clones were identified. Following two further rounds of screening, nine clones continued to hybridize to the cDNA probe (\( \lambda 5.2, \lambda 5.3, \lambda 5.4, \lambda 5.5, \lambda 5.9, \lambda 5.10, \lambda 5.11, \lambda 5.12 \) & \( \lambda 5.13 \)).
3.42. Characterization of Genomic DNA Clones.

3.42a. Identification of Human and Hamster Genomic Clones.

Small scale DNA preparations were performed on phage pure stocks of the clones, the resultant DNA was cleaved with EcoRI to release the cloned inserts from the bacteriophage vector (Charon 21A). Figure 3.25a shows the size of the inserts present in the various clones. Hybridization of Southern blotted clone DNA to radiolabelled human and hamster genomic DNA, allowed the origin of the clones to be established. Figures 3.25b & c show the hybridization patterns produced, the results of which are summarized in table 3.13.

On the basis of hybridization of the clones with human and hamster genomic DNA, clones $\lambda 5.11$ & $\lambda 5.13$ were excluded from further study, as they hybridized more strongly to the hamster genomic DNA than to the human, and were therefore assumed to be derived from the hamster component of the library. Clone $\lambda 5.2$ failed to hybridize with either probe and was also rejected. The genomic EcoRI fragments from the remaining clones were gel purified and subcloned into the plasmid vector pUC9. The plasmid subclones derived from the phage clones $\lambda 5.3$, $\lambda 5.4$, $\lambda 5.5$, $\lambda 5.9$, $\lambda 5.10$ & $\lambda 5.12$ were known as p5.3, p5.4, p5.5, p5.9, p5.10 & p5.12 respectively.
Analysis of Clones Isolated from the Chromosome Five Genomic Library.

**Figure 3.25. a:** Ethidium bromide stained TAE agarose gel. λHindIII: HindIII digested λ DNA molecular weight markers, sizes indicated (size markers were radiolabelled). λ5.2, λ5.3, λ5.4, λ5.5, λ5.9, λ5.10, λ5.11, λ5.12 & λ5.13: EcoRI digested genomic clones with released inserts ranging in size from 3 - 6.8kb. **b:** Southern blot of gel shown in a, hybridized to radiolabelled human genomic DNA. **c:** Southern blot of gel shown in a, hybridized to radiolabelled hamster genomic DNA (isolated from the cell line a23, provided by Dr Sue Povey).
Table 3.13

Analysis of Clones Isolated from the Chromosome Five Genomic Library.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Size of Insert</th>
<th>Hybridization to Human DNA</th>
<th>Hybridization to Hamster DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>λ5.2</td>
<td>3kb</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>λ5.3</td>
<td>4kb</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>λ5.4</td>
<td>3.9kb</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>λ5.5</td>
<td>3.9kb</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>λ5.9</td>
<td>3.8kb</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>λ5.10</td>
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<td>++</td>
<td>+</td>
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<td>λ5.11</td>
<td>3.7kb</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>λ5.12</td>
<td>6.5kb</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>λ5.13</td>
<td>4kb</td>
<td>-</td>
<td>++</td>
</tr>
</tbody>
</table>

Table 3.13. Size of insert refers to the approximate size of the EcoRI genomic DNA fragment cloned into the Charon 21A bacteriophage vector. - indicates that little or no hybridization was evident. + indicates low levels of hybridization, ++ indicates strong hybridization.

3.42b. Restriction Mapping the Plasmid Subclones.

The entire EcoRI insert was purified from each plasmid subclone, this was digested with a variety of restriction endonucleases, and a restriction map was constructed for each clone (figure 3.26). Southern blots of the restriction digests were hybridized to radiolabelled sonicated human genomic DNA at 65°C under standard conditions. Fragments which hybridized to the human DNA were considered to contain repeat elements, whilst those which did not hybridize to human genomic DNA were considered to be single copy. The single copy fragments, known respectively as p5.3SC, p5.4SC, p5.5SC, p5.9SC, p5.10SC & p5.12SC are indicated on figure 3.26 as hatched lines. Hybridization of these fragments to Southern blotted digests of the originals clones, allowed the restriction maps to be confirmed. Furthermore, the restriction analysis suggested that there was no overlap between any of the clones.
Figure 3.26. Restriction maps of human genomic EcoRI fragments, isolated from plasmid subclones p5.3, p5.4, p5.5, p5.9, p5.10 & p5.12. Restriction endonucleases as indicated. Hatched lines correspond to apparently single copy regions of the clones.

To confirm the chromosomal origin of the genomic clones p5.3, p5.4, p5.5, p5.9, p5.10, & p5.12, the single copy fragment from each clone was used as a probe on Southern blots of EcoRI digested human/rodent somatic cell hybrid DNA. The hybrids, which were kindly provided by Dr Sue Povey, enabled localization of the probes to human chromosome five, and the exclusion of other chromosomes (table 3.14). However, this panel did not allow subchromosomal localizations within chromosome five to be made.

The results of the hybridizations are shown in figure 3.27 and summarized in table 3.15. The single copy fragments from clones p5.3, p5.4, p5.9 and p5.12 all detected EcoRI fragments in human genomic DNA consistent with the mapping data reported in section 3.42b, in that they were the same size as the genomic EcoRI fragments from which the single copy probes were obtained. In addition, from the pattern of hybridization across the somatic cell hybrid panel it would appear that each of these four clones was derived from human chromosome five (figure 3.27 a, b, d, & f, table 3.15).

The single copy fragment from p5.5 detected a 1.35kb EcoRI fragment in the rat DNA (FAZA) and also in the rat derived hybrids (figure 3.27c, table 3.15). A 3.3kb band was also detected but only in samples with a human component (i.e.: not in FAZA or a23). The apparent presence of the 3.3kb fragment in all the hybrid cell lines, was inconsistent with the current knowledge of their genotypes (table 3.14). FG10E8B, SIF4A24E1 and FST9/10 are together mutually exclusive with respect to the human chromosomes which they contain. This result may have arisen if the p5.5SC probe identified the same size EcoRI fragment on more than one chromosome. Hybridization of p5.5SC to the chromosome five only hybrid PN/TS1 however, reveals that there were regions on chromosome five which were homologous to p5.5.

The pattern obtained with the probe p5.10SC was complex, with numerous EcoRI fragments hybridizing in each of the hybrid DNAs tested (no rodent hybridization was detected) (figure 3.27e, table 3.15). However, it was evident that the pattern differed in each hybrid, and that those
containing human chromosome five revealed a prominent band at 6.9kb, the size of the original p5.10 genomic clone. As before, hybridization of the probe with PN/TS1 confirmed that although p5.10SC detected loci on chromosomes other than five, there was a region on chromosome five which was homologous to p5.10.

**Table 3.14**

**Human Content of Somatic Cell Hybrids Used for the Localization of Genomic Clones.**

<table>
<thead>
<tr>
<th>Human / Rodent Hybrids</th>
<th>Human Chromosomal Content</th>
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<tr>
<td>FG10E8B</td>
<td>rat</td>
</tr>
<tr>
<td>FG10E8EP2.6</td>
<td>rat</td>
</tr>
<tr>
<td>STF4A24E1</td>
<td>rat</td>
</tr>
<tr>
<td>FST9/10</td>
<td>rat</td>
</tr>
<tr>
<td>PNTS1</td>
<td>hamster</td>
</tr>
<tr>
<td>Human / Rodent Hybrids</td>
<td>3 4 5 6 7 8 9 10 11 12</td>
</tr>
<tr>
<td>FG10E8B</td>
<td>- + - - + - + - - - -</td>
</tr>
<tr>
<td>FG10E8EP2.6</td>
<td>- - - + + - + + + +</td>
</tr>
<tr>
<td>STF4A24E1</td>
<td>- - + - + - + - + +</td>
</tr>
<tr>
<td>FST9/10</td>
<td>+ + - - + + - + + +</td>
</tr>
<tr>
<td>PNTS1</td>
<td>hamster</td>
</tr>
</tbody>
</table>

**Table 3.14.** The rodent background of each somatic cell hybrid is indicated, the presence or absence of a given human chromosome is indicated by + or -, ? indicates that the hybrid cannot be scored with certainty for a particular chromosome.

**Table 3.15.**

**Summary of Hybridization Results of Single Copy Probes to Somatic Cell Hybrid DNAs.**

<table>
<thead>
<tr>
<th>Probe</th>
<th>FAZA</th>
<th>FG10E8B</th>
<th>FG10E8EP2.6</th>
<th>STF4A24E1</th>
<th>FST9/10</th>
<th>a23</th>
<th>PN/TS1</th>
<th>Human</th>
<th>Size</th>
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</thead>
<tbody>
<tr>
<td>p5.3</td>
<td>-</td>
<td>+</td>
<td>NT</td>
<td>-</td>
<td>NT</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>4.2</td>
</tr>
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<td>p5.4</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>4.0</td>
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<td>+</td>
<td>(+)</td>
<td>(+)</td>
<td>+</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>3.3 (1.35)</td>
</tr>
<tr>
<td>p5.9</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>3.7</td>
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<td>NT</td>
<td>+</td>
<td>NT</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>various</td>
</tr>
<tr>
<td>p5.12</td>
<td>-</td>
<td>+</td>
<td>NT</td>
<td>-</td>
<td>NT</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>6.0</td>
</tr>
</tbody>
</table>

**Table 3.15.** FAZA and a23 were the rat and hamster cell lines respectively used as controls in the hybridizations, the size of the EcoRI fragment(s) detected by each probe is indicated in kilobase pairs, - indicates no apparent hybridization, + indicates hybridization of the probe to the sample DNA, (+) indicates hybridization of the probe to the rodent parent of the somatic cell hybrid, NT indicates not tested.
Figure 3.27.

Hybridization of Human Genomic Single Copy Fragments to Somatic Cell Hybrid DNA.

Figure 3.27. Southern blots showing hybridization of human genomic single copy fragments to a panel of EcoRI digested human/rodent somatic cell hybrid DNAs, together with rodent and human controls as indicated. a: Probed with p5.3SC. b: Probed with p5.4SC. c: Probed with p5.5SC. d: Probed with p5.9SC. e: Probed with p5.10SC. f: Probed with p5.12SC. Arrows indicate sizes of EcoRI hybridizing fragments.
3.42d Subchromosomal Localization of Chromosome Five Genomic Clones by Fluorescent In Situ Hybridization.

Subchromosomal mapping of the genomic clones was attempted using fluorescent in situ hybridization (FISH). DNA from all six original lambda clones (λ5.3, λ5.4, λ5.5, λ5.9, λ5.10 & λ5.12) was biotinylated by nick translation, and used as separate hybridizations probes on human metaphase chromosome spreads. Signals were only evident following hybridization with the clone λ5.3, the images of two chromosome spreads (figure 3.28a & b), show hybridization of λ5.3 to band q23 of chromosome five (FISH and analysis performed by D.K.Griffin). These particular lambda clones were found to be generally unsuitable for use as probes in FISH, for although the human component of each clone was less than 10kb, repetitive sequences were present which required quenching by preannealing with an excess of human genomic DNA. It was considered that preannealing reactions on relatively small genomic clones such as these, seriously impaired subsequent sequence specific hybridization. In an attempt to overcome this technical problem, human genomic clones were isolated from a cosmid library and used as probes in FISH experiments. Single copy fragments from p5.3 and p5.5 (p5.3SC & p5.5SC respectively) were used to screen a human genomic DNA cosmid library (Cachón-Gonzaléz, 1991)(library screening and clone analysis performed by S.A. Gayther & C.M. Lawson). The resultant cosmids, cos5.3iv and cos5.5iv, were then used as probes in FISH experiments. Both cosmids gave unequivocal hybridization results, with cos5.3iv (D5S321) localized to 5q35 (figure 3.28c) and cos5.5iv (D5S322) localized to 5q31 (figure 3.28d)(FISH and analysis performed by D.K. Griffin, S.A. Gayther & C.M. Lawson)(Leigh, et al, 1991). It was immediately apparent that a discrepancy existed between the localization results obtained for λ5.3 and cos5.3iv, both of which would have been expected to hybridize to the same chromosomal band, this anomaly will be discussed in section 4.11.
Figure 3.28. Fluorescent in situ hybridization of biotinylated DNA to human metaphase chromosomes. Yellow dots, indicated by arrows, represent hybridization of probe DNA to the chromosomal DNA. 

a & b: hybridization of cos5.3 to 5q23. 

c: hybridization of cos5.3iv (D5S321) to 5q35. 

d: hybridization of cos5.5iv (D5S322) to 5q31.
3.43. Summary of Chromosome Five Library Analysis.

The results presented in sections 3.41 and 3.42, represent preliminary findings in an investigation aimed at the isolation and characterization of colon specific expressed sequences originating from human chromosome five. Six genomic clones were identified and mapped back to chromosome five by hybridization to a panel of somatic cell hybrid DNAs, two of these were sublocalized to bands on chromosome five by FISH. It would have been important to confirm that the genomic clones did indeed contain expressed sequences and that they had not been identified by virtue of cross hybridization between repeat sequences in the cDNA and genomic libraries. Such confirmation could have been provided by using the single copy fragments from the genomic clones to probe either Northern blotted colon RNA or the colon cDNA library. Alternatively, hybridization of radiolabelled rodent genomic DNA to Southern blots of the human chromosome five clones under conditions of low stringency, could have revealed fragments of evolutionary conserved sequences, which in the past have been shown to be indicative of expressed gene sequences (Kinzler, et al., 1991b). Having established which clones contained expressed sequences, further analysis would have been performed on genomic clones that mapped to the FAP locus at 5q21 (Bodmer, et al., 1987, Leppert, et al., 1987). Such clones would have been used to screen the normal colonic mucosal cDNA library, with which the chromosome five genomic library was originally screened. It was hoped that this strategy would prove successful in the identification of genes from the FAP locus, which could play a role in colorectal carcinogenesis.
CHAPTER 4

DISCUSSION

4.1. Involvement of Chromosome 1 in Colorectal Carcinogenesis.

Cytogenetic abnormalities affecting chromosome 1 have been reported in all types of human malignancy and are common in colorectal carcinogenesis (Atkin, 1986, McBain, et al, 1984, Reichmann, et al, 1984, Couturier-Turpin, et al, 1992). In this study a total of 17 chromosome 1 anomalies were detected in DNA samples from 11 patients. Four of the changes were loss of short (p) arm material, as detected with the hypervariable probes p1.79 and λMS1, whilst the remaining alterations affected markers on the long (q) arm.

The results obtained for the FAP carcinoma cell line JW2, were consistent with the published cytogenetic data (Pareskeva, et al, 1984). The near diploid karyotype of JW2 was characterized by the presence of one normal chromosome 1 and an isochromosome 1q. Both of the lp markers for which JW was informative (p1.79 (lp36.3) & λMS1 (lp35-p33)), were homozygous in the cancer cell line. Thus, confirming the observation that a short arm of chromosome 1 had apparently been lost (Pareskeva, et al, 1984). The two informative markers on 1q, MUC1 and λMS32, also revealed loss of heterozygosity (LOH), suggesting that of the two original normal chromosomes, only one had been retained. Therefore, the isochromosome 1q was derived from the long arm of the retained chromosome 1. Only partial allele loss was observed at the MUC1 locus (1q21-q23), it may be that regions of 1q, including this locus, have contributed to the unidentified marker chromosomes, which were evident in some cells (Paraskeva, et al, 1984). Alternatively, the altered allele intensity observed with MUC1, could be indicative of the retention of both long arms of chromosome 1, with the duplication of one homologue in the formation of the isochromosome. If this were the case, the complete loss of one λMS32 allele, may have been brought about by a terminal deletion of 1q. Fluorescent in situ hybridization of JW2
with \( \lambda \text{MS32} \), as was performed with LIM1215 (Griffin et al., 1990), would help to resolve this issue.

Cytogenetic information was also available for the adenocarcinoma of patient 823 (J.D.A. Delhanty, personal communication). Analysis of a semi-direct preparation revealed two predominant populations of cells, one near diploid and the other with overall tetraploidy. It was proposed that the tetraploid karyotype, probably arose as a result of a duplication of the near diploid karyotype. Both karyotypes were probably represented in the DNA from the cancer. In common with the JW2 cell line, the near diploid cells of the 823 tumour, contained a single normal chromosome 1 and an isochromosome 1q. In this case however, the \( \lambda \text{MS32} \) locus (1q43-q44) remained heterozygous, suggesting that the chromosome 1 carrying the small \( \lambda \text{MS32} \) allele had remained unaltered, whilst the homologue represented by the large allele, was duplicated in the formation of the isochromosome 1q, hence, resulting in a stronger signal of hybridization in the tumour DNA. Although allele loss was observed in 823 tumour DNA at the most distal 1p marker (p1.79)(1p36.3), heterozygosity was retained at the PGD (1p36), \( \lambda \text{MS1} \) (1p35-33) and AF3 (1p35-34) loci. These findings support the karyotypic data, which suggests that in addition to the presence of a single normal chromosome 1, the other 1p homologue had been retained and was probably translocated to another site (J.D.A. Delhanty, personal communication). Karyotypic analysis had not detected the terminal deletion of 1p resulting in LOH at the p1.79 locus.

The HNPCC derived cell line LIM1215 was the only other sample with a chromosome 1 alteration, for which karyotypic information was available. In this case, the sole cytogenetic defect was reported as a duplication and translocation of the terminal region of chromosome 1q, to the short arm of chromosome 13 (Jenkyn, et al., 1987). The increased hybridization signal obtained for one of the \( \lambda \text{MS32} \) (1q43-q44) alleles, together with fluorescent in situ hybridization results, confirmed this observation (Griffin, et al., 1990).

An apparent interstitial deletion encompassing the MUC1 locus had occurred in cancers 214 and 315, as allele loss was
detected with pMUC10, but not with λMS1 or λMS32. The seven remaining chromosome 1q abnormalities were all detected as size alterations at either the MUC1 or λMS32 loci, and in one instance a third allele was detected by MUC1. The mutations responsible for the formation of such novel alleles at hypervariable loci will be considered in section 4.5.

The results obtained in this and previous reports illustrate that genes on human chromosome 1 contribute to colorectal carcinogenesis, furthermore it has been proposed that abnormalities affecting the long and short arms of chromosome 1 should be considered separately in tumourigenesis. Total or partial trisomy of 1q was the most commonly identified chromosome 1 aberration, this frequently took the form of an isochromosome 1q, however, 1q24-q44 was identified as the region most consistently duplicated (Reichmann, et al, 1984, Atkin, 1986, Oláh, et al, 1989). Although trisomy 1q was observed in at least four adenoma derived cell lines (Wilson, et al, 1987, Pareskeva, et al, 1988, 1989, Williams, et al, 1992), it would appear that this is a feature acquired after prolonged periods of in vitro culture, which was associated with an immortal but non-tumourigenic phenotype (Wilson, et al, 1987, Pareskeva, et al, 1989). The view that 1q duplications represent late and non-specific events in tumourigenesis (Atkin, 1986, Oláh, et al, 1989), was to some extent supported by the observation that in two subclones from one original adenoma cell line, the 1q isochromosomes were derived from different chromosome 1 homologues (Pareskeva, et al, 1989).

In contrast to 1q, the predominant changes affecting the short arm of chromosome 1, were deletions, resulting in either total or partial loss of 1p (Leister, et al, 1990, Couturier-Turpin, et al, 1992). Using a panel of polymorphic DNA probes from 1p22-p36, including λMS1, Leister et al (1990) detected LOH in 42% of primary colorectal tumours, with 1p35 as the most commonly deleted region. These results were at variance with those reported in this study, as no λMS1 allele loss was detected in 21 informative sporadic cancers. The non-random chromosome 1p losses reported by Leister et al (1990) suggest the possible presence of tumour suppressor genes in this chromosomal region.
The specific tumour suppressing properties of chromosome 1 have been demonstrated by both microcell mediated chromosome transfer and the fusion of normal human fibroblasts with tumourigenic cell lines (Benedict, et al, 1984, Stoler & Bouck, 1985, Yamada, et al, 1990, Kugoh, et al, 1990). Introduction of human chromosome 1 into Kirsten murine sarcoma virus transformed NIH3T3 cells, resulted not only in suppression of tumourigenicity, but also in control of the growth rate and colony forming ability in soft agar (Yamada, et al, 1990). The tumour suppression was chromosomally dosage dependent, in that fusion of normal diploid fibroblasts with tetraploid HT1080 fibrosarcoma cells, failed to achieve growth control (Benedict, et al, 1984). Furthermore, the cell lines only remained non-tumourigenic for as long as the normal human chromosomes 1 were retained (Benedict, et al, 1984, Stoler & Bouck, 1985).

A candidate tumour suppressor gene on the short arm of chromosome 1, is the ras-related K-rev-1/rap-1 gene (1p12-p13) (Kitayama, et al, 1989, Young, et al, 1992). With 50% peptide homology to the ras proteins, K-rev-1 can suppress K-ras transformed NIH3T3 cells, possibly by acting as a competitive inhibitor for GTP binding (Kitayama, et al, 1989). The significance of K-rev-1 inactivation in colorectal carcinogenesis, was assessed by searching for allele loss at the K-rev-1 locus in colorectal tumour DNAs (Young, et al, 1992). However, although K-ras activation occurs frequently in colorectal adenomas and carcinomas (Forrester, et al, 1987, Bos, et al, 1987), it would appear that this is not associated with inactivation of K-rev-1 by allele loss, as loss of heterozygosity at this locus was detected in only 1 of 20 informative samples (Young, et al, 1992).

Numerous proto-oncogenes have been assigned to chromosome 1, including N-ras (1p11-p12) (Hall, et al, 1983) and L-myc (1p32) (Nau, et al, 1985). Although L-myc expression was barely detectable in normal colonic mucosa, slightly elevated levels of the mRNA were recorded in 61% of adenomas and 16% of carcinomas (Finley, et al, 1989b). It may be however, that in common with c-myc, such modestly elevated levels of expression nearly reflect increased
cellular proliferation, rather than genuine over-expression (Viel, et al, 1990). Another proto-oncogene which maps to chromosome 1 is trk (tropomyosin receptor kinase) (1q23-q31) (Miozzo, et al, 1990). Trk was first identified as the activating oncogene in a transfection experiment between DNA from a colon carcinoma and NIH3T3 cells (Pulciani, et al, 1982). In the original and subsequent reports, activation of trk took the form of somatic rearrangements, which resulted in replacement of the external domain of the trk tyrosine protein kinase by other gene sequences (Martin-Zanca, et al, 1986, Oskam, et al, 1988, Coulier, et al, 1989). Of the 39 samples tested in this study no genomic rearrangement at the trk locus was apparent. Furthermore, when the pattern of trk expression was examined in FAP adenomas and sporadic colorectal carcinomas, little or no trk mRNA was detectable. The trk gene product is now known to be a nerve growth factor receptor (Klein, et al, 1991), whose expression is limited to certain ganglia of the peripheral nervous system (Martin-Zanca, et al, 1990). It would appear therefore, from the results obtained in this study and from a previous report (Fey & Thein, 1988), that the trk oncogene is rarely involved in colorectal neoplasia. However, it is frequently activated in papillary thyroid carcinomas (Bongarzone, et al, 1989).

In summary therefore, it is evident from cytogenetic and molecular genetic data, that genes on chromosome 1 are specifically involved in colorectal carcinogenesis. It is not yet apparent however, which genes or regions are the precise targets for the various genomic alterations, although a recent report has emphasised the importance of 1p36-34 in colorectal tumour suppression by microcell hybridization (Tanaka, et al, 1993).

4.2. Involvement of Chromosome 3p in Colorectal Carcinogenesis.

No allele loss or genomic rearrangement was detected on 3p in any of the tumour samples analysed in this study. A total of 8 FAP and 7 sporadic patients were informative for at least one of the two markers used, namely pHcA2 (c-erbA2)(3p22-p24) and H3H2 (3p21). A previous report of allele loss at H3H2 in a colorectal adenocarcinoma (Rider, 1986), together with an interstitial deletion of 3p in an
adenoma cell line (VAC0235) (Wilson, et al., 1987), led to the suggestion that the small cell lung cancer (SCLC) tumour suppressor gene at 3p21, may be involved in colorectal carcinogenesis. In SCLC and other major types of lung cancer, loss of heterozygosity at H3H2 is detected in all informative cases (Kok, et al., 1987). It would seem unlikely therefore, that this gene makes a significant contribution to colorectal carcinogenesis, as it was not deleted in any of the 10 informative samples. In a series of 25 informative colorectal carcinomas, 3p allele loss was recorded in 20% of cases (Vogelstein, et al., 1989). Vogelstein et al. (1989) observed that allelic deletions were remarkably common in colorectal tumours. Thus, the occurrence of 3p deletions may be a consequence of general allelic loss, or serve to indicate that another tumour suppressor gene on 3p is involved in a subset of colorectal carcinomas. Although the von Hippel-Lindau disease locus, responsible for an inherited cancer syndrome, has been mapped of 3p25-p26 (Maher, et al., 1991b), this tumour suppressor gene has yet to be studied in colorectal tumour samples.

4.3. Involvement of Chromosome 7 in Colorectal Carcinogenesis.

In this study genomic rearrangements of chromosome 7 were identified with the two minisatellite probes λMS31 (7p22-pter) and pλg3 (7q36-qter). Although no chromosome alterations were identified in adenomas, rearrangements of this chromosome were predominantly detected in early stage cancers, with four of the five affected tumours being Dukes stage A or B. The results obtained for two of the samples suggested that the entire chromosome 7 may have been either duplicated in one instance and lost in the other. For the tumour of patient 151 an increased hybridization signal was obtained with both λMS31 and pλg3, consistent with a chromosome 7 duplication. Whilst in the tumour of patient 200, hybridization was reduced at both loci, suggesting that one copy of a chromosome 7 may have been deleted. Of the remaining alterations detected on chromosome 7, the two at λMS31 resulted in the appearance of novel hybridizing bands. In a proportion of tumour 260 cells, it appeared that the novel fragments had resulted from the cleavage of the larger
allele at a new Hinfl recognition site. Whereas in tumour 311, the novel band was larger than the existing normal alleles and gave a comparable signal of hybridization. It would appear therefore, that additional chromosome 7 material was present in this tumour, which had resulted from a duplication and mutation at the λMS31 locus. In common with a number of other colorectal adenomas and carcinomas, the karyotype of tumour 823 revealed the presence of extra copies of chromosome 7 (J.D.A. Delhanty, personal communication) (McBain, et al, 1984, Longy, et al, 1990, Bardi, et al, 1991). This finding was not reflected in the results obtained for either λMS31 or pXg3, indeed at the pXg3 locus, allele loss was apparent. It may be however, that the amount of tumour and normal DNA loaded were not equivalent, thus, the apparent loss of the small pXg3 allele, may in fact reflect a duplication of the large allele, with the small allele remaining unaltered in tumour DNA.

Controversy exists as to whether or not the trisomy of chromosome 7 reported in colorectal and many other malignancies (Atkin & Baker, 1991), occurs in the cells of the tumour or in infiltrating normal cells (Bardi, et al, 1991, Dal Cin, et al, 1992). In kidney tumours, trisomy 7 was observed in mononuclear lymphocytes and not in the carcinoma cells (Dal Cin, et al, 1992). Similarly, in short term cultures of colorectal adenocarcinomas, the trisomy 7 was present in fibroblast like cells and not in the tumour parenchyma (Bardi, et al, 1991). However, this was not always the case, as trisomy 7 was retained in the colorectal adenoma derived cell line S/BR, after at least two years in culture (Williams, et al, 1992).

There are genes on chromosome 7 which are thought to be involved in tumour invasion and metastasis (Collard, et al, 1987). Hybrids between non-invasive, non-metastatic mouse T-lymphoma cells and activated human T-lymphocytes, were highly invasive and metastatic in nude mice (Collard, et al, 1987). After prolonged culture, it was only those hybrids retaining human chromosome 7, which continued to display the metastatic features. Further experiments sublocalized the dominantly acting metastatic locus to 7p12-cen (Habets, et al, 1992). One of the candidate genes from this region is the epidermal
growth factor receptor (EGFR) gene (7p13-p12) (Lathrop, et al, 1989). Although epidermal growth factor is known to regulate cellular proliferation (Carpenter & Cohen, 1979), and has been implicated in malignant transformation (Sporn & Roberts, 1985), the precise role of the EGFR transmembrane tyrosine kinase peptide in colorectal carcinogenesis remains unclear. Rearrangements of chromosome 7 were correlated with EGFR status in four pancreatic carcinoma cell lines (Korc, et al, 1986). The highest levels of expression were seen in those cell lines with chromosome 7p rearrangements. Up to 100% of primary colorectal tumours were reported as positive for the presence of EGFR, by immunohistochemistry, RNA quantitation and radioligand binding assays (Untawale & Blick, 1988, Steele, et al, 1990, Moorghen, et al, 1990). However, it is not yet clear whether the level of EGFR is elevated or reduced in colorectal neoplasia. Koender et al (1992), studying only stripped mucosa, reported reduced levels of EGFR in 92% carcinomas, compared with their respective normal tissues. The origin of the conflicting results, may be the great inter-individual range of EGFR expression observed by Koretz et al, (1990). Their work suggested that not only was the EGFR not involved in the adenoma-carcinoma sequence, but that EGFR expression was inconsistent in colorectal mucosa (Koretz et al, 1990).

Hepatocyte growth factor receptor (HGFR) has also been localized to chromosome 7, this is now known to be the product of the met proto-oncogene (7q31) (Bottaro, et al, 1991, Lathrop, et al, 1989). Hepatocyte growth factor (HGF, also known as scatter factor) and met, can together stimulate cultured cells into becoming motogenic and invasive, without inducing cellular proliferation (Gherardi, et al, 1989, Giordano, et al, 1993). It is thought that these features probably result from the disruption and inhibition of intercellular junctions (Gherardi, et al, 1989, Giordano, et al, 1993). Whilst NIH3T3 cells transformed with human met cDNA remained non-tumourigenic (Giordano, et al, 1993), the motogenic, invasive and angiogenic properties (Bussolino, et al, 1992) conferred by this tyrosine kinase receptor, could clearly contribute to tumour progression. The met oncogene was first identified following a chromosomal translocation,
in a carcinogen treated osteosarcoma cell line (Cooper, et al, 1984). However, as seen in the gastric tumour cell line GTL-16, met activation may also take the form of enhanced gene expression, which in this case was associated with a 10 fold amplification of a non-rearranged met gene (Giordano, et al, 1989). No genomic amplification or rearrangement was evident at the met locus, in any of the adenomas or carcinomas analysed in this study. However, met was expressed at an elevated level in 54% of sporadic colorectal cancers and in nearly all of the FAP adenomas tested. Unfortunately, because genomic and expression data was not available from the same samples, no direct correlation could be made between gene dosage and met expression in this study. Although the role of met and HGF in colorectal carcinogenesis remains uncertain, the frequent occurrence of trisomy 7 may influence the expression of these two genes, particularly as HGF has recently also been mapped to chromosome 7 (Zarnegar, et al, 1992).

Another gene associated with cellular motility which has been localized to human chromosome 7, is mts1 (7q22) (Tulchinsky, et al, 1990, Lakshi, et al, 1991). Expression of the mts1 gene, which was isolated from a highly metastatic mouse tumour cell line (Tulchinsky, et al, 1990), was found to be limited to certain metastatic tumour cells and normal cells which possess the ability to be motile (Tulchinsky, et al, 1992). Although the mts1 locus does not fall within the region of chromosome 7 defined by Habets et al (1992) as being crucial to metastasis, its role in the development of this phenotype is clearly of interest.

Genetic changes affecting chromosome 7 in neoplasia frequently result in additional material from this chromosome being present in the tumour cells. As has been seen this may be associated with the elevated expression of dominantly acting proto-oncogenes and growth factors, such as met and EGFR. However, allele loss does occur at loci on chromosome 7, and in a proportion of primary breast cancers LOH at the met locus was associated with an aggressive disease phenotype (Bièche, et al, 1992).
4.4. Involvement of Chromosome 5 in Colorectal Carcinogenesis.


In this study genomic analysis revealed loss of heterozygosity (LOH) at the ÀMS8 (5q35-qter) in 25% (4/16) of the sporadic carcinomas tested. These findings were comparable with other reports at the time (Solomon, et al, 1987, Law, et al, 1988, Vogelstein, et al, 1988). Allele loss was also detected at ÀMS8 in two adenomas from patient FAP57, together with the LOH detected in adenomas from a further two FAP patients (M. Rees, personal communication), these results constituted the first recorded chromosome 5 allele losses in precancerous adenomas (Rees, et al, 1989). Despite extensive investigation, allele loss in FAP adenomas was only reported in a few instances (Solomon, et al, 1987, Vogelstein, et al, 1988, Law, et al, 1988, Okamoto, et al, 1988). Such findings were consistent with the biology of FAP, whereby of the multiple adenomas present in the colorectum, only a small number undergo malignant change (Bussey, 1975). Miyaki et al (1990) reported loss of APC linked DNA markers in <2% of FAP adenomas with moderate dysplasia and in up to 20% of those displaying severe dysplasia. In addition, they observed the consistent loss of a specific allele from the tumour DNA of a given FAP patient, which in some instances could be identified as the normal APC allele, derived from the unaffected parent (Miyaki, et al, 1990). Further analysis of the APC gene in adenomas revealed that while mutations of both alleles were occurring, in agreement with Knudsons' hypothesis (Knudson, 1971),
detectable allele loss was seldom responsible (Miyoshi, et al, 1992b).

It would appear therefore, that the chromosome 5q allele losses observed in the mildly dysplastic adenomas of patient FAP57 were atypical. Similar results were also obtained for the adenomas of patient FAP145, where the APC locus was deleted in 4/10 adenomas tested (Rees, et al, 1993). Neither FAP57 or FAP145 had a family history of APC and were as such considered to have developed the disease as a result of a new mutation in the APC gene. With an early age of onset and a generally more aggressive phenotype, it was thought that the mutation affecting the APC gene in these patients may represent a severe variant. However, this does not appear to be the case, as both FAP57 and FAP145 exhibit the 5bp deletion in the APC gene (Vogelstein, et al & Gayther, et al, personal communication), which has been reported in a number of instances (Cottrell, et al, 1992, Miyoshi, et al, 1992b). In view of this information, it has been proposed that patients with de novo mutations at the APC locus may possess other mutations (Loeb, et al, 1991, Rees, et al, 1993), which result in increased chromosomal instability and so hasten malignant change. It is interesting to note, that consistent chromosome 5q allele losses were recently reported in the adenomas of two further FAP individuals, one of which was known to be a new mutation patient (Miki, et al, 1992, Ichii, et al, 1992).

The role of the MCC gene in colorectal carcinogenesis remains unclear. Somatic mutations have so far been identified in exons 3 and 4 in two tumours (Kinzler, et al, 1991a). The mutation in exon 3 was a C to T transition at codon 698, changing an alanine to a valine, whilst the mutation in exon 4 was a G to A transition which changed an arginine to a glutamine at codon 506 (Kinzler, et al, 1991a). Equivalent levels of tumour and normal MCC gene expression were detected in the majority of familial and sporadic tumours analysed in this study. It is of interest that the level of MCC expression was apparently normal in the case of patient FAP164, who is known to be constitutionally hemizygous at the MCC locus (Cross, et al, 1992). Cytogenetic evidence has shown that as a consequence of an
intrachromosomal insertion in 5q, this patient has lost, by microdeletion, one copy of both the MCC and APC genes (Cross, et al., 1992). Thus in this case MCC, expression would not appear to be gene dosage dependant.

Numerous germline and somatic mutations have now been reported in the APC gene, the majority of which result in a truncated gene product (Groden, et al., 1991, Nishisho, et al., 1991, Fodde, et al., 1992, Miyoshi, et al., 1992a & b, Cottrell, et al., 1992, Powell, et al., 1992). Germline variants have also been identified in the APC gene, which do not result in transcriptional termination (Powell, et al., 1992). The frequency with which these variants occurred was equivalent in FAP patients and in the general population, it was therefore suggested that such gene variants may be responsible for the commonly inherited predisposition to colorectal cancer (Powell, et al., 1992), which is thought to affect some 19% of the population (Cannon-Albright, et al., 1988). As yet no correlation has been made between precise mutations in the APC gene and the phenotype of the affected individual. However, Nagase et al., (1992) observed that in patients with profuse polyposis, germline mutations fell between codons 1250 and 1464 (exons 9 - 11), whilst no mutations were detected in this region in patients with sparse polyposis. The mouse lineage "multiple intestinal neoplasia" (Min), has recently been shown to carry a germline nonsense mutation in the murine homologue of the APC gene (Su, et al., 1992). This animal model will thus provide a valuable resource for analysing of the role of the APC gene in the development of colorectal cancer.

4.5. Mutations at Hypervariable Loci in Colorectal Carcinogenesis.

In common with previous reports (Thein, et al., 1987, Armour, et al., 1989), somatic mutations were detected at hypervariable loci in a proportion of the tumours analysed in this study. Excluding simple allele loss, mutations were detected at the MUC1, λMS32 and λMS31 loci. Mutations brought about by a change in the number of tandem repeats, were most commonly detected at the MUC1 locus in colorectal cancer DNA. Alleles with altered mobility were detected in four sporadic tumours, where the size of the hybridizing
bands were reduced in 3 cases and enlarged in 2. The tumour DNA of patient 193 was mosaic, with both the new and original alleles being present. In patient 185, the size of both normal alleles was altered in the tumour DNA. Similar results were also obtained for this tumour at the λMS32 locus, where although this patient was constitutionally homozygous, two alleles were present in the tumour DNA, neither of which corresponded in size to the original allele. Two further mutations were identified at the λMS32 locus, in the tumour of patient 195 a reduction in allele size was detected, whilst in one of the five adenomas from patient FAP70, the size of the largest λMS32 allele was increased. Finally two mutations were revealed with λMS31, in both cases the tumours were mosaic for the alteration. It appeared that a new Hinfl restriction site had developed within the VNTR of λMS31 in a proportion of the cells of tumour 260. This resulted in two novel alleles, whose combined size equalled that of the original allele, for which a reduced intensity of hybridization was apparent. The remaining mutation was an allele expansion in the tumour of patient 311.

The results presented in this study differ slightly from those of a previous report, in which the majority of mutations in gastrointestinal (GI) tumours were detected at the λMS1 locus (Armour, et al, 1989). With a heterozygosity of 99%, λMS1 is the most polymorphic of the locus specific minisatellite probes (Wong, et al, 1987), and it is also the most unstable (Jeffreys, et al, 1988). Hypervariability is therefore associated with a high mutation rate, at this and other VNTR loci (Jeffreys, et al, 1988). The germline and somatic mutation rates at a given locus may not always be comparable, as exemplified by Armour et al (1989) who observed proportionally more somatic, than germline mutations with λMS32. Although somatic mutations may occur frequently in many tissue types, it is only in a clonal growth of cells, such as a tumour, that these mutations become apparent. From their data in gastrointestinal tumours, Armour et al (1989) estimated the maximum mutation rate per mitosis at various minisatellite loci. Assuming, as they did, that an intestinal epithelial cell undergoes 14000 postzygotic mitoses prior to malignant conversion, the highest somatic

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mutation rate observed in this study, was $8.9 \times 10^{-6}$ per mitosis at the MUC1 locus. Although this rate was higher than that recorded for λMS1 ($6 \times 10^{-6}$) in the study of Armour et al (1989), it was of the same order. The rate of somatic mutation at such loci, was therefore very low (Armour et al, 1989). If similar mutation rates applied to the germline, alterations at these loci would rarely be detected. It has therefore been proposed that germline mutations occur during the final stages of meiosis, thus explaining the equal distribution of male and female derived mutations and the absence of germline mosaicism (Jeffreys, et al, 1988).

It would appear from this study that mutations at λMS32 occur early in tumourigenesis, for not only was a mutation detected at this locus in an adenoma, but none of the mutations identified in the carcinoma samples were mosaic. This was not the case at λMS31, where both of the mutations identified were apparent in the continued presence of the original alleles.

Various processes have been suggested to explain the occurrence of mutations at hypervariable loci, however, the exact mechanism(s) responsible have yet to be established with certainty (Jeffreys, et al, 1988, Armour, et al, 1989, Armour & Jeffreys, 1992). The maintenance of linkage disequilibrium between flanking polymorphic markers and λMS1 alleles before and after mutation, suggested that interallelic recombination was not responsible for the generation of new alleles (Wolff, et al, 1989). This view was reinforced when minisatellite variant repeat (MVR) mapping was first used to analyse mutations at the λMS32 locus (Jeffreys, et al, 1990). Relying upon variations within the repeat units of minisatellites, MVR mapping allows individual haplotypes to be established. The observation that such haplotypes remained essentially unaltered following deletion mutations at λMS32, implied that intra rather than interallelic recombination was occurring (Jeffreys, et al, 1990). However, a subsequent study reported mutant alleles in which recombinant and non-recombinant haplotypes were present, thereby providing direct evidence for intra and interallelic recombination at a hypervariable locus (Jeffreys, et al, 1991). The events leading to intra-allelic
recombination could be polymerase slippage at replication or unequal sister chromatid exchange, whilst gene conversion has been proposed to explain inter-allelic recombination (Armour & Jeffreys, 1992).

The MUC1 locus, and that of other mucoprotein genes, is of interest because the variability of DNA allele sizes is directly reflected in the resultant mucin peptide (Swallow, et al, 1987b). This association is based on the fact that the 60bp tandem repeat, which comprises the VNTR of MUC1, is within the coding region of the gene and encodes a 20 amino acid repeated motif in the peptide core (Swallow, et al, 1987a, Gendler, et al, 1987). Thus, the germline and somatic mutations which have been observed will alter the length of the mucin peptide (D.M. Swallow, personal communication, Fey, et al, 1989, Gendler, et al, 1990). A positive correlation has been reported between the transcriptional state of a gene and recombination (Thomas & Rothstein, 1989). This may result from either increased accessibility of the DNA to nucleases, or directly from enzymic activity associated with the transcriptional complex (Thomas & Rothstein, 1989). Such factors could influence mutation at the MUC1 locus. It would appear from the results presented in this study, that mutations at the MUC1 locus occur late in tumourigenesis, as three of the four cancers showing band shift were Dukes stage C. Furthermore, expression data revealed that during and after the transition from adenoma to carcinoma, MUC1 expression was depressed in the majority of samples. In the absence of expression and genomic data from the same samples, it is impossible to assess which event occurred first, mutation at the MUC1 locus or reduction of MUC1 gene expression. However, as 59% of the samples show reduced expression, whilst only 22% reveal mutations, it would appear the level of MUC1 expression was not dependant on the mutational status of the gene.

The significance of mutations at hypervariable loci in colorectal and other malignancies remains unclear. It could be that such mutations contribute to tumourigenesis, alternatively, the mutations may reflect the increased chromosomal instability associated with neoplasia. None the less, what is clear is that the locus specific minisatellites
are recombinational hot-spots, which may interact in various chromosomal processes, including homologue recognition and meiotic recombination (Jeffreys, et al, 1988, 1991).

4.6. Involvement of the Retinoblastoma Susceptibility Locus in Colorectal Carcinogenesis.

In accordance with the view that the retinoblastoma susceptibility gene (Rb) acts a tumour suppressor, allele losses and inactivating mutations have been identified at this locus in various malignancies, including retinoblastoma (Friend, et al, 1987, Horowitz, et al, 1989). At variance with this trend, it would appear that in a proportion of colorectal tumours the Rb gene is over-represented. In this study, no intragenic rearrangements or deletions were detected at the Rb locus in the DNA of 21 sporadic cancers, 23 adenomas from 10 FAP patients and 7 colorectal carcinoma cell lines. However, an increased copy number at the Rb locus was clearly apparent in DNA from the Dukes stage C carcinomas of patients 149 and 823. Amplification of the Rb locus has been reported in some 30% of primary colorectal carcinomas by at least two groups (Gope, et al, 1990, Meling, et al, 1991, Lothe, et al, 1992). Although amplification may be confined to the Rb gene alone, in the majority of cases it would appear that extensive regions of chromosome 13q were duplicated (Lothe, et al, 1992). Amplification of such large stretches of DNA, was reflected in the observation that 78% of the tumours in which the Rb gene was amplified, were also aneuploid (Meling, et al, 1991). Duplication of all or part of chromosome 13 has been observed in up to 50% of colorectal carcinomas cytogenetically (Reichmann, et al, 1981, Muleris, et al, 1987). In the tumour of patient 823, the long arm of chromosome 13 had been duplicated in the formation of an isochromosome, which replaced one of the normal copies of chromosome 13 (J.D.A. Delhanty, personal communication).

In addition to the genomic data, analysis of Rb gene expression in this study, revealed significantly elevated levels of the Rb transcript in the RNA of 19/20 adenomas from two FAP patients and in 10/20 sporadic colorectal carcinomas. Therefore, although genomic and expression data was not available from the same samples, it would appear likely that the increased levels of the Rb gene transcript were occurring
independently of gene dosage, in the majority of cases. Similar expression results were obtained by Gope et al (1990) and Lothe et al (1992), who reported elevated levels of a normal sized Rb transcript in 61% to 76% of primary colorectal adenocarcinomas by Northern hybridization.


Evidence exists to suggest that the Rb gene can regulate its own transcription via feedback mechanisms (Dunn, et al, 1989). In the non-tumour tissues of patients carrying
germline Rb mutations, only the normal transcript was detectable, but at a level which compensated for the lack of expression from the mutated allele (Dunn, et al, 1989). Furthermore, in tumours where both alleles were mutated, Rb expression was predominantly from the allele carrying the less severe mutation (Dunn, et al, 1989). Thus, in colorectal adenomas and carcinomas various processes could interact with the Rb transcription feedback mechanism, and thereby induce increased expression of the Rb gene. A reduction in the half life of the Rb transcript or peptide, or the presence of novel cellular components which complex with pRb and inhibit its normal function, could both influence the feedback pathways. Recently, amplification and over-expression of cyclin D2 was demonstrated in the colorectal cancer cell line 1196 (Leach, et al, 1993), this cyclin has been shown to be indirectly responsible for pRB phosphorylation (Ewen, et al, 1993). The status of cyclin gene expression in primary colorectal tumours has yet to be established. If cyclins were frequently overexpressed in colorectal neoplasia, pRB would be maintained in a perpetually hyperphosphorylated state. Transcriptional control mechanisms may operate, which detect this hyperphosphorylation and express more Rb mRNA in an attempt to provide the cell with unphosphorylated pRb. It would therefore be of great interest to study the levels of Rb and cyclin gene expression in equivalent colorectal tumour samples. Alternatively, in common with the p53 tumour suppressor gene, mutations may occur in the Rb gene during colorectal carcinogenesis, which result in enhanced expression of a potentially oncogenic product (Lothe, et al, 1992). Unlike p53, no mutational hotspots have been reported in the Rb gene (Canning & Dryja, 1989). However, several point mutations have been reported in exon 21 (Kaye, et al, 1990, Horowitz, et al, 1990), which result in defective phosphorylation of the peptide, and reduced binding of pRb to SV40 large T antigen and adenovirus E1A oncoprotein (Kaye, et al, 1990). Similar findings were reported in a bladder carcinoma cell line, where exon 21 was absent following a splice site mutation (Horowitz, et al, 1989). In light of these observations Hovig et al (1992) examined exon 21 in a
number of tumours. No mutations were detected in any of the samples analysed, including 6 colorectal cancers, five of which were amplified at the Rb locus, whilst the remaining tumour was hemizygous for Rb (Hovig, et al, 1992, Lothe, et al, 1992). Clearly these findings do not exclude the possibility that activating mutations may be occurring elsewhere in the Rb gene, or that the elevated levels of the Rb transcript simply reflect generally aberrant gene expression in the neoplastic colorectal epithelia. Indeed, it would seem unlikely that Rb expression is associated with malignant change in FAP adenomas, for although the majority of adenomas were expressing significant quantities of Rb, only a few if any of those tested in this study would have ultimately progressed to adenocarcinomas.

The discovery of Rb gene amplification and over expression in colorectal carcinogenesis is of interest and although not a widespread phenomenon is not unique, as elevated Rb expression was also reported in an adrenocortical carcinoma from a Li Fraumeni patient (Warneford, et al, 1991).

4.7. Involvement of Mucoproteins in Colorectal Carcinogenesis.

Analysis of differential mucin expression in carcinogenesis has until comparatively recently, depended upon immunological and biochemical techniques (Gold & Miller, 1975, 1978, Boland, et al, 1982, 1986). Immunological assays relied upon the specificity of antibodies to changing carbohydrate and peptide moieties, whilst biochemical studies required lengthy extraction and analytical procedures. With the recent cloning of a number of mucoprotein gene sequences, it has now become possible to assess the levels of various mucin gene transcripts in RNA from surgical and other specimens. In this study the expression levels of the genes MUC1, MUC2 and MUC3 were analysed using the cDNA probes pMUC10, SMUC41 and SIB124 respectively (Gendler, et al, 1987, Swallow, et al, 1987a, Gum, et al, 1989, 1990).

The MUC1 gene encodes a membrane bound glycoprotein, whose expression has been identified on the apical surface of all secretory epithelia so far examined (Ho, et al, 1993). Previously known by many names including PUM (peanut lectin
binding urinary mucin) and PEM (polymorphic epithelial mucin), increased immunoreactivity to MUC1 epitopes has been reported in various adenocarcinomas (Gendler, et al, 1987, Ho, et al, 1993). Indeed, although the cDNA, pMUC10 was initially cloned from the breast cancer cell line MCF-7 (Gendler, et al, 1987), MUC1 specific transcripts have subsequently been isolated from both pancreatic and ovarian tumour cells (Lan, et al, 1990, Stern, et al, 1992). In this study the generally low level of MUC1 expression in normal colorectal mucosa, was reduced further in the majority of non-mucinous adenocarcinomas. The notable exception to this trend was the Dukes stage B carcinoma from the sigmoid colon of patient 681. In this sample the level of MUC1 expression exceeded that found in the two MUC1 producing mucinous cancers, 517 and 570, it was perhaps surprising therefore, that tumour 681 had not been classified as mucinous. For an adenocarcinoma to be described as mucinous or colloid, more than 50% of the tumour mass must be composed of extracellular mucin (Jass & Sobin, 1989). Hence it may be, that over-expression of MUC1 alone does not result in a tumour which was recognisably mucinous, and it is only over-expression of secreted mucins such as MUC2 and MUC3, as in tumours 517 and 570, which allows this classification to be applied. Alternatively, although the level of MUC1 RNA was elevated in tumour 681, this might not have been reflected in the amount of MUC1 peptide present in the cells, mutations in the MUC1 gene or elsewhere may have disrupted translation or post-translational processing of the MUC1 peptide. In contrast to the results obtained in the adenocarcinomas, the level of MUC1 RNA was elevated in the majority of the adenomas (16/21) from FAP patients 136 and 139.

The products of MUC2 and MUC3 genes are both intestinal mucopolypeptides, which are secreted into the intestinal lumen as long homopolymers (Gum, et al, 1989, 1990). MUC2 would appear to be expressed chiefly by the goblet cells of the colon and small intestine (Ho, et al, 1993), whilst the principle sites of MUC3 expression are the columnar cells of the small intestine (Ho, et al, 1993). In this study the normal level of MUC2 expression was dramatically reduced in all of the non-mucinous adenocarcinomas tested. The pattern
of expression in the adenomas from patients FAP136 and 139 were discordant, with 8/10 FAP136 adenomas showing increased MUC2 expression and 8/11 FAP139 adenomas showing reduced, there was no apparent clinicopathological correlation to these findings. The only remaining samples in which MUC2 expression was increased were the two mucinous tumours 517 and 570. Although MUC3 expression was reduced in 58% of the non-mucinous adenocarcinomas analysed in this study, elevated levels of expression were apparent in 30% of tumours and equivalent tumour and normal expression was found in 12% of samples. It was interesting to note that none of the mucinous cancers tested expressed significant quantities of MUC3. Although genomic and expression data were not available from the same samples in this study, it would seem unlikely that genomic events influence the levels of mucin gene expression, as genomic alterations at the MUC1 and MUC2 loci occurred less frequently than changes in expression. However, it would be interesting to see whether or not the novel MUC1 alleles found in some tumour samples were expressed.

Overall the results obtained in this study were in agreement with those of other reports, in that expression of the MUC1, MUC2 and MUC3 genes was depressed in the majority of non-mucinous colorectal carcinomas (Byrd, et al, 1991, Yonezawa, et al, 1991, Ogata, et al, 1992, Ho, et al, 1993). Loss of specific mucin expression may well be due to the general de-differentiation which accompanies colorectal carcinogenesis, whereby cells become depolarized, and the numbers of clearly defined goblet and columnar cells decline (Bussey, 1975, Muto, et al, 1975). In contrast to data obtained by RNA analysis, immunoreactivity of colorectal adenocarcinomas to mucin specific antibodies, was reported to be increased (Boland, et al, 1982, Ho, et al, 1993). It was suggested that aberrant glycosylation of the mucoproteins, may allow greater accessibility of antibodies to the peptide epitopes (Boland, et al, 1982, Burchell, et al, 1989, Ho, et al, 1993). Splice site variants of the MUC1 gene have been identified in cDNA isolated from breast and other cancer cell lines (Ligtenberg, et al, 1990, Wreschner, et al, 1990, Stern, et al, 1992). The products of such alternate splicing
would have different signal peptides and altered carboxy-terminal hydrophobicities (Ligtenberg, et al, 1990, Wreschner, et al, 1990). These observations led to speculation that the variant forms of the MUC1 peptide could localize to different cellular compartments and may follow different glycosylation pathways, thus resulting in tumour associated peptides with altered immunoreactivity (Ligtenberg, et al, 1990, Wreschner, et al, 1990). However, the single base pair transition responsible for the alternate splicing, was shown to be a polymorphism present in the normal population, at a frequency comparable to that of the originally identified form (Ligtenberg, et al, 1991). Hence suggesting that alternate splicing of the MUC1 transcript does not significantly alter the function of the mature peptide (Ligtenberg, et al, 1991).

Although mucin expression was predominantly reduced in colorectal carcinogenesis, approximately 15% of all colorectal tumours may be classified as mucinous (Symonds & Vickery, 1976). Associated with an early age of onset, a high metastatic potential and a generally poor prognosis, mucin expression in such cancers clearly influences tumour biology (Umpleby, et al, 1985, Kuan, et al, 1987). In this study elevated levels of MUC1 and MUC2 RNA were detected in two of the five mucinous carcinomas analysed. Expression of the three mucin genes MUC1, MUC2 and MUC3 in the remaining samples, was either below or equivalent to normal. It may be that these samples were expressing aberrantly a mucin gene other than those tested, possibly MUC4, MUC5 or MUC6. Therefore, it would be of interest, to analyse the expression of these additional mucin genes in the samples tested in this study. However, the findings reported here do demonstrate that mucinous carcinomas are heterogeneous with respect to mucin gene expression. Some clues to the role of inappropriate mucin expression in colorectal tumourigenesis, have been eluded to from studies of mucin secreting cancer cell lines. In contrast to the parental cells, a high mucin producing variant of the colon adenocarcinoma cell line HT29, lacked contact inhibition and grew as aggregates in culture (Hanski, et al, 1992a). This variant, which grew at a fast rate in vitro, and was tumourigenic in athymic mice, was
shown to be expressing MUC3 and not MUC2 mRNA (Hanski, et al, 1992a, Niv, et al, 1992). Thus, although none of the mucinous tumours analysed in this study over-expressed MUC3, this gene can apparently contribute to the mucinous tumour phenotype. However, this was not always the case, as over-expression of MUC3 was found in seven non-mucinous tumours analysed in this study. MUC2 over-expression was reported in the mucinous variant (LS-LiM6) of the colon cancer cell line LS174T (Bresalier, et al, 1991). LS-LiM6, was found to adhere to and invade basement membranes in vitro, more readily than its parental counterpart (Schwartz, et al, 1992). Furthermore, this effect could be reproduced in the parental line, by addition of colonic mucin to the culture medium (Schwartz, et al, 1992). In common with the mucinous HT29 line, LS-LiM6 was also tumourigenic in nude mice, and the extent of liver metastasis was found to be proportional to mucin production (Bresalier, et al, 1991). Taken together, these studies suggest that the poor prognosis associated with mucinous colorectal tumours depends largely on their metastatic potential.

At present little information is available concerning the etiology of mucinous carcinomas. However, it would seem likely that they develop from adenomas, as prolonged in vitro culture of the adenoma cell line PC/AA, results in a carcinoma cell line with a mucinous phenotype (Pareskeva, et al, 1992). In this study mucin expression was elevated in the majority of adenomas. Pilbrow et al (1992) were able to detect increased expression of mucin antigens in adenomas at the earliest stages of development. Furthermore, they were able to correlate increased reactivity to small intestinal mucin antibodies with increasing adenoma size. No such associations were found in the results obtained from this study, even amongst the adenomas of patient FAP139, where MUC2 expression was variable. It would seem however, that elevated MUC1 and MUC2 expression is a transient feature in the adenoma-carcinoma sequence, as their expression was generally depressed in carcinomas.

The regulation of mucin gene expression is clearly disrupted during colorectal and other carcinogenesis. Although some of the effects of aberrant mucin expression
have been revealed, analysis of tumour specimens and cancer cell lines with all of the presently available cDNA probes, may help to further characterise the role of mucin expression in carcinogenesis.

4.8. Involvement of c-myc in Colorectal Carcinogenesis.


In this study c-myc expression was assessed in 17 sporadic colorectal carcinomas and 21 adenomas from two FAP patients. Elevated levels of c-myc RNA were detected in 53% of carcinomas and 52% of adenomas. These results were broadly in agreement with published reports of c-myc expression in colorectal tumours (Erisman, et al, 1985, 1988, Rothberg, et al, 1985, Sikora, et al, 1987, Sugio, et al, 1988, Finlay, et al, 1989b, Viel, et al, 1990). No clear association has been established between c-myc expression and clinical outcome in colorectal neoplasia (Erisman, et al, 1988, Viel, et al, 1990). However in this study, three of the four Dukes stage C carcinomas tested over-expressed c-myc, whilst over-expression was only evident in 3/9 Dukes B cancers. Similarly, levels of c-myc RNA were elevated in 71% of the FAP adenomas that showed signs of moderate dysplasia, but in only 50% of those with mild dysplasia. These findings suggest that a correlation may exist between c-myc expression and cellular dysplasia and tumour invasiveness, although the significance of this association is limited by the small sample size.

normal mucosa, c-myc staining was limited to the nuclei of approximately 25% of cells in the lower third of the crypts of Lieberkühn, thereby mirroring the distribution of proliferating cells (Stewart, et al, 1986, Royds, et al, 1991, 1992, Melhem, et al, 1992). Concomitant with expansion of the proliferative zone during adenoma formation, the distribution of c-myc staining cells also spread, such that p64c-myc was detected in 50-100% of adenoma cells, including some on the luminal surface (Royds, et al, 1992, Melhem, et al, 1992). In addition to a greater distribution of c-myc expressing cells, some of the adenoma cells were stained more intensely than their normal counterparts, implying either an increase in c-myc expression, or a reduction in c-myc RNA or p64c-myc breakdown (Melhem, et al, 1992). As in the normal mucosa, p64c-myc was chiefly restricted to the nucleus in adenoma cells (Melhem, et al, 1992). This pattern was not maintained in the carcinoma cells however, where cytoplasmic staining was also apparent, suggesting that the nuclear targeting of p64c-myc may have been disrupted (Royds, et al, 1992, Melhem, et al, 1992). Although c-myc positive cells were identified in all of the carcinomas analysed, the pattern of staining was heterogeneous, with a few cells stained in some tumours and the majority stained in others (Melhem, et al, 1992). One surprising observation, was abnormal p64c-myc staining in the normal mucosa surrounding some carcinomas, it was suggested that the increased c-myc expression may have been in response to growth factors released from the cancer cells (Melhem, et al, 1992). This expression of c-myc in normal colorectal epithelia, reinforces the view that de-regulated c-myc expression alone is not sufficient to bring about cellular transformation (Land, et al, 1983b, Melhem, et al, 1992).

has been reported between c-myc expression and the site in the colorectum from which tumours arise, such that cancers from the descending (left) colon over-express c-myc more frequently than those of the ascending (right) colon (Rothberg, et al, 1985, Erisman, et al, 1988). This association could not be tested in the samples examined in this study, as they all originated in the sigmoid colon and rectum. The observation that FAP-associated carcinomas also arise predominantly in the descending colon (Bussey, 1975), led to speculation that the APC tumour suppressor gene on chromosome 5q may contribute to the regulation of c-myc expression (Rothberg, et al, 1985). It appeared that c-myc expression could be modulated by trans-activating factors, as fusions between cells which expressed c-myc normally and those that did not, resulted in hybrids with regulated c-myc expression (Erisman, et al, 1989, Rodriguez-Alfageme, et al, 1992). Furthermore, introduction of human chromosome 5 by microcell fusion, into cells which were aberrantly expressing c-myc, led to reregulation of c-myc expression (Rodriguez-Alfageme, et al, 1992). Together with the observation that chromosome 5 allele losses were only found in those tumours with elevated c-myc expression (Erisman, et al, 1989), these data provide tantalising evidence for the presence of a gene on chromosome 5 which directly or indirectly modulates c-myc expression (Erisman, et al, 1989, Rodriguez-Alfageme, et al, 1992). Although in this study genomic data was not available from the sporadic carcinomas for which c-myc RNA levels had been established, no chromosome 5 allele losses were observed in any of the FAP136 or FAP139 adenomas (M. Rees, personal communication). Now that the APC tumour suppressor gene from 5q21, has been cloned (Joslyn, et al, 1991, Kinzler, et al, 1991b), it may be possible to establish whether or not this gene influences c-myc expression.

One way in which the transcription of c-myc may be controlled, is by the degree of DNA methylation at the CpG dinucleotide in the CCGG sequence within exon 3 of the gene (Cheah, et al, 1984). In many cases hypomethylation of CpG dinucleotides at the 5' end of genes has been associated with gene expression (Bird, 1986). For c-myc though, these sites would not appear to be important, as they were unmethylated
even in cells which were not expressing c-myc (Mango, et al, 1989). However, the CpG in exon 3 was methylated in normal tissue, but became hypomethylated in a number of cancer cell lines (Cheah, et al, 1984). Analysis this site in colorectal neoplasia, revealed a gradual increase from low levels of hypomethylation in normal epithelia, to progressively higher levels in adenomas, carcinomas and metastases (Sharrard, et al, 1992). This pattern would appear to follow that of increasing c-myc expression in the various stages of colorectal carcinogenesis. General hypomethylation of DNA in premalignant colorectal lesions was reported as an early event in carcinogenesis (Goelz, et al, 1985). The results of Sharrard et al (1992) therefore, provide evidence that one of the targets for this general hypomethylation and consequent deregulated gene expression may be c-myc. It is of interest to note that a number of chemical carcinogens specifically inhibit DNA methylation (Wilson & Jones, 1983), thus the presence of such agents in the diet could contribute to deregulated oncogene gene expression in colorectal epithelia.

Although c-myc is over-expressed in a proportion of colorectal cancers, the significance of this finding has yet to be fully understood. In vitro over-expression of c-myc increases the responsiveness of cells to the mitogenic effects of growth factors, such as platelet derived growth factor (PDGF) and epidermal growth factor (EGF) (Armelin, et al, 1984, Sorrentino, et al, 1986, Stern, et al, 1986). Furthermore, in experiments where v-myc was introduced into a small number of cells in a reconstituted organ, areas of hyperplasia developed (Thompson, et al, 1989). However, carcinomas only arose when H-ras was also introduced into the cells (Thompson, et al, 1989), thus reinforcing the view that ras and myc oncogenes can cooperate to bring about cellular transformation (Land, et al, 1986, Weinberg, et al, 1985). Activating mutations in the ras oncogenes have been reported in approximately 58% of adenomas and 40% of carcinomas (Vogelstein, et al, 1988). The adenomas of FAP136 and FAP139 were analysed for mutations in K-ras (M. Rees, personal communication). A mutation in codon 12 was identified in P4 of FAP139, although the level of c-myc expression was elevated in this sample it was not particularly high. It
would be interesting examine the ras genes in the other samples for which c-myc expression levels were known, as a correlation may exist between ras mutations and c-myc expression. One further factor which should be taken into account when considering the role of c-myc in carcinogenesis is Max (myc associated 'X' factor). Like p64\(^{c-myc}\), the Max peptide has basic helix-loop-helix (bHLH) and leucine zipper (LZ) domains, which are thought to be involved in peptide dimerization and sequence specific DNA binding (Blackwood & Eisenman, 1991, Prendergast, et al, 1991). Recently it has been demonstrated that the oncogenic activity of c-myc depends upon dimerization between p64\(^{c-myc}\) and Max (Amati, et al, 1993), thus alterations in Max could also influence c-myc activity in carcinogenesis.

4.9. Involvement of p53 Tumour Suppressor Gene in Colorectal Carcinogenesis.

Although a number of studies have examined mutations in the p53 gene, and analysed expression of the p53 gene product with specific antibodies, few have assessed the levels of p53 mRNA present in normal and neoplastic colorectal mucosa. In this study p53 expression was investigated by hybridization of a p53 cDNA probe (pR4.2) to mucosal RNA samples. Elevated levels of p53 RNA were detected in 50% of 38 sporadic carcinomas and 94% of 36 adenomas from six FAP patients, by dot and slot blot hybridizations. Northern hybridizations also demonstrated the presence of significant quantities of a normal sized p53 transcript in a number of tumour samples. No clear correlation was identified between cellular dysplasia and p53 expression in this study. However, the highest levels of p53 RNA were detected in FAP adenomas and Dukes stage B carcinomas, whereas levels equivalent to or below normal were detected in 70% of Dukes stage C cancers. These results, which were in agreement with those presented by Lothe et al (1992), suggest that p53 gene expression declines as tumours take on an invasive phenotype. In contrast to this finding, the incidence of allele loss and point mutation at the p53 locus increases with tumour progression (Vogelstein, et al, 1988, Miyaki, et al, 1990, Baker, et al, 1990a, Kikuchi-Yanoshita, et al, 1992). Consequently, p53 allele losses were reported in less than 10% of adenomas, and although over a hundred mutations have been reported in colorectal carcinomas, only 14 have been identified in adenomas (Vogelstein, et al, 1988, Nigro, et al, 1989, Miyaki, et al, 1990, Baker, et al, 1990a, Rodrigues, et al, 1990, Han, et al, 1991, Shirasawa, et al, 1991, Ishioka, et al, 1991, Lothe, et al, 1992, Kikuchi-Yanoshita, et al, 1992b). The combined occurrence of mutations and allele losses at the p53 locus imply that an association may exist between these two events, indeed although p53 gene mutation appears to precede allele loss, it has been suggested that tumour cells gain a further growth advantage by loss of the remaining normal p53 allele (Baker, et al, 1990a, Rodrigues, et al, 1990). Of the samples examined in this study, the adenomas from patients FAP136, 172
FAP139, FAP146, FAP147 and FAP164 were analysed with chromosome 17p polymorphic DNA markers (M. Rees, personal communication). Loss of heterozygosity was found solely in the adenoma of FAP146, the level of p53 RNA in this sample was only moderately elevated.

Cancer associated missense mutations in the p53 gene, have been shown to result in a mutant peptide with an extended biological half life (Kopelovich & DeLeo, 1986, Finlay, et al, 1988). The increased stability of the p53 gene product, allows mutant forms of the peptide to be readily identified in surgical specimens using polyclonal and monoclonal antibodies. Immunocytochemical analysis of colorectal carcinomas revealed positively staining regions in 42% to 70% of cases (van den Berg, et al, 1989, Rodrigues, et al, 1990, Campo, et al, 1991, Scott, et al, 1991, Cunningham, et al, 1992, Starzynska, et al, 1992). The variable extent to which p53 was detected by immunological methods, was probably due to differences in the specificities of the antibodies used. Although no consistent correlation was observed between p53 staining and clinicopathological factors, levels of the p53 peptide generally rose as tumours progressed from adenoma to carcinoma (van den Berg, et al, 1989, Rodrigues, et al, 1990, Campo, et al, 1991, Scott, et al, 1991, Cunningham, et al, 1992, Starzynska, et al, 1992). In some cases p53 staining was associated with high grade dysplasia and a poor prognosis (van den Berg, et al, 1989, Starzynska, et al, 1992). Additionally, non-mucinous cancers or those originating from the right colon, were found to be stained with p53 specific antibodies more frequently than, mucinous or left sided tumours (Scott, et al, 1991, Hanski, et al, 1992b). These results serve to further emphasise the negative relationship that is becoming apparent between increased p53 gene expression and p53 gene mutation. As 4/5 mucinous tumours analysed in this study, expressed significant levels of p53 RNA. Cunningham et al (1992) found that 76% of p53 positively staining cancers had lost one p53 allele. Furthermore, unlike the heterozygous tumours, those with allele loss were frequently stained with all three of the monoclonal antibodies used. It was suggested therefore, that oligomerization between the normal and mutant peptides

The p53 gene product is a 393 amino acid nuclear phosphoprotein, which can oligomerize to form homotetramers (Lane & Crawford, 1979, Lamb & Crawford, 1986, Stenger, et al, 1992, El-Deiry, et al, 1992). With sequence specific DNA binding properties (Kern, et al, 1991a, Bargonetti, et al, 1991), wild-type p53 acts as a transcription factor, activating the transcription of genes adjacent to p53 binding sites (Raycroft, et al, 1990, Kern, et al, 1992, Farmer, et al, 1992). It is thought that this transcriptional activation may be regulated by cellular factors which facilitate sequence specific binding of p53 to DNA (Hupp, et al, 1992). Although the exact targets of p53 activation have yet to be identified, a consensus p53 DNA binding sequence has now been defined in human genomic DNA (El-Deiry, et al, 1992). Functionally; the p53 peptide may be divided into three domains, the amino-terminal region appears to be responsible for transcriptional activation (Fields & Jang,

this view, a comparison of stabilized p53 peptide levels and chromosomal abnormalities in a series of colorectal cancers, revealed that the majority of those with immunocytochemically detectable p53 were also aneuploid (Carder, et al, 1993).

Unfortunately, the mutational status of the p53 expressed in the colorectal samples examined in this study was not established. However, in view of the published data, it would seem unlikely that the p53 expressed in the adenomas carried mutations. The marked overexpression identified in these samples, could therefore represent a response to the presence of genetic lesions, such as those responsible for adenoma formation. If this were the case, adenoma cells would be expected to undergo growth arrest or apoptosis, as this apparently does not occur, the p53 in these cells may be functionally inactive. In addition to point mutation and interaction with viral oncoproteins, p53 bioactivity can also be ablated by association with the cellular oncoprotein MDM-2 (murine double minute 2 gene) (Momand, et al, 1992). Amplification and overexpression of MDM-2, has been reported in at least a third of human sarcomas (Oliner, et al, 1992). It would be interesting therefore, to determine whether or not MDM-2 was involved in colorectal carcinogenesis, possibly inactivating the p53 apparently overexpressed in adenomas.

effect (Levine, et al, 1991). Recently, Dittmer et al (1993) have shown that in the absence of normal p53, some mutant forms actually gain tumourigenic function, they suggest that such forms could be responsible for more aggressive and tumourigenic cancers.

Release from p53 growth control clearly plays an important role in colorectal carcinogenesis. The results presented in this study suggest that an inverse relationship may exist between p53 gene expression and missense gene mutation. It would seem that once a cell has acquired a p53 mutation, the rate of p53 gene transcription declines, possibly by some mechanism of autoregulation which depends upon the presence of functional p53. Further analysis of the p53 gene in the samples examined in this study would help to establish whether or not a relationship does exist between p53 mutation and gene expression.

4.10. Involvement of Carbonic Anhydrase 1 in Colorectal Carcinogenesis.

Carbonic anhydrase 1 (CA1) catalyses the reversible hydration of CO\(_2\) and consequently plays a crucial role in pH regulation and CO\(_2\) excretion (Tashian, 1989). Although carbonic anhydrases are expressed along the length of the gastrointestinal tract, CA1 expression occurs predominantly in the large intestine (Lonnerholm, et al, 1985). In this study, levels of the CA1 transcript were significantly reduced or undetectable in all of the sporadic colorectal cancers tested. In FAP adenomas, CA1 expression was similarly reduced, however, in three mildly dysplastic adenomas the levels of CA1 expression were equivalent to those found in normal mucosal RNA. Indeed, it appeared that CA1 expression was influenced by the degree of cellular dysplasia in the colorectal samples. These findings contributed to an ongoing interest in the control of CA1 expression, and prompted further analysis of CA1 in normal and neoplastic colorectal mucosae (Sowden, et al, 1993).

As mentioned earlier, CA1 has two promotors, the distal promotor is specific for erythroid cells, whilst the proximal one is specific for colonic epithelium (Brady, et al, 1991). Although transcription from both promotors results in identical peptides, the 5' leader sequences differ. Based on
these differences, primers were designed to distinguish between the two transcripts in polymerase chain reactions (PCR) (Sowden, et al, 1993). Thus, although it was impossible to identify which transcripts were present in the colorectal RNA samples by slot blot hybridization, transcript specific PCR showed that only the colon specific promotor was active in these samples (Sowden, et al, 1993).

In an attempt to understand how neoplastic transformation influenced CA1 expression, in situ hybridization and immunohistochemistry were used to precisely define the sites of CA1 expression in colorectal epithelia (Sowden, et al, 1993). In situ hybridization with a CA1 specific RNA probe to normal colon tissue sections, revealed an absence of CA1 transcripts in proliferating cells at the base of the crypts of Lieberkühn. CA1 expression rose to maximal levels in the differentiating cells of the mid-crypts, and then declined as migrating cells approached the luminal surface. Thus, CA1 gene expression appeared to be a feature of differentiating colonic epithelial cells (Sowden, et al, 1993). Immunohistochemistry however, showed that the CA1 protein was not abundant in these differentiating cells, but accumulated instead in mature cells on the luminal surface of the colonic epithelium. It would appear therefore, that in addition to the transcriptional controls which were obviously governing CA1 expression in the colon, post-transcriptional mechanisms were also operating (Sowden, et al, 1993). Analysis of tumour tissue sections not only revealed a loss of cellular differentiation and crypt morphology, but also showed that loss of CA1 expression directly accompanied this dedifferentiation (Sowden, et al, 1993).

The levels of CA1 expression found in adenoma RNA varied between that seen in normal mucosa and carcinomas, this appeared to be dependent upon the degree of cellular dysplasia in the samples, in that the highest levels of CA1 expression were seen in mildly dysplastic adenomas. Thus although reduced CA1 expression was the earliest and most consistent change seen in the samples analysed in this study, it would seem likely that loss of CA1 expression was a consequence of cellular dedifferentiation, and was not
directly associated with malignant transformation (Sowden, et al., 1993).

4.11. Isolation of Chromosome 5 Specific Clones.

The familial adenomatous polyposis (FAP) locus was assigned to human chromosome 5q21 by linkage analysis (Bodmer, et al., 1987, Leppert, et al., 1987) and tumour deletion mapping (Solomon, et al., 1987). Taking into account the resources available, the novel approach of Wong et al. (1989) was adopted in this study, in an attempt to contribute the search for APC candidate genes. The strategy outlined by Wong et al. (1989), allows expressed sequences derived from genes in a specific chromosomal region to be identified. Initially the method relies upon cross hybridization between radiolabelled cDNA molecules, present in a tissue specific cDNA library, and genomic clones from a chromosome specific genomic library. Once genomic clones have been identified and mapped back to the relevant chromosomal region, they are in turn used to identify individual clones from the cDNA library.

In this project therefore, the cross hybridization strategy required a normal colonic cDNA library and a chromosome five genomic library. Although normal colon cDNA libraries were available commercially, they were all derived from normal mucosa adjoining neoplastic lesions. As such apparently normal tissue may have undergone some preliminary malignant changes, or may be influenced by the nearby tumours, these libraries were considered unsuitable for use in this study. Fortunately, a sample of stripped colonic mucosa was available in the laboratory, which was known to have no association with any colorectal disorder. Hence, RNA from this sample was used to construct a cDNA library in the bacteriophage vector λgt10. The chromosome five specific genomic library was purchased from the ATCC, ideally a library containing only 5q or even 5q21 alone would have been used, however such a library was not available. Alternatively, a genomic library would have been constructed in this study, had a somatic cell hybrid line been available, which contained the relevant region of chromosome 5 as its sole human component.
It is important to note that the clones isolated in this study may have been identified solely on the basis of cross hybridization between repeat sequences in the cDNA and genomic libraries. This could be the case as no unlabelled human genomic DNA was used in the screening hybridizations, to quench non-specific DNA associations. Confirmation that the chromosome 5 genomic clones contained expressed sequences could have been obtained had the resultant single copy fragments been hybridized either to Northern blotted colon RNA or to the colon cDNA library. Alternatively, conserved sequences may have been identified in the genomic clones by the use of rodent DNA as a hybridization probe under conditions of low stringency (Kinzler, et al, 1991b). Although the clones isolated in this study require further characterization to establish whether or not they contain expressed sequences, this strategy has been applied successfully to a study aimed at the identification of genes from the X chromosome which may be involved in various ophthalmic disorders.
Six independent clones were isolated from the chromosome 5 genomic library, following screening with the colon cDNA library. Preliminary characterization of these clones revealed that they were all indeed derived from human chromosome 5. Fluorescent in situ hybridization (FISH) was attempted with all six original genomic clones, however results were only obtained with one clone, λ5.3, which was assigned to 5q23 (FISH performed by D.K. Griffin). It was considered that clones such as those isolated from the chromosome 5 genomic library, were probably unsuitable for use as probes in FISH experiments, because although the insert size was generally small (<7kb), repetitive sequences in the clones required quenching by preannealing with excess human genomic DNA. Thus, effectively reducing further the amount of probe DNA available for sequence specific hybridization. Cosmid clones were isolated from a genomic library using the single copy fragments from λ5.3 and λ5.5 (library screening performed by S.A. Gayther & C.M. Lawson). Unequivocal FISH results were obtained for the cosmid clones, with the λ5.3 derived cosmid (D5S321) mapping to 5q35 and the λ5.5 derived cosmid (D5S322) mapping to 5q31 (FISH performed by D.K.Griffin, S.A. Gayther & C.M. Lawson). The discrepancy between the FISH results obtained for λ5.3 and cos5.3iv (D5S321), may testify to the unreliability of FISH assignments made for small genomic clones, such as λ5.3 (4kb), indeed attempts to repeat the FISH with λ5.3, proved unsuccessful (D.K. Griffin, personal communication). These results may therefore indicate that the clones isolated from the chromosome five library, come within the lower limit of resolution obtainable for genomic probes by FISH. Had time permitted, the remaining genomic clones would have been precisely localized to chromosome 5, and the normal colon cDNA library would have been screened with any clones that mapped to 5q21.

Recently, a partial cDNA clone (XEH.8c) was isolated from a choroid/retina cDNA library using this approach (Wong, et al, 1993). XEH.8c maps to the region Xp11.3-q12, which
encompasses the loci for retinitis pigmentosa type 2, congenital stationary night blindness and Aland Island eye disease. The role of this cDNA in these conditions is currently under review. It would appear therefore that this approach does represent a valid method for the isolation of expressed sequences from a given chromosomal region.

Ultimately, the APC gene was identified by two groups using positional cloning (Joslyn, et al, 1991, Kinzler, et al, 1991b). Joslyn et al (1991) isolated expressed sequences from yeast artificial chromosomes (YAC) spanning the germline deletions carried by two unrelated FAP patients, whilst Kinzler et al (1991b) identified expressed sequences in the YACs which made up three contigs representing a total of 5.5mb of DNA between the markers YN5.64 and YN5.48 on chromosome 5q21.


The results presented in this study and elsewhere, demonstrate that colorectal carcinogenesis is a complex multistage process, involving both proto-oncogene activation and the disruption of tumour suppressor gene function. As a disease, colorectal cancer may be considered to be heterogeneous, in that a range of different genetic defects can be detected in individual tumours. Although a number of the genes commonly associated with this process have now been identified, namely the K-ras and c-myc oncogenes, and the APC, MCC, p53 and DCC tumour suppressor genes, many more remain to be discovered or implicated in colorectal carcinogenesis. Of the other genes involved in the initiation and promotion of colorectal neoplasia, evidence exists both from this study and published reports, that a tumour suppressor gene on chromosome 1p may play an important role in a subset of colorectal tumours (Leister, et al, 1990, Tanaka, et al, 1993). Furthermore, two recent studies individually implicate unidentified tumour suppressor genes on chromosomes 8p and 14q, in at least 50% of colorectal carcinomas (Cunningham, et al, 1993, Young, et al, 1993). Together with the allele losses detected at loci on chromosomes 1q, 7p, 7q and 11p in this study, much work is still required to identify all of the tumour suppressor genes associated in colorectal carcinogenesis.
Although the identity of some of the genes involved in colorectal carcinogenesis is now known, the precise functions and interactions of these genes have yet to be fully understood. As more information becomes available, other genes are also implicated in colorectal carcinogenesis, for example, activation of c-myc is now known to be dependent upon dimerization between the p64-c-myc peptide and another protein, Max (Amati, et al, 1993). Thus, control of Max expression in colorectal carcinogenesis becomes of interest. In addition, interactions between normal and mutated gene products can influence the manifestation of a given gene defect, such as occurs with p53, where oligomerization between the mutant and wild type peptides result in a dominant negative effect (Milner & Medcalf, 1991). Preliminary data suggests that similar interactions arise in vivo, between the wild type APC peptide and truncated mutant forms (Su, et al, 1993), the functional implications of this oligomerization have yet to be explored.

Following a genome wide search with 345 microsatellite markers, genetic linkage has recently been established in a number of families, between hereditary nonpolyposis colorectal cancer (HNPCC) and loci from chromosome 2p15-16 (Peltomäki, et al, 1993, Aaltonen, et al, 1993). Supposing that in common with other cancer predisposing genes, the HNPCC gene had tumour suppressing activity, Aaltonen et al (1993), analysed matched tumour and normal DNA samples for allele loss at this locus. However, rather than allelic deletions, they detected changes in the electrophoretic mobility of the dinucleotide repeat alleles at this locus in the majority of HNPCC tumours. The finding that all microsatellite loci analysed, were similarly affected, prompted speculation that the HNPCC gene may be involved in the control and fidelity of DNA replication (Aaltonen, et al, 1993, Thibodeau, et al, 1993, Ionov, et al, 1993). In addition to tumours arising in patients with a family history of cancer, widespread microsatellite instability was also detected in between 12% and 28% of apparently sporadic tumours (Aaltonen, et al, 1993, Thibodeau, et al, 1993, Ionov, et al, 1993). However, in common with the HNPCC cancers, the majority of these sporadic tumours were derived
from the proximal colon and had developed at an early age. Such tumours may therefore represent, the familial component of colorectal malignancies in the general population (Burt, et al, 1985, Cannon-Albright, et al, 1988, Aaltonen, et al, 1993, Thibodeau, et al, 1993, Ionov, et al, 1993). Although it is difficult to establish what effect somatic microsatellite instability has on normal cellular function, the activity of those genes which actually contain dinucleotide repeats, such as the DCC tumour suppressor, may well be disrupted (Fearon, et al, 1990, Thibodeau, et al, 1993). In addition to the instability identified at dinucleotide microsatellite repeats, bands shifts were also reported in stretches of adenosines at the end of Alu repeat sequences and in a trinucleotide repeat sequence (Ionov, et al, 1993, Aaltonen, et al, 1993). The size changes observed at various minisatellite loci in colorectal tumours (Thein, et al, 1987, Armour, et al, 1989), may also be caused by mutation of the HNPCC gene on chromosome 2. In this study, size shifts and novel alleles were detected in tumour samples at the MUC1, λMS31 and λMS32 hypervariable loci. A single locus was affected in one FAP adenoma and 5 sporadic cancers, whilst two loci were mutated in a further 2 carcinomas. It would be interesting therefore, to assess the level of microsatellite instability in these samples and those in which no mutations were identified, in an attempt to establish whether or not, replication errors brought about by mutations in the HNPCC gene, were also responsible for minisatellite alterations.

REFERENCES


Eva, A., Robbins, K.C., Andersen, P.R., Srinivasan, A.,
Tronick, S.R., Reddy, E.P., Ellmore, N.W., Galen, A.T.,
Lautenberger, J.A., Papas, T.S., Westin, E.H., Wong-Staal,
analogous to retroviral onc genes are transcribed in human

Ewen, M.E., Sluss, H.K., Sherr, C.J., Matsushime, H., Kato,
the retinoblastoma protein with mammalian D-type cyclins.
Cell, 73, 487-497.

Farmer, G., Bargonetti, J., Zhu, H., Friedman, P, Prywes, R.

Fearon, E.R., Cho, K.R., Nigro, J.M., Kern, S.E., Simons,
J.W., Rupert, J.M, Hamilton, S.R., Preisinger, A.C., Thomas,
G., Kinzler, K.W. & Vogelstein, B. (1990). Identification of
a chromosome 18q gene that is altered in colorectal cancers.
Science, 247, 49-56.


Ferti-Passantonopoulou, A., Panani, A., Avgerinos, A. &
Raptis, S. (1986). Cytogenetic findings in a large bowel

Fey, M.F. & Thein, S.L. (1988). Somatic rearrangement of the
tropomyosin-receptor-kinase (trk) oncogene is rare in

Fey, M.F., Hesketh, C., Wainscoat, J.S., Gendler, S. & Thein,

transcription activating sequence in the p53 protein.
Science, 249, 1046-1049.

radiolabelling DNA restriction endonuclease fragments to high

Fienberg, A.P. & Vogelstein, B. (1983b). Hypomethylation of

by p53 produce a gene product that forms as hsc70-p53 complex

proto-oncogene can act as a suppressor of transformation.
Cell, 57, 1083-1093.

Finlay, G.G., Schulz, N.T., Hill, S.A., Geiser, J.R., Pipas,
in different stages of human colorectal cancer. *Oncogene*, 4, 963-971.


p53 primary amino acid sequence are implicated in stable complex formation with simian virus 40 T antigen. J. Virol., 62, 3903-3906.


APPENDIX.

1. Materials.

1.1. Reagents.

1.1a Chemicals.

All chemicals were purchased from either British Drug House, Poole, Dorset, or from Sigma Chemical Company, Poole Dorset, except for the following: bovine serum albumin (BSA) nucleic acid enzyme grade; Bethesda Research Laboratories (BRL), Paisley, Scotland, TEMED; Biorad Laboratories Ltd., Herts., Saran-Wrap, Dow Chemical Company, Michigan, USA.

1.1b. Radiochemicals.

All radiochemicals were obtained from Amersham International Plc., Amersham, Bucks. On occasion 5'[^32P]dCTP was purchased from ICN Biomedicals Inc., California, USA.

1.1c. Enzymes.

All restriction endonucleases (which were supplied with the appropriate reaction buffer) and large fragment DNA (Klenow) polymerase were purchased from BRL, Paisley, Scotland. Other DNA and RNA modifying enzymes were purchased as specified in the text.

1.2. Media.

All Bacterial growth media was prepared in deionized water and was autoclaved at 15 lb/sq.inch, 120°C for 20 minutes. Thermolabile ingredients were filter sterilized using 0.2mm nitrocellulose filters (Flow Laboratories, Rickmansworth, Herts) and were added to the media after autoclaving. Bacto-agar, Bacto-tryptone and Bacto-yeast extract were all purchased from Difco Laboratories, East Molesly, Surrey.

1.2a. Recipes.

L-broth: 1% tryptone (w:v), 0.5% yeast extract (w:v), 0.5% NaCl (w:v).
L-agar: (for plates) L-broth with 1.5% agar (w:v).
L-top agar: L-broth with 0.8% agar (w:v).
L-glycerol broth: L-broth with 15% glycerol (v:v).

SM buffer (phage dilution buffer): 50mM Tris.HCl pH 7.5, 100mM NaCl, 10mM MgSO₄.7H₂O, 0.1% gelatin.

1.2b. Antibiotics.

Ampicillin: 200mg/ml stock solution was stored at -20°C and diluted to 200µg/ml for use.

Tetracycline: 50mg/ml stock solution in 1:1 methanol:water was stored at -20°C and diluted to 50µg/ml for use.

1.3. Buffers.

TE: 10mM Tris.HCl, 1mM EDTA adjusted to the required pH with concentrated HCl.

20xSSC: 3M NaCl, 0.3M sodium citrate pH 7.

20xSSPE: 3.6M NaCl, 0.2M sodium phosphate pH 7.7, 20mM EDTA

10xTBE: 50mM Tris.HCl pH 8.3, 50mM boric acid, 1mM EDTA.

PBS: phosphate buffered saline tablets made up as recommended (Sigma Chemical Co) pH 7 with 266µg/ml phenol red.

All other buffers are given in the text.

1.4. Organic Solvents.

1.4a. Phenol.

Phenol was purchased in crystalline form. It required further purification both to remove denaturing contaminants and to raise the pH. The phenol was melted at 60°C and 0.1% 8-hydroxyquinoline (w:v) was added. This acts as an antioxidant, a weak chelator of metal ions and a partial inhibitor of ribonucleases. It also gives a yellow colour to the phenol so aiding recognition of the organic phase during extractions. Several volumes of 0.1M EDTA were mixed with the phenol to form an emulsion, this was allowed to settle into aqueous and organic phases. The upper aqueous phase was siphoned from the phenol and replaced with an excess of 0.1M Tris.HCl pH 8, 10mM EDTA, an emulsion was formed, allowed to settle and again the aqueous phase was removed as before. This procedure was repeated with TE pH 8 until the pH of the aqueous phase reached 7.5 after extraction. Finally the TE
was replaced with 10mM Tris.HCl pH 7.5 mixed with the phenol to form an emulsion and frozen in aliquots at -20°C for storage.

1.4b. Chloroform.

Iso-amyl alcohol was added to chloroform at a ratio of 1:24 prior to use.

2. General Methods Associated with Nucleic Acid Manipulation.

2.1. Quantitative Analysis of Nucleic Acids.

The concentration of DNA and RNA in solution was determined spectrophotometrically. The absorbence of a dilution (1/100 or 1/500) of the sample was measured at 260nm with a path length of 1cm. A reading of 1 at this wavelength was equivalent to a concentration of 50μg/ml for double stranded DNA, 40μg/ml for RNA and 20μg/ml for single stranded oligonucleotide DNA. The absorbence was also measured at 280nm, if the ratio A\textsubscript{260}/A\textsubscript{280} was below 1.5 the sample was considered to be contaminated with proteins and was purified further by phenol and chloroform extractions and reprecipitation.

2.2. Electrophoresis of DNA.

2.2a Agarose Gel Electrophoresis.

Agarose gels were of the horizontal slab type. The concentration of the agarose (0.5 - 2.5% (w:v)), length of the gel (7, 14, 25cm), voltage and duration of electrophoresis were dependent on the size of the DNA fragments to be analysed.

Agarose was melted in TAE (40mM Tris.Acetate pH 8, 1mM EDTA, 0.2μg/ml ethidium bromide (MacDonell et al, 1977)), allowed to cool and cast into the appropriate mold. TAE was also the electrophoretic running buffer. The samples were loaded into the gel in 0.025% bromophenol blue (w:v), 0.025% xylene cyanol FF(w:v), 2.5% Ficoll type 400 (w:v)(Pharmacia LKB, Milton Keynes, Northants.).

After completion of electrophoresis the DNA was visualised by observing the fluorescence of the intercalated ethidium bromide on an ultraviolet transilluminator.
(wavelength 302nm), and a photograph was taken with either Polaroid 667 or 665 type black and white film. Latterly a video camera was also used (Video Copy Processor, Mitsubishi, Japan.).

2.2b. DNA Molecular Weight Markers.

DNA molecular weight markers for use in gel electrophoresis of DNA and RNA were purchased from BRL (Paisley, Scotland.) The markers were: 1kb DNA ladder with bands of 12.2, 11.2, 10.2, 9.2, 8.1, 7.1, 6.1, 5.1, 4.1, 3.0, 2.0, 1.6, 1.0, 0.5, 0.4, 0.34, 0.3, 0.2, 0.15, 0.1, 0.075kb; 123bp ladder with resolvable bands of 123, 246, 369, 492, 615, 738, 861, 984bp and HindIII digested bacteriophage λ DNA with fragments of 23.1, 9.4, 6.7, 4.4, 2.3, 2.0, 0.56, 0.12kb. In addition bacteriophage PM2 DNA (Boehringer Mannheim, Lewes, Sussex.) was digested to completion with HindIII to give fragments of 5.4, 2.3, 1.0, 0.47, 0.45, 0.27, 0.11kb.

2.2c. Radiolabelling DNA Molecular Markers.

Radiolabelled DNA molecular weight markers were used on gels which were to be analysed ultimately by autoradiography. The 3' recessed ends of HindIII digested DNA were filled in by the 5'→3' polymerizing activity of large fragment *E.coli* DNA polymerase (Klenow enzyme) using deoxynucleotide triphosphates including 5'-[α-32P] dCTP as the substrates (Drouin, 1980).

10μg HindIII digested DNA were incubated with 50mM Tris.HCl pH 7.8, 5mM MgCl₂, 10mM 2-mercaptoethanol, 1mg/ml BSA, 100μM dATP, dGTP, dTTP, 20μCi 5'-[α-32P] dCTP (3000 Ci/mMol) and 10 units Klenow polymerase in a volume of 40μl, at 20°C for 30 minutes. The reaction was stopped by dilution to 200μl with H₂O and the addition of SDS to 0.2% (w:v), and EDTA to 10mM, followed by phenol:chloroform and chloroform extractions. The radiolabelled molecular weight markers were stored frozen at -20°C.

2.2d. TBE Gradient Urea Polyacrylamide Gel Electrophoresis.

The products of DNA sequencing reactions were fractionated on thin (0.4mm) TBE gradient urea polyacrylamide gels. A 40% acrylamide stock solution was used which
comprised: 38% acrylamide (w:v), 2% N,N'-methylene-bis-acrylamide (w:v), this was prepared by dissolving the preweighed solids in water and deionizing with Dowex mixed bed resin for an hour. After removal of the resin by filtration the solution was degassed by application of a vacuum and stored at -20°C in aliquots. The two TBE gel mixes were: 0.5xTBE (150ml 40% acrylamide solution, 50ml 10xTBE, 460g urea/litre) and 5xTBE (150ml 40% acrylamide solution, 500ml 10xTBE, 460g urea/litre).

5ml of 5xTBE mix (to which a small quantity of bromophenol blue was added for visualisation) and 45ml 0.5xTBE mix were prepared for polymerization by the addition of 10 and 90μl 25% ammonium persulphate (w:v) respectively, and 10 and 90μl TEMED respectively, both solutions were mixed well. 5ml of the 0.5xTBE gel mix were taken up into a 10ml pipette, the 5xTBE mix was taken up into the same pipette and the solutions were mixed by the admission of an air bubble into the pipette. The contents were then poured between the plates of the sequencing gel apparatus (Sequi-Gen DNA Sequencing Cell, Bio-Rad Laboratories, California, USA.), the remainder of the 0.5xTBE mix was added and a flat edge was obtained at the top of the gel by placing the sharks tooth comb upside down between the plates. The acrylamide was allowed to set for at least 30 minutes. The comb was removed and the sequencing apparatus was assembled for a pre-run at 2000 volts, to allow the gel to warm to approximately 50°C before the samples were loaded.

2.3. Isolation of Insert DNA.

Fifty microgrammes of plasmid or cloned DNA were digested to completion with 40 units of the appropriate restriction enzyme according to the manufacturers specifications, for at least an hour. The reaction was heat inactivated and the whole digest was loaded onto a TAE agarose gel (appendix 2.2a) of a concentration suitable to separate the vector and insert fragments. After electrophoresis for an appropriate length of time, the gel was viewed under long wavelength ultraviolet light and the section of the gel containing the insert was carefully excised. The insert was purified as described by Heery et al (1990), the section of agarose was placed into a 0.5ml
microcentrifuge tube whose base had been pierced with a 21 gauge hypodermic needle and plugged with a small piece of siliconized glass wool, this tube was placed in a 1.5ml microcentrifuge tube and the whole assembly was spun at 6000g for 10 minutes. The eluant collected in the larger tube was stored at -20°C.

2.4. Hybridization of Radiolabelled DNA Probes to Immobilised Nucleic Acids.

2.4a. Transfer of DNA to Nylon Hybridization Membranes.

The routine analysis of DNA by Southern transfer and subsequent hybridization to radiolabelled DNA probes was essentially as described by Southern (1975).

DNA was transferred from agarose gels to Gene Screen Plus hybridization membrane (NEN DuPont, Southampton, Hants.) or Hybond-N+ (Amersham International Plc., Amersham, Bucks.) by capillary blotting.

Transfer was achieved as follows: for Gene Screen Plus the DNA was denatured by soaking for 30 minutes in 0.4N NaOH, 0.6M NaCl on a rocking platform, the gel was then neutralised in 1.5M NaCl, 0.5M Tris.HCl pH7.5 for 30 minutes. The pre-wetted Gene Screen Plus membrane was placed on top of the gel and the DNA was transferred to the membrane using 10X SSC as the transfer buffer, as detailed in the Gene Screen Plus specifications. Genomic DNA was blotted overnight, whereas cloned DNA could be blotted for as little as 10 minutes and up to three filters were taken from a single gel. After blotting, the membrane was denatured in 0.4N NaOH for 45 seconds, rinsed in 0.2M Tris.HCl pH 7.5 and allowed to air dry.

For Hybond-N+ the gel and the membrane were both rinsed briefly in 0.4N NaOH, this was also the transfer medium. Genomic DNA blots were left for at least 3 hours whilst cloned DNA could be transferred in as little as 10 minutes. After blotting the membranes were neutralised by a wash in 2xSSPE and either added directly to the prehybridization solution or stored still wet at 4°C until required.
2.4b. Prehybridization, Hybridization, and Posthybridization Treatment of Nylon Hybridization Membranes.

Gene Screen Plus membranes were prehybridized in sealed plastic sandwich boxes at 65°C for at least 15 minutes, in a solution of 1M NaCl, 1% SDS (w:v), 5% dextran sulphate (w:v) (Pharmacia LKB, Milton Keynes, Northants.), with constant agitation. Hybridization followed addition of the denatured radiolabelled DNA probe (50-200ng) (appendix 2.4d), together with denatured salmon testes DNA (final concentration 100μg/ml) (appendix 2.4e) to the prehybridization mixture. After at least 16 hours hybridization at 65°C, the membranes were subjected to a series of washes (according to manufacturers specifications) to remove excess unhybridized probe. The level of radioactivity remaining on the filters was monitored with a Geiger counter after each wash, and successive washes were only performed if significant quantities of signal were detected. Residual buffer was blotted from the filters (they were never allowed to dry completely), and they were wrapped in Saran-Wrap for autoradiography.

Hybond-N+ and Hybond-N membranes were treated in a similar fashion to the Gene Screen Plus filters. In addition to using plastic sandwich boxes, Hybond filters were also successfully hybridized in the bottles of the Hybaid Hybridization oven (Hybaid Ltd., Teddington, Middlesex.). The prehybridization was performed at 65°C for least 15 minutes in 5xSSPE, 0.5% SDS (w:v), 5xDenhardt's solution (0.1% BSA fraction V (w:v), 0.1% Ficoll 400 (Pharmacia LKB) (w:v), 0.1% polyvinylpyrolidone (w:v)). The denatured radiolabelled probe and salmon testes DNA were added as above and hybridization proceeded for 16 hours or more. The washing regime for the Hybond membranes was followed in a similar way to that for Gene Screen Plus membranes, as specified by the manufacturers.

2.4c. Rehybridization of DNA and RNA.

Probe DNA was removed from hybridized Gene Screen Plus and Hybond-N+ filters to allow rehybridization to new probes. At no point were the filters allowed to dry out, as this would result in irreversible binding of the probe to the membranes. The treatments depended on the type of membrane
and whether it was DNA or RNA that was immobilised on them. Following probe removal all filters were autoradiographed overnight, to check that the probe DNA had been successfully removed. The filters were then either placed into prehybridization solution or were stored at -20°C until required. Gene Screen Plus filters with DNA bound to them were boiled for 30 minutes in 0.01xSSC, 0.01% SDS (w:v). Whilst probe DNA was removed from RNA bound Gene Screen Plus membranes by five 3 minute washes in boiling 0.01% SDS (w:v), 0.01xSSC on a rocking platform. DNA and RNA bound Hybond N+ filters were both treated in the same way, boiling 0.1% SDS (w:v) was poured over the membranes and allowed to cool to room temperature.

2.4d. Radiolabelling DNA Probes.

Isolated inserts of cloned DNA and genomic DNA (50-200ng) (appendix 2.3) were radiolabelled by the random oligonucleotide priming method, exactly as outlined by Fienberg & Vogelstein (1983a).

Random oligonucleotides (6mers) were purchased from Pharmacia LKB (Milton Keynes, Northants.) and the isotope used was 5'([α-32P] dCTP (3000 Ci/mMol). Sephadex G-50 (Pharmacia LKB) 1ml spin columns were used to separate labelled DNA from unincorporated nucleotides. The excluded fraction was collected and denatured together with salmon testis DNA (final concentration in the hybridization solution of 100μg/ml (appendix 2.4e)) by boiling for 5 minutes and quenching on ice. The denatured probe was then added to the prehybridization fluid.

2.4e. Salmon Testes DNA.

Salmon testis DNA was dissolved in H₂O to a final concentration of 10mg/ml using a magnetic stirrer in a beaker. The DNA was sheared by passage several times through a 19 gauge hypodermic needle, it was then denatured by boiling for 10 minutes and finally stored frozen at -20°C.

2.4f. Human Competitor DNA.

Sonicated human placental DNA was used in some hybridizations, to inhibit hybridization between human repetitive sequences in the probe DNA and the DNA on the
hybridization membrane. The DNA was dissolved in water to 2.5mg/ml and subjected to four 10 second bursts of sonication at 30Hz using MSE Soniprep 150 sonicator. It was then denatured by boiling for 10 minutes and stored frozen at -20°C. Prior to use the DNA was boiled for 5 minutes and 200μl were added to 50ng denatured radiolabelled probe DNA (plus salmon testes DNA). Preannealing was then allowed to proceed at 65°C for between 5 and 10 minutes, and the probe and competitor DNA were added to the prehybridization solution.

2.5. Autoradiography.

Nylon hybridization membranes, DEAE blotted agarose gels, SDS:PAGE dried gels were all autoradiographed in the same way. The samples were wrapped in Saran-Wrap and sandwiched between fast tungsten intensifying screens and Fuji RX or RXL X-ray film in light proof metal cassettes. The films were exposed at -70°C overnight in the first instance and for longer if necessary. The X-ray film was developed in Phenisol (Ilford Ltd, Mobberley, Cheshire) for up to 5 minutes, and fixed in Hypam fixer (Ilford Ltd). Dried sequencing gels were exposed without Saran-wrap and at room temperature.

2.6. Computerised Densitometry.

Quantitative analysis of autoradiographs was possible using a Sigma FTR20 densitometer and Shimadzu C-R3A integrator. This was essential for quantitative analysis of RNA dot blot hybridization autoradiographs.


The reaction buffer was supplied as a 10x concentrated stock (10mM MgCl₂) by the enzyme manufacturer (Promega), ultrapure deoxynucleotide triphosphates were purchased as 100mM solutions buffered to pH 7.5 (Pharmacia LKB), oligonucleotide DNA primers were purchased from Oswell DNA Service (Dept of Chemistry, University of Edinburgh, Edinburgh, Scotland) and used at 50pM in a 100μl reaction volume. Dimethylsulphoxide and formamide were sometimes used at 10% and 5%(v:v) respectively to improve the specificity of the reaction (Sarkar, et al, 1991).
A typical reaction comprised an initial denaturation step at 95°C for 5 minutes, followed quickly by the addition of 1 unit Taq DNA polymerase and 30 repeats of the following three step cycle:

**denaturation:** 90-92°C for 0.5-1 minute  
**primer annealing:** annealing temperature for 0.5-1 minute  
**elongation:** 72°C for 0.5-2 minutes  

Where the annealing temperature equals:

\[ 69.3 + (0.41 \times G+C\%) - 650/bp \text{ of primer} \] - 12  

(Maniatis, et al, 1982)

The length of time for the denaturation and annealing depend on the nature of the sequence to be amplified, the elongation period is dependent on the length of the sequence to be amplified with approximately 1000 bases being copied in 1 minute.

All PCR reactions were performed in the Hybaid Thermal Reactor-HB TR1 (Hybaid Ltd, Teddington, Middlesex.).
PUBLICATIONS
FAMILIAL POLYPOSP COLI

Sir,—The gene for familial polyposis coli (adenomatous polyposis coli [APC]) is linked to the probe C11p11 (D5S71), which maps to chromosome 5 in the region q21-q22.\(^1\) No recombinants have been reported. We have investigated the linkage relation between C11p11 and the APC gene in seven previously untested families from the polyposis registry at St Mark's Hospital, London, and have found an example of paternal recombination.

DNA was extracted from blood or normal colonic mucosa by standard methods and Southern blot analyses were done.\(^2\) C11p11 reveals a TaqI polymorphism with alleles A1 at 1.4 kb and A2 at 3.9 kb. The frequencies for A1 and A2 were initially estimated as 0.08 and 0.92, respectively.\(^3\)

Two families were informative; one has at least one recombinant, II 3 in the figure. The C11p11 typing was confirmed in more than one tissue for this individual. Hence the probe is not as closely linked to the disease gene as was originally hoped.

Initial use of the probe in polyposis patients in this laboratory resulted in a heterozygote frequency approximately double that expected from reported allele frequencies.\(^1\) Subsequent investigation of randomly ascertained individuals revealed an almost identical allele frequency of 0.2 for A1 and 0.8 for A2 in the two groups. The 60 controls and 26 patients examined were mainly Caucasian. These gene frequencies agree with those reported from the United States.\(^2\) The probe C11p11 is thus likely to be more informative in Northern European families than at first predicted.

We thank the following for financial support: Medical Research Council (M.R.C.), Quest for a Test for Cancer (K. T.), and Cancer Research Campaign (S. E. A. L.).

<table>
<thead>
<tr>
<th>Pedigree of APC family</th>
<th>A1 and A2 alleles of probe C11p11. * = the recombinant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A1: 0.8, A2: 0.2</td>
</tr>
</tbody>
</table>

Initial use of the probe in polyposis patients in this laboratory resulted in a heterozygote frequency approximately double that expected from reported allele frequencies.\(^1\) Subsequent investigation of randomly ascertained individuals revealed an almost identical allele frequency of 0.2 for A1 and 0.8 for A2 in the two groups. The 60 controls and 26 patients examined were mainly Caucasian. These gene frequencies agree with those reported from the United States.\(^2\) The probe C11p11 is thus likely to be more informative in Northern European families than at first predicted.

We thank the following for financial support: Medical Research Council (M.R.C.), Quest for a Test for Cancer (K. T.), and Cancer Research Campaign (S. E. A. L.).


CLOBAZAM FOR EPILEPSY

Sir,—We agree with Dr Scott (Aug 6, p 337) that clonazepam is a useful drug for the treatment of chronic intractable epilepsy, but have found tolerance to be a drawback. In a study of 41 chronic drug-resistant adolescent or adult patients, 25 (61%) showed an initial dramatic response (over 90% reduction of seizures) at one month of follow-up.\(^1\) However, 45% of these responders were tolerant at 6 months and 64% at a year of follow-up. Nevertheless, for 9 patients (22% of the study group) to be seizure-free, or nearly so, after a year of treatment is a considerable achievement. Patients with a known cause of their epilepsy, with complex partial seizures without secondary generalisation, and without mental retardation were more likely to have a sustained striking response to the drug.

We also agree that clonazepam treatment in epilepsy is not associated with the usual features of dependency. Withdrawal seizures occur, as with all antiepileptic drugs, but no more so in patients who have become tolerant than in those whose epilepsy remains well controlled. Tolerance to the antiepileptic action of the drug is not therefore related to dependency, which seems to be determined by the dose, supported by Dr Feely and Dr Haigh (June 25, p 1460), that tolerance and dependency result from the same adaptive mechanisms.

Department of Neurology, King's College Hospital, London SE5 9SR

E H REYNOLDS
A J HELLER
H A RING


USE OF MIDAZOLAM IN CHILDREN

Sir,—Your July 10 editorial on midazolam calls attention to its side-effects, especially in elderly patients. We frequently use midazolam as an anxiolytic and sedative in children before bone marrow biopsy and other invasive procedures. It provides good sedation, causes less local irritation with intravenous injection, and has a shorter elimination half life (1-4 h)\(^2\) than other benzodiazepines. Furthermore, its antegrade amnesia effect confers no recollection of the unpleasant and painful procedures.

Children are, however, resistant to the action of midazolam, and there is considerable individual variation in the dose requirement, which is age-related and may partly be affected by emotional state. But its rapid onset of action following intravenous administration allows titration of the dose to achieve the optimum effect. Total doses for adequate sedation range from 0.2 to 1.25 mg/kg.\(^3\) We followed these requirements in our children and most of them needed 0.3-0.5 mg/kg. Although the dose is greater than that recommended by the manufacturer, it is much the same as that commonly used for anaesthetic premedication.\(^4\)

We have not seen complications such as arrhythmia or cardiorespiratory suppression on more than 100 occasions in the past two years. Therefore we think that midazolam is a safe and effective agent for sedation in children. However, we agree that precautions should include careful monitoring of the patient's condition and the provision of facilities for resuscitation. Great care should also be taken when giving the drug to patients in respiratory distress or who have impaired conscious states.

Department of Paediatrics, Chinese University of Hong Kong, Prince of Wales Hospital, Hong Kong

TAI KWAN LAM
WENCHEUNG NG
YUMING CHAN


GLYCERYL TRINITRATE IN SKIN NECROSIS CAUSED BY EXTRAVASATION OF PARENTERAL NUTRITION

Sir,—Skin necrosis due to extravasated intravenous infusions, which cause intense local vascular constriction, causes morbidity in infants treated in special care units and is a common reason for litigation by their parents. Where skin loss occurs over a wide area or over a joint, grafting may be necessary.

In a newborn baby with extravasated parenteral nutrition solution we applied glyceryl trinitrate, a powerful local vasodilator,\(^5\) to an area about 2 × 3 cm on the dorsum of the foot, which still appeared white and ischaemic 24 h after removal of the intravenous cannula. A patch containing glyceryl trinitrate 25 mg (releasing 5 mg per 24 h) was applied for an hour. On removal, the superficial epidermis blanched but the area beneath appeared healthy and well perfused and has healed without scarring. A small
Chromosome 5 allele loss in familial and sporadic colorectal adenomas

M. Rees¹, S.E.A. Leigh¹, J.D.A. Delhanty¹ & J.R. Jass²

¹Gatton Laboratories, Department of Genetics and Biometrics, University College London; 4 Stephenson Way, London NW1 2HE, UK and ²St Mark’s Hospital, City Road, London EC1V 2PS, UK.

Summary. DNA extracted from familial and sporadic colorectal neoplasms was compared with constitutional DNA using a range of hypervariable DNA probes to assess the extent of allele loss during conversion to malignancy. Chromosome 5 allele loss was observed in 23% of carcinoma samples, as previously found by others. However, we have been able to show for the first time loss of the D5S23 locus on chromosome 5 in adenomas from three patients, two of whom had the preneoplastic condition adenomatous polyposis coli (APC). These results suggest significant genetic changes involving chromosome 5 are occurring in benign adenomas. Probes for chromosome 3 (D3S17 and D3S48) and for chromosome 7 (D7S21 and D7S22) revealed no notable alterations in the adenoma samples. Complete loss of alleles for loci on chromosome 7 was not observed in carcinomas but reduced intensity of one parental allele was found in three specimens one of which was known to have multiple copies of this chromosome. Results using probes for chromosome 3 suggest that deletion of the D5S7 or D5S28 loci is not a common event in colorectal carcinogenesis. Loss of chromosome 5 alleles in adenomas from APC patients provides evidence in support of Knudson’s hypothesis.

The observed characteristics of the autosomal dominant condition, adenomatous polyposis coli (APC Human Gene Mapping 9, 1987), are hyperproliferation of epithelial and mesenchymal tissues (Bussey, 1975; Bulow, 1987) and widespread spontaneous chromosome instability (Gardner et al., 1982; Delhanty et al., 1983). Expansion of the proliferative compartment of the colonic crypts and shift of this region to the mucosal crypt surface (Lipkin, 1988) results in the production of hundreds of adenomatous polyps by the second decade of life. Without surgical intervention progression to malignancy occurs in all cases (Muto et al., 1977).

The gene for APC (also called FAP-familial adenomatous polyposis) has been mapped to chromosome 5 region 5q21-22 (Bodmer et al., 1985; Leppert et al., 1987). According to Knudson’s hypothesis (Knudson, 1971), exemplified classically by retinoblastoma (Cavenee et al., 1983), inheritance of one mutant form of the gene should be followed by loss or inactivation of the normal allele in tumorigenesis. Loss of chromosome 5 alleles relative to non-malignant tissue has indeed been found in three out of five informative APC cancers (Okamoto et al., 1988). Following the retinoblastoma model in sporadic cases of colorectal cancer, reduction of heterozygosity for loci on chromosome 5 should also be observable evidence for this in at least 20% of cases which has been gathered (Solomon et al., 1987; Okamoto et al., 1988). However, these previous studies investigated of adenomas from APC patients revealed no loss of DNA restrictions fragments from chromosome 5 compared with normal tissue. We wish to report the first examples of such loss in adenomas from polyposis patients and from a normal individual, shown by the use of highly informative locus specific minisatellite probes (Wong et al., 1987).

Materials and methods

Tissue samples

Tissue was obtained from 26 sporadic colorectal carcinomas, three sporadic adenomas from two patients, 48 adenomas from 21 APC patients, two colorectal cancers and a desmoid tumour from APC patients, together with corresponding normal mucosa or blood in all cases. With certain exceptions, the material came from patients at St Mark’s Hospital, London. Carcinoma specimens had been flash frozen in liquid nitrogen, adenomas were received fresh. Samples from patients 26, 49 and 52 were from Ashington Hospital, Northumberland, no. 29 came from the Royal Victoria Infirmary, Newcastle upon Tyne and adenomas from patient 50 were received from the Royal Naval Hospital, Plymouth. All these samples came as fresh tissue.

In addition, cells were cultured from a colon carcinoma cell line established from an APC patient, no. 27 (Paraskeva et al., 1984). The corresponding normal fibroblasts were grown from a skin biopsy in this laboratory.

Adenomas from the majority of APC patients were 5 mm or less in diameter — the exceptions are listed in Table II. The two sporadic adenomas were 5 mm and 1 cm in size. None had any macroscopic evidence of malignant change.

DNA extraction and hybridisation

DNA was prepared from tissue samples and cultured cells by standard methods (Maniatis et al., 1982). Samples were digested with the appropriate restriction endonuclease and size fractionated by electrophoresis through 1% agarose gels. The DNA was transferred to Gene Screen Plus-hybridisation membrane (NEN, Dupont) according to the manufacturer’s specifications. DNA probes were radio-labelled with 32P-dCTP (3,000 Ci mmol-1) by the random hexanucleotide primer method (Feinberg & Vogelstein, 1983) to a high specific activity. Hybridisations were performed at 65°C in 1% SDS, 1 M NaCl and 5% dextran sulphate (w/v) for 16 h. Filters were washed to a stringency of 2× SSC, and were autoradiographed at -70°C using Fuji RX-1 X-ray film.

DNA probes

The locus-specific hypervariable DNA probes used (obtained from ICI Diagnostics) were: ZMS1, chromosome 1 (p33-p35); ZMS8 (5q35-qter); ZMS1 (7p22-ter), pvg3 (7q36-qter), all of which show polymorphisms with HindIII restriction digests of genomic DNA, and ZMS32 (1q42-q43) which requires AluI digests.

Results

The great advantage of the minisatellite probes is that they detect extremely variable loci with heterozygosities ranging from 90 to 99% (Wong et al., 1987). However, if the locus detected by the probe is not close to the critical region of interest (as is the case for chromosome 5) loss of the whole or a substantial part of the chromosome will be detected but not small deletions which may allow expression of recessive
Table I  Allele changes in sporadic and APC colorectal carcinomas

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**APC**

<table>
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<tr>
<th>Patient no.</th>
<th>No. of adenomas</th>
<th>Size</th>
<th>iMS1</th>
<th>iMS32</th>
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</table>

**Homogygosity in the constitutional DNA is indicated as a dash; where the normal tissue was informative the tumour genotype is shown in the table. Heterozygosity is indicated by 1.2 even though some probes recognise multi-allelic systems. The continued presence of the larger allelic restriction fragment is indicated by '1' and '2' indicates continued presence of the smaller allelic restriction fragment. Reduction of intensity is indicated by ( ). Absence of an entry indicates not tested or no result.**

Table II  Allele changes in familial and sporadic colorectal adenomas

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>No. of adenomas</th>
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**Homogygosity in the constitutional DNA is indicated as a dash; where the normal tissue was informative the tumour genotype is shown in the table. Heterozygosity is indicated by 1.2 even though some probes recognise multi-allelic systems. The continued presence of the larger allelic restriction fragment is indicated by '1' and '2' indicates continued presence of the smaller allelic restriction fragment. Reduction of intensity is indicated by ( ). Absence of an entry indicates not tested or no result.**

*Adenomas <5 mm diameter unless otherwise stated; *altered band size DNA from largest polyp; *reduced intensity of larger allele in two separate polyp DNA samples.*
mutations in the APC gene. Hence the number of changes detected may be a gross underestimate.

The results obtained by hybridisation of the probes to the matched normal and carcinoma pairs are shown in Table 1 and those for the adenomas in Table II. A total of 23 carcinoma patients were informative for the probe /MS1, which recognises the locus D1S7 on chromosome 1; all the cancer samples retained heterozygosity. Thirteen adenoma patients (11 of them APC) were also informative with this probe; none showed any changes with adenoma formation. Heterozygosity for the second chromosome 1 probe, /MS32 (locus D1S8), was revealed in 22 carcinoma patients; clear allele loss was found in one case (no. 27), the cancer cell line derived from an APC patient, while DNA from two sporadic cancers (nos. 8 and 11) showed different sized bands compared with the normal counterpart (Figure 1). Of eleven informative adenoma patients (10 of them APC) a single adenoma from a total of five from one APC patient (no. 32) showed an altered band size; this specimen was a 2 cm diameter sessile villous polyp (Figure 1).

With the chromosome 5 probe, /MS8 (for locus D5S43), 22 carcinoma patients proved informative and allele loss was seen in two cancers (nos. 15 and 25) with decreased intensity of one allele in a further three (nos. 12, 19 and 26); all these were sporadic cancers (Figure 2a). Among 19 informative adenoma patients (17 of them APC) three gave evidence of allele loss in DNA from adenoma tissue. A clear reduction in intensity of the larger allele was seen in two of the three adenomas examined from one APC patient (no. 34). DNA extracted from a single adenoma from a second APC patient (no. 40) had complete loss of the smaller allele, while DNA from the sporadic polyp of patient 52 showed a similar loss (Figure 2b).

Two probes were available for chromosome 7. A total of 20 carcinoma and 10 adenoma patients were informative with /MS81 (D7S21). One of the carcinomas (no. 23) exhibited reduced allele intensity together with the appearance of two new bands (Figure 3a); additional bands were also seen in a further carcinoma (no. 24). No obvious changes were detected in the adenomas. Finally, the p/g3 probe (D7S22) detected heterozygosity in 22 carcinoma patients, of which two (12 and 26, both sporadic cases) showed a definite reduction in intensity of one allele in cancer DNA (Figure 3b). Chromosomes prepared from a short-term culture of the cancer from patient 26 revealed four copies of chromosome 7 in diploid cells. With this probe the 15 informative adenoma patients remained heterozygous in all samples tested.

The desmoid tumour (a benign neoplasm of mesenchymal origin) from an APC patient (no. 53) who was informative at one locus for each tested chromosome showed no change from the constitutional type.

Reduced intensity of one of a pair of allelic fragments rather than complete loss probably reflects the presence of normal stromal tissue in the neoplasm, the coexistence of more than one clone, or duplication of one allele at the expense of the other.

Discussion

We have compared DNA extracted from a number of colorectal neoplasms with constitutional DNA using a range of highly informative locus specific probes.
In accordance with the findings of Solomon et al. (1987) and Okamoto et al. (1988) chromosome 5 allele loss was observed with /MS8 (DSS43) in 23% of carcinoma samples. However, we have been able to show for the first time loss of the DSS43 locus in adenomas from familial polyposis patients (Table III), demonstrating probable APC allele loss in not only the APC precancerous condition, but also in an adenoma from a non-APC individual. These results suggest that significant genetic changes involving chromosome 5 are occurring in benign adenomas, whereas the other probes tested revealed no notable alterations in the adenoma samples. In view of the number of polyps typically present in APC patients, an average of just over 1,000 in counted colectomy specimens, the rate of conversion to malignancy is low (Bussey, 1975). Allele loss in three of 38 informative adenomas thus appears to be significant. While this paper was in preparation Law and colleagues published the results of their study on non-syndromic allelic loss in colorectal carcinomas and adenomas (Law et al., 1988). They found no allele loss from chromosome 5 in 40 adenomas from APC patients who were informative for at least one chromosome 5 probe; no information on size of the adenomas was given. The difference between their findings and ours may simply be due to sampling from the multitudes of polyps available or may reflect differences between patients. Two of the losses we observed were from adenomas of a single patient, a 15-year-old with exceptionally well developed adenomas considering his age. Chromosomes prepared from a 48-h culture of a smaller adenoma from this same patient showed random loss or gain (sometimes both) of chromosomes in 11 of 26 cells analysed.

Trisomy of chromosome 7 in colorectal carcinogenesis has been reported previously (Reichmann et al., 1985). In this study, while complete loss was not seen, reduced intensities of alleles on this chromosome was observed in DNA from three separate carcinomas, one of which was known to have multiple copies of chromosome 7. The DNA results indicate duplication of one parental chromosome at the expense of the other in the latter case.

Increased copy number of this chromosome is thought to be important in carcinogenesis of solid tumours in general (Van Der Berghe, 1987). The various proto-oncogenes mapped to chromosome 7 are obvious candidates for a role in this process (Human Gene Mapping 9, 1987).

In common with most malignancies, chromosome 1 structural alterations are frequent in colorectal cancer (Reichmann et al., 1984). Before this study we had evidence for loss of expression of the z-fucosidase gene (located at Ip22) in two of six informative colorectal cancers, although phosphoglucomutase 1 (at Ip22) expression remained, suggesting loss or deletion of part of chromosome 1p (our unpublished observations using isoenzyme analysis, S.H. Rider, M.B. Davis & J.D.A. Delhanty). Use of the hyper-variable probe /MS1 in 23 informative colorectal cancers failed to detect allele loss in the region Ip33-p35 in this larger sample.

The appearance of additional or altered sized bands in the samples when probed with both /MS2 and 31 may be due to the high somatic mutation rate known to be detected in this type of material with these probes (J.A.C. Armour, I. Patel, S.L. Thein, M. Fey & A.J. Jeffreys, manuscript submitted). The significance of such mutations with respect to oncogenesis is unknown at present.

Loss of a normal gene product is thought to play a critical role in the generation of several embryonal tumours (Cavenee et al., 1983; Koufos et al., 1984; Orkin et al., 1984) and certain adult cancers (Koufos et al., 1985, Fearon et al., 1985, Kok et al., 1987). APC is unusual in that heterozygosity for the deficiency gives rise to local growth excesses, possibly through a threshold effect produced by fluctuating levels of gene product (Solomon et al., 1987). The smallest adenomas in this condition can be viewed as simply a manifestation of hyperproliferation. Post-colectomy regression of rectal polyps has been observed (Feinberg et al.,

<table>
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<th>Table III Summary of results obtained with locus-specific probes</th>
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<td>Probe</td>
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*Two adenomas from one patient
1988), which suggests that no irreversible genetic change has occurred. Demonstration of the clonal origin of these adenomas (Fearon et al., 1987) is not incompatible with this hypothesis since the colonic crypts are known to be maintained by a single stem cell (Griffiths et al., 1988). Larger adenomas would be expected to have undergone one or more genetic changes of a clonal nature; we have provided evidence for this in three adenomas from two polyposis patients. In view of the multistage nature of carcinogenesis it is probable that large adenomas will have undergone several gene or chromosome mutations before reaching the fully malignant state.

In normal people loss or mutation of one copy of their two normal alleles of the APC gene would be expected to be an early event to initiate the requisite hyperproliferation for adenoma formation. Loss of chromosome 5 alleles would thus be expected in some small sporadic adenomas, of which we have one example. Chromosome instability would presumably be conferred by the heterozygous state, providing a mechanism for further genetic change by means of deletion or somatic crossing over leading to homozygosity or functional hemizygosity for critical loci on chromosome 5 or on chromosomes 17, 18 and 22. The latter chromosomes have recently been implicated in colorectal cancer by cytogenetic (Muleris et al., 1987) and molecular data (Fearon et al., 1987; Okamoto et al., 1988). Use of polymorphic DNA probes which are closer to the critical region of chromosome 5 as well as those assigned to chromosomes 17, 18 and 22 will enable us to obtain a more complete picture of the genetic events leading from adenoma to carcinoma in both polyposis patients and normal individuals.

We wish to thank the following: all the staff of the Polyposis Registry, St Mark's Hospital, London, Mr A. Gunn, Ashington Hospital, Northumberland and Cmdr A.R. Margaret, The Royal Naval Hospital, Plymouth for supplying material; Dr C. Paraskeva, The Medical School, Bristol for the cell line JW2, and Dr A. Jefferyes and IC1 Diagnostics for the minisatellite probes. M. Rees was supported by the Medical Research Council of the UK and S.E.A. Leigh by the Cancer Research Campaign.

References


Use of Fluorescent In Situ Hybridisation to Confirm Trisomy of Chromosome Region 1q32-qter as the Sole Karyotypic Defect in a Colon Cancer Cell Line

Darren K. Griffin, Sarah E.A. Leigh, and Joy D.A. Delhanty

Department of Genetics and Biometry, University College London, London, England

The sole chromosome defect in a colon cancer cell line derived from a patient with inherited nonpolyposis colorectal cancer was karyotypically designated as 46,XY,-13,+der(13)t(1;13)(q32.1;p11) on the basis of banding homology. We have obtained molecular confirmation that the additional chromosome material is derived from chromosome region 1q32-qter by the use of a highly specific fluorescent in situ hybridisation technique on G-banded chromosomes and also by Southern hybridisation.

INTRODUCTION

The cell line, LIM 1215, derived from a patient with inherited nonpolyposis colorectal cancer, was established from a poorly differentiated adenocarcinoma of the ascending colon (Whitehead et al., 1985). The sole karyotypic abnormality appeared to be the ubiquitous presence of a marker 13p+ chromosome. Careful G band comparison led to the tumour karyotype subsequently being designated as 46,XY,-13,+der(13)t(1;13)(q32.1;p11) (Jenkyn et al., 1987). We have now been able to confirm that the trisomic material is derived from chromosome region 1q32-qter by in situ hybridisation (ISH) of a locus-specific minisatellite probe to metaphase chromosomes from LIM 1215 by using a nonradioactive fluorescent technique. Further confirmation was obtained by using the same probe in Southern hybridisation.

MATERIALS AND METHODS

The hypervariable minisatellite probe λMS32, which recognises the locus D1S8, was previously assigned to chromosome 1, region 42-43, by Royle et al. (1988). Probe DNA was biotinylated by nick translation using the Bethesda Research Laboratories kit.

Chromosomes, prepared by standard methods, were G banded prior to ISH by treatment with 2× SSC (1× SSC is 0.15M sodium chloride and 0.015M trisodium citrate, pH 7.2) and trypsin (Gallimore and Richardson, 1973). Metaphases were then photographed in order that they could be relocated for subsequent analysis after the ISH procedure had been performed.

The following fluorescent ISH technique was adapted and developed from Pinkel et al. (1986) by Tim Kievits and Joop Wiegant of the Department of Human Genetics, University of Leiden. Slides were destained and dehydrated in an alcohol series and then air dried. Pretreatment with RNase (100 μg/ml in 2× SSC) under a coverslip in a moist chamber for one hour at 37°C preceded washing three times in 2× SSC, dehydration in alcohol, and air drying. Washing with proteinase K buffer (20 mM Tris·HCl, 2 mM CaCl₂, pH 7.4) was followed by a 7 minute treatment with proteinase K (500 ng/ml) at 37°C. Slides were then washed in paraformaldehyde buffer [0.9% PBS (phosphate-buffered saline) + 50 mM MgCl₂], fixed in 4% paraformaldehyde for 10 minutes, washed again in the buffer, dehydrated, and air dried. (Paraformaldehyde in solution is hazardous and should be handled in a fume hood.) Thirty-five microliters of biotinylated probe DNA in hybridisation buffer (50% formamide, 20% dextran sulphate, 50 mg/ml denatured salmon sperm DNA, 1 mM EDTA, 2 mM Tris·HCl in 2× SSC) was applied at a concentration of 30-40 ng/slide and sealed under a coverslip with rubber solution. Denaturation of probe and genomic DNA was done simultaneously in a preheated 80°C oven for 4 minutes. Hybridisation proceeded overnight in a moist chamber at 37°C.

Coverslips were gently removed and post-hybridisation washes were carried out at 42°C as follows: three times for 5 minutes in 50% formamide in 2× SSC and 5 times for 2 minutes in 2× SSC. Preparatory incubation steps were 5 minutes in 4× SSC + 0.05% Tween 20 detergent and 10 minutes in 4× SSC + 5% non-fat dry milk (Mar-
Biotin detection was facilitated by sequential layers of Avidin-FITC (fluorescein isothiocyanate) conjugate and biotin-anti-avidin D conjugate, both from Vector Laboratories, U.S.A. In each case conjugate was applied at a concentration of 5 μg/ml (in 5% milk in 4x SSC), 100 μl per slide for 20 minutes under a coverslip and washed off with 0.05% Tween 20 in 4x SSC three times for 5 minutes. Three multilayers were applied to obtain maximum signal and minimum background. Final washes were once in 0.05% Tween 20 in 4x SSC and twice in 0.9% PBS (5 minutes each). Slides were dehydrated, air dried, and then mounted and sealed (with nail varnish) under a coverslip in "anti-fade medium." Anti-fade medium: 9 parts glycerol containing 2% 1.4 diazabicyclo-(2.2.2.)-octane (DABCO) and 1 part 0.2 M Tris – HCl + 0.02% sodium azide pH 7.5. DAPI was added to a concentration of 0.5 μg/ml. DABCO is extremely hazardous and should only be handled in a safe fume hood wearing nitrile gloves.

Slides were observed under a Reichert polyvar microscope fitted with ultraviolet fluorescence and filters for DAPI and FITC. Photographed metaphases were relocated and scored for hybridisation by using the FITC filter. Hybridised areas are recognised as bright fluorescent spots. The position of these spots was marked on the original G-banded photograph to give the band location. LIM1215 was also examined at the D1S8 locus by Southern hybridisation. DNA prepared from LIM1215 cells was digested to completion with AluI, size fractionated by electrophoresis through a 1% agarose gel, and transferred to Gene Screen Plus hybridisation membrane (NEN, DuPont) according to the manufacturer’s recommendations. The AMS32 probe was labelled with α-32P dCTP by the random priming method of Feinberg and Vogelstein (1983). Hybridisation and posthybridisation washes were performed as specified (NEN, DuPont). The hybridisation membranes were autoradiographed at −70°C by using Fuji RX-L film.

RESULTS

Twenty cells were analysed, a total of 87 hybridised areas (seen as bright fluorescent spots) were observed, of which 48 (53.1%) were located on chromosomes 1, at region q32-qter and 23 (26.1%) were on the p arm of the 13p+ chromosome (Fig. 1). This gives molecular confirmation of the chromosome 1 origin of the extra material on the marker chromosome 13 and also confirms the assignment of the locus D1S8, recognised by the probe, AMS32, to region 1q42-43 where the peak occurs (Fig. 1). The presence of additional chromosome 1q material was further demonstrated by the relative intensities of the two allelic bands obtained by Southern hybridisation of AMS32 to LIM1215 genomic DNA (Fig. 2). Densitometric measurements reveal that it is the 10.3 kb allele which has been duplicated in the segment translocated to 13p. Unfortunately, normal tissue from the same patient was not available for comparison.

DISCUSSION

We have confirmed by fluorescent ISH to pre-G-banded metaphases that the sole chromosome

![Figure 1](image1.png)

![Figure 2](image2.png)
TRISOMY CONFIRMED BY IN SITU HYBRIDISATION

anomaly in the colorectal cancer cell line LIM 1215 is trisomy of chromosome segment 1q32-qter. This accords with previous observations of minimal chromosome changes in some colorectal cancers of right-sided origin (Muleris et al., 1989; Delattre et al., 1989; and our own unpublished observations). Rearrangements of chromosome 1 are frequent in malignant cells, including those of colorectal origin (Mitelman, 1988). Isochromosome 1q formation may be accompanied by loss of material from 1p but this is not universal; regions of consistent duplication have been observed to be 1q24-qter (Reichmann et al., 1984).

Several recent studies implicate human chromosome 1 in the process of cellular immortalisation. PC/AA is an adenoma cell line derived from a familial polyposis patient which originally displayed a normal diploid karyotype; two separate derivatives of this cell line became established in culture and each displayed an isochromosome of 1q, with the origin from different homologues of chromosome 1 (Paraskeva et al., 1988, 1989). Willson et al. (1987) reported a villous-adenoma-derived established cell line with a deletion of the short arm of chromosome 1 and an isochromosome 1q.

Experiments with hybrids between immortal Syrian hamster cells and normal human fibroblasts indicated that each hybrid clone which escaped senescence had lost both copies of chromosome 1 (Sugawara et al., 1988), again implicating this chromosome in immortalisation. It has been proposed that tumorigenicity results from a balance between genes which suppress malignancy, possibly on Ip, and others which allow expression of the transformed phenotype (Benedict et al., 1984). Perhaps this latter type resides on the terminal region of 1q.

ACKNOWLEDGMENTS

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REFERENCES


Two Cosmid Clones containing expressed sequences mapping to 5q31 & 5q35.

Leigh SEA, Gayther SA, Lawson CM, Griffin DK, Delhanty JDA

The Galton Laboratory, Dept of Genetics & Biometry, University College London, Wolfson House, 4 Stephenson Way, London, NW1 2HE

Two human genomic clones were isolated from a chromosome 5 specific lambda library (ATCC: LA05NS01) on the basis that they contained sequences expressed in normal colonic mucosa(1). These were in turn used to screen a total human genomic cosmid DNA library. We report here the identification of two cosmid clones, cos5.3iv (D5S321) and cos5.5iv (D5S322), which have been mapped to 5q35 and 5q31 respectively by fluorescent in situ hybridisation. These localizations are currently being confirmed with a panel of somatic cell hybrids.

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Expression from the proximal promoter of the carbonic anhydrase 1 gene as a marker for differentiation in colon epithelia

Jane Sowden¹, Sarah Leigh², Ian Talbot¹, Joy Delhanty¹, Yvonne Edwards¹

¹ MRC Human Biochemical Genetics Unit, The Galton Laboratory (UCL), Wolfson House, 4 Stephenson Way, London, NW1 2IE, UK
² Genetics Department, University College London, 4 Stephenson Way, London, NW1 2IE, UK
³ Pathology Department, St Marks Hospital, City Rd, London EC1V 2PS, UK

Abstract. Carbonic anhydrase 1 (CA1) catalyses the reversible hydration of CO₂ and is important for cellular diffusion of CO₂, ion transport and pH regulation. The gene encoding CA1 (CA1) has two promoters. In adult colon epithelium the proximal promoter determines high levels of expression and the distal erythroid promoter is repressed. RNA in situ hybridisation shows that CA1 mRNA is abundant in differentiating cells of the colonic crypt as they migrate to the luminal surface, but is not present at the base of the crypts and levels are low on the luminal surface. It is likely that CA1 gene expression in these cells is regulated by differential transcription and/or mRNA stability. In contrast CA1 protein is localised predominantly on the luminal surface. Since CA1 mRNA and protein do not exactly co-localise it can be inferred that CA1 gene expression is also subject to post-transcriptional control. CA1 mRNA is significantly reduced in colon carcinoma and in adenomas from familial adenomatous polyposis patients. Loss of CA1 expression is associated with the disappearance of differentiated epithelial cells. Out of twelve colon carcinoma cell lines three, LIM1215, LIM1899 and HT115, expressed CA1 and nine did not. This variation in expression may also be associated with cell type differentiation.

Introduction

The intestinal mucosa is a dynamic cellular system which is renewed every 5 days in humans [23]. In the large intestine the mucosal epithelium lacks villi and is organised as crypts within which columnar, goblet and isolated endocrine cells are found. These cells differentiate from a proliferating pool of cells in the lower third of the crypt and migrate towards the luminal surface [26]. On reaching the lumen the cells, which are by now fully differentiated, are extruded and replaced by a new population.

The epithelium of any one crypt arises clonally, comprising cells of a single genotype [22]. In the case of the intestine little is known of the mechanisms which lead to differentiation of multiple cell types from the pluripotent stem cell. There is evidence from other continuously renewing cell systems, such as the haematopoietic cycle, that commitment to a particular lineage is in part regulated by tissue-specific protein factors. For example the transcription factor GATA-1 plays a key role in the establishment of the erythroid phenotype by activating erythroid-specific genes [21].

Progress has been made towards characterising the intestinal mucosa cell lineages in terms of their protein profile and more recently their mRNA levels. Intestinal-specific genes show complex patterns of expression along both the crypt-villus axis and the length of the intestine from duodenum to colon. For example both the enterocyte-specific brush border enzyme sucrase isomaltase (SI) and the liver fatty acid binding protein (L-FABP) have their highest levels of activity in the villus cells of the small intestine [27, 31]. In situ hybridisation has allowed the localisation of specific mRNAs in tissue sections and determination of the point at which their genes are activated during epithelial differentiation [13, 19].

Only a relatively small number of genes have been characterised whose expression is limited to the large intestinal epithelia. Amongst these is the carbonic anhydrase 1 gene (CA1); the product of this locus catalyses the reversible hydration of CO₂ and plays a vital role in CO₂ excretion, pH regulation and ion exchange [30]. CA1 protein is present at high levels in adult colon epithelia and in trace amounts in other regions of the gastrointestinal tract [15] and in the eye, salivary gland and myoepithelial cells [30]. In fetal colon, 19 to 21 weeks gestation, the levels of CA1 protein are much lower [16].

We have recently demonstrated that the CA1 gene [17] has two distinct promoters which give rise to CA1 protein in colon epithelia and erythroid cells [7]. In colon cells CA1 is transcribed from a promoter that is proximal to the start of the protein coding sequence. Transcription from a distal erythroid-specific promoter which lies
36 kb upstream of the start of the protein coding sequence occurs in erythroid cells. The erythroid transcription unit is flanked by binding sites for the erythroid GATA-1 transcription factor [5]. The colon and erythroid mRNAs have unique 5' leader sequences but the same protein coding sequences.

We have used RNA in situ hybridisation to show the spatial organisation of CAIL mRNA within adult colon tissue sections. This was contrasted with the localisation of the CAIL protein determined by immunohistochemical analyses in adjacent sections. RNA polymerase chain reaction (PCR) was used to distinguish colon CAIL mRNA from erythroid CAIL mRNA and colon-specific transcription of CAIL was investigated in various human tissues, colon carcinoma cell lines and in tumours from colorectal cancer patients. CAIL colon promoter activity appears to be cell type specific and characteristic of the normal adult colon phenotype.

Methods

Isolation of RNA. Surgical tissue samples were provided by the Polyposis Registries of St Marks Hospital, London, and of the Northern Region (Prof. J. Burn and Mr A. Gunn). The samples were obtained during elective surgery either for colectomy in familial adenomatous polyposis (FAP) patients or for resection of carcinomas in non-FAP patients. Thirty-eight adenoma samples were obtained from 8 FAP patients (aged 21 to 37 years) and 29 tumour samples were obtained from 9 female patients and 20 males, aged from 52 to 86 years with sporadic colorectal cancer. Tumour samples were characterised histologically according to Dukes stage of progression A (n = 3), B (n = 14), C1 (n = 9) and C2 (n = 2) and graded as, poorly differentiated (n = 4), moderately differentiated (n = 23) and well differentiated (n = 2). Material was obtained from the tumour adenoma and from normal mucosal tissue in the same region of the colorectum. Surgical samples to be used for RNA analysis were flash frozen in liquid nitrogen shortly after surgery. A 50% homogenate of each sample was diluted 1:20 into a Gene Screen Plus hybridization membrane (NEN DuPont) in duplicate using a slot blot manifold (S&S Minifold II, Schleicher & Scheull, FRG). The membrane was baked at 80°C for 2 h before hybridisation. Filters were hybridised with 32P-labelled CAIL cDNA at 65°C in 1 M NaCl, 1% sodium dodecyl sulfate (SDS) (w/v), 5% dextran sulphate (w/v), 100 mg ml⁻¹ denatured salmon testes DNA for 16 h and washed to a stringency of 0.1 x SSC (0.015 M NaCl, 0.0015 M sodium citrate) at 65°C. Filters were subsequently de-probed (in boiling 0.01% SDS w/v, 0.01 x SSC) and hybridized with a cDNA probe for the ubiquitously expressed actin [12]. The level of actin mRNA was used to standardise the quantities of RNA loaded in each sample. Steady state RNA levels were compared using scanning densitometry of the autoradiographs from both hybridisations.

Histological in situ analysis. Formalin saline fixed tissue was mounted in paraffin blocks and sections were analysed using CAIL specific RNA probes and CAIL polyclonal antibodies. A 530 bp PstI–HindIII fragment from the 3' region of the CAIL cDNA was subcloned into pGEMZ+ plasmid. The recombinant plasmid was digested with HindIII and EcoRI and cRNA was synthesised and labelled with [35S]UTP (>1000 Ci mmol⁻¹) using T7 and Sp6 polymerase to generate both sense and antisense RNA probes respectively. This probe is specific for CAIL comprising mainly non-conserved 3' untranslated sequence and coding sequence for 23 amino acids of which only 2 are conserved between CAIL, CA2 and CA3. The hybridisation and post-hybridisation procedures were as described by Wilkinson et al. [36]. Immunostaining of paraffin tissue sections was carried out using a specific rabbit anti-CAIL antibody [1, 2] (a kind gift from Dr. Nick Carter). The specificity of the antisera for CAIL was confirmed using Ouchterlony double diffusion and immunoblotting of isoelectric focusing gels to analyse red cell lysates and muscle extracts. No cross reactivity was detected against purified CA2 and antisera sensitivity to CAIL was maintained in 200 fold dilutions of hemolysates. Sections were deparaffinized in xylene and hydrated through graded alcohol series.

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68
In the tissue sections was quenched by preincubation in 0.3% H$_2$O$_2$/methanol for 30 min. The CAI antiserum was applied at a concentration of 50 μg ml$^{-1}$ for 1 h in conjunction with the Vectastain ABC (Vector laboratories) kit which employs a biotinylated second antibody and then a preformed avidin and biotinylated horseradish peroxidase macromolecular complex.

**Results**

**CAI expression in the adult colon and other sites**

Histological in situ analyses using a CAI-specific $^{35}$S-labelled RNA probe and a CAI-specific antibody were carried out to determine the precise localisation of CAI mRNA and protein in adult human colon tissue sections. Samples from several different individuals were analysed, in all cases CAI mRNA was clearly detected in differentiating cells in the upper half of the crypts, but was absent or present in trace amounts in the lower proliferating region of the crypt and in the cells on the luminal epithelium. In contrast CAI protein was predominantly localised in the epithelial cells on the luminal surface and was not detected in any part of the crypt. An example of sections from normal colon showing mRNA and protein localisation is shown in Fig. 1. These results suggest that transcription of the CAI gene is activated as the cells of the crypt differentiate and migrate to the luminal surface of the colon. Although abundant the CAI mRNA is apparently not translated until the cells of the luminal epithelia mature and at this stage mRNA transcription is apparently halted.

The transcripts from the CAI colon and erythroid promoters share a common protein coding sequence but differ in their 5' leader sequences [7]. In order to discriminate between the two CAI mRNAs we have carried out RNA PCR amplifications using transcript specific oligonucleotides.

The diagram shown in Fig. 2 indicates the position of these primers; the colon specific PCR primers are colol1F in combination with primer 1R, while the erythroid specific PCR primers are ery2F in combination with primer 1R. In addition a pair of primers was designed which amplify exon 2 and exon 3 sequence common to both transcripts. Using the colon specific primers a 208 bp product was amplified from RNA isolated from colon tissue which was not amplified from erythroid cell (HEL, [33]) RNA. Conversely using the erythroid specific primers a 239 bp product was amplified from HEL but not colon RNA (Fig. 2). A 270 bp product was amplified from both HEL and colon RNA using the internal primers 3F and 2R. These data show that in colon cells which transcribe CAI from the colon promoter the erythroid promoter is completely repressed, whereas in erythroid cells the opposite situation occurs and no transcripts from the colon promoter are detected.

It is well known from earlier studies that the erythroid promoter is strictly erythroid-specific [7, 11] but much less is known about the specificity of the colon promoter. In order to investigate this, analyses were carried out using the colon specific PCR primers and RNA from

Fig. 1. Histochemical analyses of paraffin sections of human colon biopsies. A and C Binding of carbonic anhydrase 1 (CAI) specific polyclonal antibodies, detected using a peroxidase conjugated second antibody and biotin-avidin signal amplification. B and D Dark field micrograph of a serial section to that in A and C hybridised with a CAI-specific $^{35}$S-labelled riboprobe as described in the Methods and developed after 7 days exposure. Micrographs A and B, 250 x magnification; C and D, 10 x magnification. An area of luminal epithelia is indicated by arrows. Hybridisation of serial sections with a sense CAI RNA probe as a control did not show any specific signal.
Fig. 2. A diagrammatic representation of the CAI gene. Coding exons are indicated by solid boxes and non-coding exons by open boxes. The position of primers used for polymerase chain reaction (PCR) and transcription start sites are indicated by arrows below and above the gene respectively. Also shown is an electrophoretic separation of PCR products amplified using primers specific for CAI colon mRNA (1F/1R), erythroid mRNA (2F/1R) and for both species of mRNA (3F/2R). The template RNA was prepared from adult colon (C) and human erythroleukemia cells (H). Markers (M) were 1 kb ladder from BRL.

Fig. 3. Autoradiograph of a Southern blot hybridised with a CAI cDNA probe, showing PCR products amplified from RNA prepared from various human adult (a) and fetal (f) tissues and the colon carcinoma cell line LIM1215. 16w and 28w indicates the weeks gestation; C indicates a no RNA control amplification and M a track where markers were visualised with ethidium bromide. The primers were the colon-specific 1F/1R pair (Fig. 2). In each case 5 μg of total RNA was reverse transcribed except in the case of the fetal muscle sample indicated by an asterisk where 5 μg poly(A) was used.

Fig. 4. Autoradiograph of a Southern blot hybridised with a CAI cDNA probe, showing PCR products amplified from RNA prepared from various colon carcinoma cell lines, adult human colon and the human erythroleukemic cell line, HEL. Aliquots of reverse transcribed RNA were amplified using both the colon specific primers, 1F/1R and the erythroid specific primers 2F/1R. C indicates a no RNA PCR.

Levels of CAI mRNA were relatively much lower in fetal 16 and 20 week gestation colon than in the adult tissue and could only be detected using labelled CAI cDNA as probe; CAI RNA could not be detected in fetal jejunum (Fig. 3).

In addition to the RNA studies a series of colon sections from human fetuses (25–33 weeks gestation) were analysed using the anti-CAI antibody. Significant levels of CAI protein were not apparent until 29 weeks gestation. At this stage of development the CAI protein was confined to the luminal epithelium as in the adult material (not shown).

CAI expression in colon carcinoma cell lines

Expression of CAI from the colon specific promotor was also investigated in a variety of human colon carcinoma cell lines. These cell lines display, to a variable extent, typical features of intestinal cell differentiation and are likely to provide useful model systems to investigate factors involved in intestinal-specific gene expression.

RNA PCR was used to screen twelve cell lines for CAI expression using initially the primers 2R and 3F (see Fig. 2) which amplify a 270 nt fragment across exons 2 and 3. Three colon carcinoma cell lines, LIM1215, LIM1899 and HT115 were identified which express CAI whereas the other nine cell lines HCA7, KMS-4, JW2, HT29, SW620, SW480, CCD841, colo320 and Caco-2 do not express CAI. The CAI mRNA present in LIM1215, LIM1899 and HT115 is transcribed solely from the colon promotor since the colon specific PCR primers 1R and colo1F amplified the expected 208 nt product, identical to that found in colon tissue (Fig. 4 and see LIM1215 in Fig. 3). There was no evidence for transcription from the erythroid promotor in these cells (Fig. 4). The full length (1.4 kb) CAI transcript is relatively abundant in LIM1215, LIM1899 and HT115 cells since it could be detected by the less sensitive Northern procedure (data not shown).
CAI expression in neoplasia

It is well documented that malignant cells often 'de-differentiate' and exhibit a fetal-like biochemical phenotype [37], and it seems possible that the absence of CAI expression in many colon carcinoma lines is a consequence of such changes. If this is the case then CAI may be a useful marker for malignant transformation and de-differentiation in the colon. In order to explore the potential of CAI in this regard we have investigated the pattern of CAI expression in colon neoplastic tissue.

Total cellular RNA was isolated from a panel of tumour and normal mucosal samples, derived from the rectum and sigmoid colon of 29 patients with sporadic colorectal cancer, and from a series of adenomas from eight familial adenomatous polyposis (FAP) patients. Levels of steady-state CAI mRNA were assessed by hybridisation of 5 µg total RNA samples applied as slots with 32P-labelled CAI cDNA (Fig. 5). Some variation in the levels of CAI mRNA in normal samples from different individuals was observed which may reflect differences in CAI expression along the length of the colon. For this reason each tumour sample was compared with an adjacent normal sample taken from the same patient and scanning densitometry of autoradiographs was used to compare signal intensities. Hybridisations of the same or parallel samples with an actin probe were used to control for RNA loading (Fig. 5). The results were striking. Out of 29 sporadic tumours 15 showed no CAI mRNA compared with the normal colon mucosa and 11 other tumours showed substantially lower levels (1-40% of normal). The remaining 3 showed reduced levels (40-80% of normal). Figure 5 shows some examples. Most of the tumours were graded histologically as moderately differentiated although these included a range of Dukes stages. No clear cut relationship between the Dukes stage, the histological grading and the amount of CAI mRNA present was apparent (Fig. 5).

Generally, benign polyps from FAP patients showed reduced levels of CAI mRNA, intermediate between the normal and tumour samples. Out of 38 adenomas from 8 FAP patients, 29 showed 1-40% of the normal level of CAI mRNA, 6 showed 40-60% of normal and 3 were near normal. Results obtained with multiple adenomatous polyps taken from two patients are shown in Fig. 5.

PCR amplifications of the tumour/normal colon RNA samples using the CAI colon specific primers demonstrated that CAI mRNA in these samples was transcribed specifically from the colon promoter (Fig. 6). The CAI erythroid specific primers did not amplify CAI erythroid transcripts in the tumour or normal samples. Thus the down regulation of CAI expression in colorectal neoplastic tissue is due to a reduction in transcription from the colon promoter and there is no evidence for the use of the erythroid promoter in neoplastic tissue. As an additional control in these experiments primers which amplify RNA for the glycolytic enzyme phosphoglucomutase 1 [35] were used in order to illustrate
the presence of amplifiable RNA. In each case PGM1 RNA was amplified, to give the expected 417 bp product, in both tumour and normal samples.

RNA in situ hybridisations and immunohistochemical analyses of tumour/normal sections showed that in the tumour sections the highly organised morphology of the epithelial crypts was, in general, no longer visible and the levels of both CAI mRNA and protein were greatly reduced (Fig. 7). It seems reasonable to suppose that this suppression of CAI transcription is concomitant with the neoplastic dedifferentiation process.

Discussion

Carbonic anhydrase (CA) is found in the colon of all mammals where it has a role in electroneutral NaCl reabsorption, secretion of HCO₃⁻ to alkalise the luminal content and absorption of short chain fatty acids [29]. We have shown that in the developing human fetus the CAI gene is transcribed at low levels from 16 weeks onwards, although levels of mRNA and protein do not become significant until much later during gestation at week 28-29. This is in line with the observation made by Lonnerholm et al. [16] using immunohistochemistry that CA activity in fetal colon at 19-21 weeks gestation is around 7% of that found in the adult. These observations suggest that the proximal colon promoter is inactive in early fetal life. It may be significant that the distal erythroid promoter of CAI is similarly inactive at this stage of development; neither erythrocyte CAI protein or mRNA are detectable before birth [4, 6]. It will be of interest to establish whether the same sequence elements could be responsible for the repression of both promoters. The identification of three cell lines, LIM1215, LIM1899 and HT115, expressing CAI from the colon promoter provides the basic ingredient for an in vitro transfection system for direct investigation of those gene sequences and nuclear factors which regulate CAI expression.

The very sensitive procedure of RNA in situ hybridisation has allowed us to focus in detail on the pattern of CAI expression within the colonic epithelium. These analyses show that CAI transcripts are absent from the proliferating cells at the base of the crypt, accumulate to high levels in differentiating cells in the mid-region of the crypt and decrease as the cell approach the luminal surface. This variation in the abundance of CAI mRNA may reflect differential transcription from the colon promoter and/or variable mRNA stability. A further mechanism of control appears to be operating post-transcriptionally since CAI protein does not accumulate in those cells containing most mRNA, but in the mature cells on the luminal surface where mRNA levels are relatively low. The details of these regulatory mechanisms are at present uncertain. Nevertheless it is clear that CAI is a marker for colon epithelia and furthermore is specific to a particular stage in the differentiation of the epithelial cells as they mature in the crypt.

Several proteins that define differentiated phenotypes in the cells of the gastrointestinal tract have been identified but only a few have been characterised in detail. Amongst these sucrase isomaltase (SI) in the small intestine presents a similar picture to that which we have outlined for CAI. Recent work has described abundant SI mRNA in the crypt-villus junction but low levels in villus tip cells and this distribution has been attributed to either differential transcription of the SI gene or mRNA stability [31]. Using a sensitive procedure of sequential immunoprecipitation with monoclonal antibodies it has been shown that the distribution of SI protein does not correspond exactly to that of the mRNA, but is found in both villus and crypt cells [3]. In this case only the protein in the villus is enzymatically active and it has been suggested that SI gene expression along the crypt-villus axis may also be regulated post-transcriptionally [3]. In addition the expression of lactase mRNA and protein do not correspond in newborn rat small intestine indicating a post-transcriptional control mechanism for this gene in fetal development [25].

In neoplasia the colonic epithelium loses, in a progressive manner, its organised cryptal structure and differentiated cellular phenotypes, and malignancy is associated with loss of CAI expression. Adenomas from FAP patients, which represent a benign intermediary stage in this process showed reduced levels of CAI mRNA, intermediate between normal and adenocarcinoma. This
points to the loss of CA1 transcripts as an early event occurring prior to a malignant transformation. Out of 12 colon carcinoma cell lines investigated only three, LIM1215, LIM1899 and HT115 express CA1. It can be concluded that these particular cell lines have retained or re-established some aspects of their colonic epithelial phenotype. There is evidence that at least two of the cell lines which do not express CA1, Caco-2 and HT-29 have undergone some reversion to a more fetal phenotype [38], expressing genes whose products are transiently observed in colon at mid-gestation [32, 37]. Thus in summary the abundant expression from the colon promoter of CA1 is a specific marker for the adult colon epithelial phenotype and its loss in neoplasia appears to be related to loss of differentiation rather than transformation.

Acknowledgement. Sarah Leigh was supported by the Cancer Research Campaign. The cell lines LIM1215 and LIM1899 were kindly provided by Robert Whitehead, Ludwig Institute, Melbourne. The authors are grateful to Virginia Sams, UCIU, for providing colon sections nd the MRC Tissue Bank at the Royal Marsden Hospital for supplying fetal tissues.

References


Molecular genetic evidence for the delineation of a more severe form of familial adenomatous polyposis which results from fresh mutation

M. REES, S. E. A. LEIGH, J. D. A. DELHANTY, L. BOWLES AND I. C. TALBOT

1 The Galton Laboratory, Department of Genetics and Biometry, University College London.
4 Stephenson Way, London NW1 2HE, UK
2 The Polyposis Registry and ICRF Colorectal Cancer Unit, St Mark's Hospital, City Road.
London EC1V 2PS, UK

SUMMARY

Familial adenomatous polyposis, an inherited pre-malignant condition, is caused by mutation in the adenomatous polyposis coli (APC) gene at chromosome 5q22. The lifetime risk of carcinoma approaches 100%, with an average age at death from cancer of 40 years, allowing most patients to complete reproduction. Since there is no evidence for a rising incidence, this is at variance with an apparently high mutation rate. We present evidence for the delineation of a severe form which hitherto has largely been maintained by fresh mutation. An atypically high frequency of loss of heterozygosity at chromosome 5q22 in small adenomas correlated with an early age of onset or malignancy in two patients, both due to fresh mutation. In both cases, the mutation in APC was shown to be a commonly occurring deletion, leading us to postulate the co-existence of a modifying gene.

INTRODUCTION

The inherited premalignant condition, familial adenomatous polyposis, is due to dominant mutation in a recently isolated gene, APC, which maps to chromosome 5q22 (Groden et al. 1991; Kinzler et al. 1991a). Germline mutation of APC has been demonstrated in approximately 70% of families (Cottrell et al. 1992; Miyoshi et al. 1992a). The majority of changes are 1 to 5 bp deletions leading to frameshifts, with the remainder being point mutations; all are predicted to lead to non-functioning protein products. Somatic mutations occurring in both familial and sporadic colorectal carcinogenesis are of a similar nature, with an approximately equal frequency of frameshift and point mutations (Miyoshi et al. 1992b; Powell et al. 1992). Both somatic and germline changes are clustered in the 5’ half of the last exon, 15.

The presence of at least 100 adenomas in the colon and rectum is usually taken as diagnostic of the classical form of familial polyposis, although the average number in counted colectomy specimens is about 1000 (Bussey, 1975). Extra-colonic neoplastic lesions are frequent and include osteomas, epidermoid cysts, desmoid tumours and the diagnostically important congenital hypertrophy of the retinal pigment epithelium (CHRPE) (Gardner, 1951; Chapman et al. 1989). Due to the number of adenomas present the lifetime risk of colorectal (CR) cancer approaches 100% (Muto et al. 1977), but for each adenoma the chance of progression to malignancy is low.

* To whom correspondence should be addressed.
It is frequently stated that the mutation rate is high, based on the figure for the proportion of 'isolated' cases with no apparent family history (33% in various studies: Reed & Neel, 1955; Veale, 1965; Bülow, 1987). There are some obvious possible causes for the occurrence of isolated cases apart from fresh mutation. Polyposis-related cancer can be of late onset (Bussey, 1975); such patients may die from other causes before the malignancy manifests itself, and non-paternity is not uncommon. Without prophylactic colectomy the average age at death from carcinoma in APC patients is 40 years (Bussey, 1975), which suggests that most would by then have completed their families. A factor which would affect reproduction is genetic heterogeneity; if a subset of patients is prone to malignancy of early onset this may have precluded reproduction in previous generations. Such severe forms may thus be maintained by fresh mutation. Based on molecular genetic data we present evidence for the delineation of a more severe form of APC in two patients from the St Mark's Polyposis Registry, both of whom appear to be new mutations.

MATERIALS AND METHODS

Patients studied

The first patient was APC 34 in the paper by Rees et al. (1989). He presented with symptoms of diarrhoea and some bleeding at the age of 15. Examination of the colon after removal revealed a high density of polyps, with an overall estimated number in excess of 3500. There was no family history of polyposis: both parents, aged 44 and 40 respectively, and a sister were examined and found to be unaffected. The maternal grandparents were alive and well; those on the paternal side died at the ages of 62 and 86 with no evidence of colorectal malignancy. The second patient studied in detail (APC 145) was a 23-year-old woman who was initially thought to have Crohn's disease. Further investigation revealed the presence of a rectal carcinoma, and at operation the diagnosis of APC was made. On examination the colectomy specimen was found to have an estimated 2500 polyps and a second cancer, arising in an adenoma, was present. Within a year a third rectal carcinoma was found. There was again no positive family history: the mother aged 53 and the father, 54, were examined by sigmoidoscopy and found to be unaffected. Ophthalmological examination showed 4 typical CHRPEs in the patient but none in the parents. Both paternal grandparents are alive and well at the age of 75; the maternal grandparents died of a heart attack and chest ailments respectively.

Tissue samples

Normal colonic mucosa and three 6 mm adenomas, one from the transverse colon and two from the descending colon, were obtained from the first patient, APC 34. From the second, APC 145, in addition to normal mucosa, 10 adenomas (2 from the ascending, 4 from the transverse and 4 from the descending colon) ranging in size from less than 5 mm to 15 mm, were studied. All tissues were received fresh, and portions were later flash frozen in liquid nitrogen for further study.

Blood samples were obtained from the parents of no. 145 in order to check paternity. The parents of no. 34 were unavailable for this study. Further analysis was also undertaken on the APC adenoma series previously studied (Rees et al. 1989).
### Table 1. Informative DNA probes used for Southern hybridization

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**DNA extraction and Southern hybridization**

DNA was extracted from tissue samples by standard methods (Maniatis et al. 1982). Samples were digested with the appropriate restriction endonuclease and size-fractionated by agarose gel electrophoresis. DNA was transferred to either Gene Screen Plus (NEN, DuPont) or Hybond X Plus (Amersham, UK) membrane and hybridized according to the manufacturer's specifications to DNA probes radiolabelled with [32P]dCTP (3000 Ci mmol⁻¹) by the random hexanucleotide primer method (Feinberg et al. 1983). Membranes were washed to a stringency of 2 x SSC, 1% SDS at 65 °C and autoradiographed at -70 °C using Fuji RX X-ray film.
Detection of K-ras mutations

A 111 bp fragment of exon 1 of the K-ras gene (including the mutation hot-spot codons 12 and 13) was amplified by the polymerase chain reaction (PCR) and a tenth of the PCR product was dot-blotted in duplicate on to Hybond N+ (Amersham) in accordance with the manufacturer's specifications. These dot blots were hybridized to a series of T4 kinase-labelled mutant oligonucleotides each specific for the various first and second base point mutations possible at codons 12 and 13. To reduce the number of hybridizations required to produce a positive result, four mutation 'cocktails' were prepared, one for each of base positions 1 and 2 of codons 12 and 13, respectively, and each consisting of three oligonucleotides, each one specific for one of the three base substitutions possible at that position. Oligonucleotide hybridizations were carried out according to the method of Farr et al. (1988) and autoradiography performed overnight at −70 °C. To check that the PCR products were equally loaded, all filters were finally hybridized to one of the PCR primers which had also been end-labelled with T4 kinase. Samples proving positive in the dot-blot assay were verified by direct sequencing using the method of Ruano et al. (1990), which also enabled the exact base substitution to be determined.

DNA probes

Informative polymorphic DNA probes used to detect loss of heterozygosity are listed in Table 1.

Histological examination

Frozen sections were cut from the adenomas of patient 145. They were prepared and stained by standard methods and analysed to determine the degree of dysplasia and to estimate the percentage of DNA contributed by the adenomatous tissue.

RESULTS

To detect loss of heterozygosity (LOH), DNA prepared from 10 adenomas from patient 145 was compared with that from normal mucosa using DNA probes hybridizing to chromosomes 5p17p and 18q. A search for mutation in K-ras, codons 12 and 13, was also performed. Results for the adenomas are shown in Table 2 and Fig. 1. The four largest, 7.5–15 mm in size, but displaying no more than moderate dysplasia, all showed LOH for the same region of chromosome 5q, extending from the MCC gene to 5q35–qter and thus encompassing APC. The same allele was lost in each adenoma. Evidence for a non-random event, K-ras mutation was demonstrated in one 9 mm adenoma only.

Previously, we had studied LOH in APC adenomas using the hypervariable probe, pMS8 (5q35–qter) (Wong et al. 1987) and found evidence of loss in 3 only of 39 informative samples (Rees et al. 1989); two of the three were from patient 34. Further investigation of his adenomas using intragenic and flanking probes for APC revealed that the deletion in both polyps extended to include the whole APC gene region (Table 2). An additional 20 of our original APC adenoma series were informative for APC flanking probes; only a single extra example of LOH for this region was found.

All DNA samples from patient 145 were also studied for LOH on chromosome 17p and 18q.
Table 2. Chromosome 5 allele loss and K-ras activation in adenomas from two APC patients

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<td>5</td>
<td>Tub. ad. mild dyspl.</td>
<td>55</td>
<td>1/2</td>
<td>1/2</td>
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<td>12</td>
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<tr>
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<td>50</td>
<td>1/2</td>
<td>1/2</td>
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<td>1/2</td>
<td>1/2</td>
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<tr>
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<td>Hom</td>
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<td>1/2</td>
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<td>60</td>
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<td>1/2</td>
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<tr>
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<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>Hom</td>
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</tr>
</tbody>
</table>

* Histol., histology; tub. ad., tubular adenoma; mild, mod. dyspl., mild or moderate dysplasia. (2) Mutation at position 12-2. GGC > GAC, heterozygous; NT, not tested. Hom, homozygosity in the constitutional DNA. Where the normal tissue was informative, neoplastic genotype is shown in the table. Heterozygosity is indicated by 12 even though some probes recognize multi-allelic systems. Reduction of intensity is indicated by ( ); complete absence of an allele by a dash.
sites of the p53 and DCC genes, frequently found to be involved in colorectal cancer (Vogelstein et al. 1988). No loss at these sites was found in the adenomas.

Paternity testing for APC 145 revealed no inconsistency with three VNTR probes.

**DISCUSSION**

Since most, if not all, CR carcinomas are thought to arise from pre-existing adenomas in the adenoma carcinoma sequence (Muto et al. 1977), adenomas from polyposis patients provide a useful model system for the study of adenomas of various sizes and grades of dysplasia. The normal allele of the APC gene is considered to belong to the class of genes known as "tumour suppressors", in that loss or inactivation of one or possibly both copies of the gene allows progression to the malignant state (Knudson, 1971). Loss of the remaining normal allele during tumorigenesis might thus be expected to occur in polyposis patients. Japanese (Miyoshi et al. 1992b) and our own unpublished LOH data have confirmed that this is true of the carcinoma stage in at least some APC patients. Using markers on chromosome 5q which map close to the APC locus, many groups sought evidence for loss of heterozygosity in the region of the APC gene in the adenomas of these patients with no positive result (Solomon et al. 1987; Vogelstein et al. 1988; Law et al. 1988), giving rise to the idea that allele loss occurs at or near the time of transition to a carcinoma (Solomon, 1990). In a definitive study, Miyaki and colleagues reported that LOH occurred in less than 2% of polyposis adenomas with moderate dysplasia (Miyaki et al. 1990). The rarity of chromosome deletion leading to detectable LOH in familial adenomas is confirmed by recent somatic mutation studies (Miyoshi et al. 1992b). Although 5 of 8 polyposis adenomas exhibited somatic alteration of the normal allele of the APC gene this was due to LOH in only a single specimen. The observed high frequency of allele loss in adenomas with at most mild to moderate dysplasia in our two patients suggests that they are manifestly atypical in this respect.

Our LOH data on adenomas together with the results of the somatic mutation studies (Miyoshi et al. 1992b; Powell et al. 1992) provide evidence that inactivation of both alleles of APC can be an early event in the progression to malignancy and hence is likely to provide a growth advantage. One only of the adenomas showing chromosome 5 LOH had K-ras activation and there was no evidence for LOH at other sites commonly involved in CR carcinogenesis, in support of the suggestion (Miyaki et al. 1990) that these are later events in the polyposis adenoma carcinoma sequence.
Severe familial adenomatous polyposis

The early age at which our patients presented with symptoms or multiple cancers is at variance with the findings in classical polyposis (Bussey, 1975). So far as we are able to ascertain, both patients are the result of fresh mutation. It is of interest that an interstitial 5q deletion was found in 5 of 7 adenomas from a Japanese patient with polyposis which was also the result of fresh mutation (Miki et al. 1992).

We first thought that these mutations resulting in a severe phenotype would be due to allelic variants of the classical polyposis mutation. But work by Vogelstein and colleagues (personal communication) has shown that APC 145 has the common 5 bp deletion in the ‘G’ region of exon 15 that is found in 10–15% of patients worldwide (Cottrell et al. 1992; Miyoshi et al. 1992a) and we have found the same deletion to be present in APC 34 (Gayther & Delhanty, unpublished observation). Information on phenotype–genotype correlation in APC is being gathered, but it seems unlikely that all patients with this same 5 bp deletion are severely affected. We propose therefore that in patients like the two reported here an additional, modifying mutation is present, of the ‘mutator’ type (Loeb, 1991), which causes increased chromosomal instability and hence speedier progression to malignancy. In previous generations genetic fitness is likely to have been severely impaired and this form of polyposis maintained solely by fresh mutation.

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REFERENCES


