Mutation Analysis of the Adenomatous Polyposis Coli Gene

by

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The Galton Laboratory Department of Biology University College London
To Joan, Lindsay, Julian and Becky
Acknowledgements

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Abstract

Familial adenomatous polyposis (FAP) is a dominantly inherited syndrome characterised by the development of hundreds to thousands of adenomatous polyps throughout the colon and rectum. These usually arise during the second or third decades of life, and in the absence of surgical intervention it is inevitable that one or more will progress from a benign to a malignant state. The underlying cause of FAP is mutation of the adenomatous polyposis coli (APC) gene, a tumour suppressor gene located on chromosome 5q21-22.

Analysis of the APC gene undertaken during this study has provided data on the type and distribution of APC mutations in FAP patients. Fifty two unrelated patients were investigated and mutation was detected in 35 (67%) of them. Of the different types of mutation identified deletions and insertions were the most frequently noted accounting for 85% of all mutations. Nonsense and splice consensus sequence mutations were also detected, all of which are predicted to result in premature truncation of the APC protein. Mutations were identified in most regions of the APC gene assessed but showed a marked clustering within the 5′ half of exon 15. Mutations at codon 1061 and codon 1309 were found to be responsible for 10% and 19% of FAP cases respectively. In addition to analysis of APC sequence variants chromosomal rearrangements and deletions in the vicinity of the APC gene were investigated using fluorescence in situ hybridisation (FISH).

Aspects of the FAP phenotype were considered and compared to the site of mutation within the APC gene. A correlation between severity of phenotype, defined by number of polyps and age of disease onset, and location of APC mutation was identified. Mutation between codons 1309 and 1464 was found to be associated with early onset of cancer and the development of thousands rather than hundreds of colorectal polyps. Interestingly the majority of patients with no family history of FAP (new mutations) were found to have mutation within this 'severe' region of APC.

Presymptomatic diagnosis of FAP was performed for members of FAP families that were of uncertain gene status. This was accomplished by direct mutation detection or by linkage analysis. Additionally a strategy for preimplantation genetic diagnosis of this disorder was also developed. This involved detection of APC
mutation in single cells biopsied from human preimplantation embryos at the cleavage stage. After extensive preliminary testing this method reached clinical application, but no pregnancy was achieved on this occasion.
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## Abbreviations used in this thesis

Abbreviations commonly found in this thesis. Additional abbreviations are defined where appropriate in the text.

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<td>A</td>
<td>adenine residue in a DNA sequence</td>
</tr>
<tr>
<td>ADO</td>
<td>allele drop-out</td>
</tr>
<tr>
<td>APC</td>
<td>adenomatous polyposis coli gene</td>
</tr>
<tr>
<td>APC</td>
<td>adenomatous polyposis coli protein</td>
</tr>
<tr>
<td>ARMS</td>
<td>amplification refractory mutation system</td>
</tr>
<tr>
<td>ASO</td>
<td>allele specific oligonucleotide</td>
</tr>
<tr>
<td>AT</td>
<td>ataxia-telangiectasia</td>
</tr>
<tr>
<td>bcr</td>
<td>breakpoint cluster region</td>
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<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BS</td>
<td>Bloom syndrome</td>
</tr>
<tr>
<td>C</td>
<td>cytosine residue in a DNA sequence</td>
</tr>
<tr>
<td>CDGE</td>
<td>constant denaturing gel electrophoresis</td>
</tr>
<tr>
<td>cdk</td>
<td>cyclin dependent kinases</td>
</tr>
<tr>
<td>CGH</td>
<td>comparative genomic hybridisation</td>
</tr>
<tr>
<td>CHRPE</td>
<td>congenital hypertrophy of the retinal pigment epithelium</td>
</tr>
<tr>
<td>CMC</td>
<td>Chemical mismatch cleavage</td>
</tr>
<tr>
<td>CVS</td>
<td>chorionic villus sampling</td>
</tr>
<tr>
<td>Δ</td>
<td>deletion of DNA sequence</td>
</tr>
<tr>
<td>DCC</td>
<td>deleted in colon cancer gene</td>
</tr>
<tr>
<td>DGGE</td>
<td>denaturant gradient gel electrophoresis</td>
</tr>
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<td>DMD</td>
<td>Duchenne muscular dystrophy</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>FA</td>
<td>Fanconi’s anemia</td>
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<tr>
<td>FAP</td>
<td>familial adenomatous polyposis</td>
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<tr>
<td>FISH</td>
<td>fluorescent in situ hybridisation</td>
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<tr>
<td>G</td>
<td>guanine residue in a DNA sequence</td>
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</table>
GSK 3β  glycogen synthase kinase 3β
GTP  guanine triphosphate
GDP  guanine diphosphate
HMPS  hereditary mixed polyposis syndrome
HNPCC  hereditary nonpolyposis colorectal cancer
HPRT  hypoxanthine phosphoribosyl transferase
HSR  homogeneously staining regions
IGF-BP3  insulin-like growth factor-binding protein-3
ins  insertion of a DNA sequence
IVF  *in vitro* fertilisation
kb  kilobase pairs
λ  Bacteriophage lambda
LFS  Li-Fraumeni syndrome
LOH  loss of heterozygosity
Mb  Megabase pairs
MCC  mutated in colon cancer gene
MEN2A  multiple endocrine neoplasia type 2A
Min  multiple intestinal neoplasia
NSAID  nonsteroidal anti-inflammatory drug
PCR  polymerase chain reaction
PGD  preimplantation genetic diagnosis
PFGE  pulsed field gel electrophoresis
PKC  protein kinase C
PTT  protein truncation test
RER⁺  replication error positive
RER⁻  replication error negative
RFLP  restriction fragment length polymorphism
RNase  ribonuclease
SSCP  single-strand conformation polymorphism
T  thymine residue in a DNA sequence
U  units
UPD  uniparental disomy
v/v  volume for volume
w/v  weight for volume
XP  xeroderma pigmentosum
YAC  yeast artificial chromosome
Chapter 1

Introduction
Introduction


1.1. Epidemiology of cancer.

There is a wealth of evidence suggesting that cancer should be considered a genetic disease, an uncontrolled proliferation of cells caused by mutation of genes that normally regulate cell division, differentiation, and cell death. The existence of inherited disorders that predispose to cancer is, in itself, indicative of a genetic involvement. The majority of cancer syndromes show a pattern of inheritance consistent with highly penetrant autosomal dominant genes, however low penetrance genes are also thought to exist. Almost every form of cancer can be found in both familial and nonhereditary (sporadic) forms (Knudson, 1986) and in most cases the two cannot be separated by histological assessment. Individuals presenting with cancer as a result of an inherited gene are believed to account for a small, but significant portion of total cancer cases, probably less than 5% (Ponder, 1990).

A genetic basis for cancer is also suggested by the high level of chromosome abnormalities observed in many tumours. Microscopically visible deletions, translocations, and structures such as homogeneously staining regions (HSR) and double minutes, indicate loss of some genes and amplification of others. That DNA damage is critical for the development of cancer is highlighted by experiments demonstrating the carcinogenic nature of mutagens (Ames, 1979). Excessive exposure to ultraviolet light for example can cause the formation of pyrimidine dimers, which result in cytosine to thymine transitions, and frequently lead to the development of skin cancer.

Approximately $10^{16}$ cell divisions occur during the course of a human lifetime. Given the fundamental limitations on DNA replication and repair it is estimated that mutations will occur spontaneously even in an environment free of mutagens at a rate of $10^{-6}$ mutations per gene per cell division. Therefore it is likely that every gene
undergoes mutation on approximately $10^{10}$ separate occasions during a lifetime. From this perspective the most compelling question is not why cancer occurs, but why it occurs so infrequently.

Clearly, carcinogenesis is a multistep process requiring an accumulation of mutations in various genes. This is implied by the increase in cancer prevalence that occurs with age. Age represents the most significant single risk factor for the development of cancer (Miller, 1980). If cancer were the consequence of a single mutational event then individuals of different age groups should have an equal probability of developing cancer, this is not so. In the majority of cases cancer mortality increases proportionately with age (Nordling, 1953), although there are exceptions, principally those cancers influenced by endocrine secretions (Armitage and Doll, 1954). Mathematical approaches have been employed in an attempt to predict the number of mutations required to give rise to a cancer, estimating that between four and seven individual mutations are required (Nordling, 1953; Armitage and Doll, 1954). Complementary data comes from analysis of exposure to carcinogenic agents. A long time period separating initial exposure and tumour formation has been reported, suggesting that further mutations need to accumulate to transform normal cells to a malignant phenotype (Boice and Monson, 1977). The incidence of lung cancer, for example, does not begin to rise steeply until after 10-20 years of heavy smoking, while industrial workers exposed to chemical carcinogens for limited periods sometimes take more than 20 years to develop cancers characteristic of their occupation (Horton and Bingham, 1977). Considering the specific case of colorectal cancer Ashley (1969) also concluded that a number of mutations are necessary for tumour formation, and hypothesised that individuals who are genetically predisposed to colorectal cancers, develop colorectal cancer at an early age because they do not need to acquire so many mutations.

Recently genes involved in the regulation of cell growth, differentiation and division have been isolated and demonstrated to contain mutations in many cancers (Bishop, 1991). Furthermore inherited predisposition to cancer has been shown to result from inactivation or inappropriate expression of some of these genes (Bishop, 1991). As well as mutation of the genes directly involved in the control of cell proliferation, a tendency to develop cancer can also result from mutation of genes that
influence the rate at which mutations accumulate. Mutation in any one of several genes functioning in DNA mismatch repair can be responsible for the dominantly inherited cancer susceptibility syndrome hereditary non-polyposis colorectal cancer (HNPCC) (Watson and Lynch, 1993). Examples of autosomal recessive diseases in which defects in DNA repair lead to cancer predisposition also exist and include ataxia-telangiectasia (AT), Fanconi's anemia (FA), xeroderma pigmentosum (XP), and Bloom syndrome (BS) (Shroeder, 1982; Lehman, 1981). In the absence of efficient DNA repair mutations that cause a malignant phenotype are less likely to be corrected and consequently cancers are formed more rapidly. The study of a gene involved in an inherited cancer is of interest not only in terms of the syndrome it produces but also because the causative gene may be involved in the much more common sporadic form of the tumour. Once the functions of critical genes are understood it may be possible to devise new therapies for the treatment of cancer.

It is widely believed that tumours are monoclonal in origin, developing from a single cell that has acquired a combination of mutations that allow it, and it's descendants, to escape the normal regulatory constraints on cell growth. The probability that any given cell will accumulate such a set of mutations without being eliminated by the immune system or undergoing apoptosis, is extremely low. However, an initiating mutation may produce a limited amount of cellular proliferation, in turn providing a larger population of cells in which a second mutational event can occur. This may allow an even greater degree of proliferation, and so on until a malignant phenotype is produced (Nowell, 1976). Evidence supporting the monoclonal theory includes the observation that identical chromosome rearrangements are often found in every cell within a given tumour. Furthermore, investigation of the phenomenon of X-chromosome inactivation has shown that in the vast majority of benign and malignant tumours developed by women all the tumour cells have the same X-chromosome inactivated (Noguchi et al., 1992; Li et al., 1996).

Thus a cancer follows an evolutionary path with progressively more aggressive cells outgrowing earlier zones of dysplasia, made up of less rapidly dividing cells, and ultimately giving rise to a malignant metastatic tumour. Even following this hypothesis the probability of a cell developing a malignant phenotype
remains small, and in the human situation usually requires many years to occur

An alternative hypothesis argues that tumours are not clonal in origin, requiring the interaction of multiple cells. In this case the apparent monoclonality of tumours is explained by the emergence of a dominant clone that develops after the initiation of the tumour and proceeds to outgrow other clones (Rubin, 1985; Alexander, 1985). In support of this theory it has been noted that few tumours are composed entirely of one tissue type. Furthermore, a recent serendipitous discovery of an X0/XY mosaic patient with familial adenomatous polyposis (FAP) has also provided support for a polyclonal origin of cancer (Novelli et al., 1996). Patients with FAP develop hundreds of adenomas throughout the colorectum, each of which has generally been thought to derive from a single cell. However, individual adenomas found throughout the mosaic patient’s colon contained dysplastic crypts composed of both X0 and XY cell lineages, rather than the expected single cell type (Novelli et al., 1996). Although the most compelling conclusion appears to be a polyclonal origin of colorectal adenomas in this FAP patient, environmental changes in a small region of tissue could elicit epigenetic changes (alterations of the pattern of gene expression without a change in DNA sequence) effecting a number of cells in a discrete area. Heritable epigenetic changes are a feature of normal development, and have been implicated in the formation of teratocarcinoma.

1.2. Molecular genetics of cancer.

The genes which govern the complex negative and positive feedback systems controlling cell growth and division fall into two categories: proto-oncogenes and tumour suppressor genes (anti-oncogenes). Each class of gene encompasses members with diverse biological functions. Broadly speaking tumour suppressor genes act negatively on cell growth and must be entirely inactivated if progression to cancer is to occur. In the case of proto-oncogenes mutation does not cause inactivation, rather it typically confers a dominant positive influence on cell growth, transforming the proto-oncogene into an oncogene. It is the subtle balance of growth promotion and growth suppression, provided by these genes, that is responsible for normal cellular behaviour and tissue homeostasis (Weinberg, 1991).
1.2.1. Oncogenes.

Proto-oncogenes form a heterogeneous group of genes which normally function in the control of cell proliferation. The localisation and function of their protein products is varied. They may be found in the nucleus, the cytoplasm, or associated with cellular membranes. Oncogenic proteins act either as growth factors (e.g. the sis oncogene that produces platelet derived growth factor), growth factor receptors (e.g. v-erbB), transducers of growth factor responses (e.g. the ras family of oncogenes), or transcription factors (e.g. the myc gene family) (Hunter, 1991). The evidence currently available suggests that proto-oncogenes exert their effects by acting in one of three possible pathways: protein phosphorylation with serine, threonine, and tyrosine as substrates (Hunter et al., 1987); signal transmission by GTPases (Bourne et al., 1990); or control of transcription (e.g. Ariga et al., 1989).

The first oncogene isolated was v-src found in the tumourigenic Rous sarcoma virus, a retrovirus of the chicken. It was subsequently discovered that v-src is an activated form of a normal cellular gene (a proto-oncogene) from the chicken genome (Stehelin et al., 1976; Bishop, 1987). This led to the hypothesis that other cellular genes might have transforming potential if their expression or coding sequence is disturbed. Since these initial findings an increasing number of proto-oncogenes that are mutated in human tumours have been isolated. In some cases identification of the same oncogene has been achieved from two different retroviral species, suggesting that proto-oncogenes are not numerous in the genome (Eva et al., 1982).

Although critical for malignant transformation mutation of a proto-oncogene has never been reported as the sole genetic defect in a cancer, suggesting that such mutations alone are incapable of causing transformation (Weinberg, 1989). Experiments conducted in vitro have demonstrated that activation of at least two proto-oncogenes is necessary for transformation. One of the first such experiments showed that transformation of rat fibroblasts could be achieved by the action of two viral oncogenes together, whereas the tumourigenic phenotype was not produced by either of the genes acting alone (Rassoulzadegan et al., 1982). Similar experiments using the cellular oncogenes myc and ras demonstrated that the requirement for two or more activated oncogenes is not unique to virus mediated transformation (Land et al.,
1983). The fact that ras and myc have different cellular localisations led to the suggestion that oncogenes collaborating in transformation might be confined to different biochemical pathways regulating cell growth (Weinberg, 1985). Cytoplasmic oncoproteins such as the products of the ras genes and src allow anchorage independent growth and alter cellular morphology and growth factor requirements, however they are not capable of immortalising cells (Weinberg, 1985). The converse appears to be true of oncoproteins with a nuclear localisation such as those produced by fos, jun and the myc genes (Weinberg, 1985). This hypothesis holds true in as much as the co-operation of two oncogenes is required for expression of the full tumourigenic phenotype, however there are examples of two nuclear (Ruppert et al., 1990) or two cytoplasmic oncoproteins (Reed et al., 1990) interacting to induce transformation.

Like the oncogenes themselves the genetic changes that lead to activation of proto-oncogenes are diverse. Alterations include: point mutations; small deletions and insertions; increases in copy number (amplification); and juxtaposition of oncogenes to other chromosomal regions by translocation or inversion (Solomon et al., 1991 and references therein). The most frequently noted mutation of ras proto-oncogenes is point mutation occurring at one of a number of hot-spots. Members of the highly conserved ras family are mutated in a wide range of human malignancies (Bishop, 1991).

Chromosomal rearrangements effecting oncogenes have been observed in a number of malignancies and are particularly common in certain leukaemias and lymphomas (Mitelman et al., 1990). Translocations that move growth-promoting genes into the vicinity of strong promoters or enhancers can have oncogenic potential, while those that interrupt proto-oncogenes can affect their function or expression. The creation of a fusion protein underlies the frequent appearance of a reciprocal translocation involving chromosome 9q band 34 and chromosome 22q band 11 in chronic myeloid leukaemia, the so called Philadelphia chromosome (Nowell and Hungerford). This rearrangement most frequently moves the c-abl proto-oncogene from 9q34 to a location within a 5.8 kb region of 22q11 termed the breakpoint cluster region (bcr) (de Klein et al., 1982; Bartram et al., 1983). The break point cluster region actually lies within an intron of a gene also termed bcr. Because the transcriptional orientation of c-abl and bcr is the same the Philadelphia translocation
creates a fusion hybrid gene that produces a 210 kDa $bcr-abl$ fusion protein with oncogenic properties (Shtivelman et al., 1985; Ben-Neriah et al., 1986; Clark et al., 1987).

Another cytogenetic feature frequently observed in cancer cells is the appearance of double minute chromosomes and homogeneously staining regions. These structures are associated with gene amplification and are particularly common in more advanced aggressive tumours, and in tumours that have survived some forms of chemotherapeutic treatment (Alitalo and Schwab, 1986; Tlusty et al., 1989). Lower levels of gene amplification may involve more straightforward karyotypic changes such as the simple increase in the number of copies of a given chromosome. Certain forms of cancer show characteristic amplification of specific proto-oncogenes occurring in a significant proportion of tumours. Examples include adenocarcinoma of the breast and ovary, which frequently display amplification of $c$-$erbB-2$, particularly in more advanced tumours (Liberman et al., 1985; Slamon et al., 1987), and amplification of the proto-oncogenes $c$-$myc$ and $L$-$myc$ in carcinomas of the breast, cervix, and lung (Bishop et al., 1991). Such amplifications occur only rarely if at all in normal cells (Wright et al., 1990).

In contrast to tumour suppressor genes, mutant forms of proto-oncogenes are rarely inherited. Presumably the dominant mode of action of the oncogene is less tolerable during development than the recessive nature of tumour suppressor genes (Scrable et al., 1990). The only proto-oncogene implicated in an inherited cancer predisposition syndrome is $ret$, germline transmission of which results in multiple endocrine neoplasia type 2A (MEN2A), a disease characterised by predisposition to thyroid cancer (Mulligan et al., 1993).
1.2.2. Tumour suppressor genes.

The existence of genes which act negatively on cell growth and other aspects of the malignant phenotype has been indicated by several lines of research. Somatic cell hybridisation experiments showed that tumour cells fused with normal cells adopt a non-tumourigenic phenotype, suggesting that the normal cell provides factors, no longer functional in the tumour cell, which act negatively on cell growth and division. It is now known that these factors are genes which normally restrain proliferation (Harris et al., 1969; Stanbridge, 1976). Chromosomes are lost at random from the unstable hybrids created by fusion experiments, and consequently it is possible to correlate loss of specific normal chromosomes with reversion to a tumourigenic phenotype, and to propose probable locations for important tumour suppressing genes. Since these early studies transfection experiments have refined this approach, allowing the introduction of individual normal chromosomes (e.g. Weismann et al., 1987; Huang et al., 1988), and even specific genes (e.g. Casey et al., 1991). Remarkably Boveri predicted that elimination of growth suppressing chromosomes might be responsible for cancer as early as 1914 (cited in Bishop, 1991). However, it has only been possible to show that this hypothesis is correct since the development of molecular techniques.

Work by Knudson (1971) had also provided support for the existence of tumour suppressor genes. He devised a scheme that explains differences between familial and sporadic forms of retinoblastoma, a rare childhood eye tumour. He postulated that the tumours observed in retinoblastoma are caused by two mutations of the cell genome, the now classic ‘two-hit’ hypothesis. In sporadic retinoblastoma both genetic lesions must, by chance, occur in the same somatic cell. This is a very unlikely event, and consequently sporadic retinal tumours are rare and always unilateral. In familial retinoblastoma one of the two mutations necessary is already present in every retinal cell, having been inherited from an affected parent. As a consequence only one mutation need occur as a somatic event, and therefore the probability of cancer is much higher than in the sporadic case. This explains the high frequency of bilateral tumours and early onset of malignancy observed in familial retinoblastoma (Knudson et al., 1971). The targets of mutation were eventually identified as the two copies of the RB1 gene on chromosome 13q14 (Friend et al.,...
1986). Inactivation of both copies of \textit{RBI} were found in tumours associated with retinoblastoma (Benedict \textit{et al.}, 1983; Godbout \textit{et al.}, 1983; Cavanee \textit{et al.}, 1983; Sparkes \textit{et al.}, 1983). Thus, although retinoblastoma is inherited as an autosomal dominant, it is actually recessive at the cellular level, a single mutation being insufficient for progression to malignancy (Comings \textit{et al.}, 1973).

Independent mutation of both copies of a tumour suppressor can be responsible for inactivation, but more often the first mutation is followed by deletion of the remaining wild-type allele by mechanisms such as chromosomal nondisjunction or deletion, mitotic recombination, or gene conversion (Cavanee \textit{et al.}, 1983; Vogelstein \textit{et al.}, 1988; Mackay \textit{et al.}, 1988). These forms of mutation usually involve chromosomal regions that flank the gene as well as the gene itself, and consequently the whole region becomes homozygous (or hemizygous). By comparing polymorphic DNA sequences in normal and cancerous tissues from the same individual it is possible to identify regions that have undergone loss of heterozygosity. Chromosomal regions frequently displaying loss of heterozygosity (LOH) can be correlated with sites of tumour suppressor genes (Vogelstein \textit{et al.}, 1988).

There are many mechanisms by which tumour suppressor genes function. Some tumour suppressors are components of signalling pathways, responsible for receiving and processing growth-inhibitory signals from other cells. Such signals normally prevent further cell division by eliciting one of several possible responses governed by other tumour suppressors. These include: blocking the cell cycle at a defined point, preventing DNA replication and therefore cell division; promoting the terminal differentiation of cells leaving them too highly specialised to divide; or more dramatically by mediating entry into a pathway culminating in programmed cell death (apoptosis), an essential feature of tissue homeostasis. A growing number of tumour suppressor genes, including \textit{TP53} and \textit{RBI}, have been shown to interact with apoptotic pathways (Herwig and Strauss, 1997). Disruption of any of these mechanisms is likely to promote tumour formation.
Part 2. Colorectal cancer.

1.3. Epidemiology of colorectal cancer.

Cancer of the colon and rectum shows a significant geographic variation in incidence and is primarily considered a disease of developed industrialised countries (Boyle et al., 1985). In North America and the United Kingdom it is the most common form of cancer after lung cancer, with 155,000 new cases per year in the United states alone (Doll and Peto, 1981; Henderson et al., 1991). The 25,000 new cases of colorectal cancer reported in the UK annually are ultimately responsible for 19,000 deaths (Northover, 1989).

In genetic terms colorectal cancer has become one of the best understood forms of neoplasia. Inherited syndromes that predispose to colorectal cancer have been characterised at the DNA level, and have shed considerable light on the role of various genes and biological processes in the evolution of this type of tumour (e.g. Kinzler et al., 1991; Fishel et al., 1993). Furthermore, the existence of a defined premalignant lesion, the adenomatous polyp, allows mutations that occur early in tumour development to be assessed more easily than in many other forms of malignancy (Muto et al., 1975).

The specific environmental factors underlying the aetiology of malignant disease may vary considerably from one form of cancer to another. A substantial volume of population data suggests that diet is the principal factor influencing the development of cancers of the gastrointestinal tract. The association between colon cancer and diet was originally noticed in an investigation of cancer incidence in rural South Africans (Bantu and Cape), which revealed that they were ten times less likely to develop colon cancer than their North American (both black and white) counterparts (Higginson and Ottle, 1960). That this variation has an environmental and not a genetic basis is evidenced by migrant populations that adopt the colorectal cancer incidence of their new locality within a single generation (Shottenfeld and Haas, 1978).
The simple high fibre diet of the rural South Africans compared to the higher fat content diet of the Americans appears to be the most significant difference between the two populations. Burkitt, who found a lower incidence of colon cancers in several agricultural African populations, suggested that the concentration of carcinogens and the duration of their contact with the gut would be less if a diet had a high roughage content (Burkitt, 1971). Such diets produce a faster passage of material through the gut and a larger stool volume. The majority of studies support a protective effect of fibre against colorectal cancer, however not all studies are in agreement (Wasan and Goodlad, 1996). Indeed most studies in which rodents have been fed significant quantities of dietary pectin, alfalfa, or guar gum show either an increased rate of colorectal cancer or no effect (Roberfroid et al., 1993; Hill and Leeds et al., 1996).

This illustrates that the complexity of fibre, which has no universally accepted scientific definition, makes it difficult to deduce its effect on colorectal cancer development. The source of fibre and the proportion of resistant starch compared to non-starch polysaccharides varies considerably between different studies (Trock et al., 1991). Factors such as total calorific intake and affluence are positively correlated with both colorectal cancer and reduced fibre intake further confusing matters (Armstrong and Doll, 1975).

A less contentious relationship exists between fat intake and risk of colorectal cancer. That increasingly high levels of fat, animal fat in particular, cause an increase in the chance of developing colorectal cancer has been well documented (Morgan et al., 1988; Willet et al., 1990). Results of epidemiological studies suggest that a 50% reduction in the consumption of animal fat would cause a similar reduction in colorectal cancer risk (Morgan et al., 1988; Willet et al., 1990). Mice genetically predisposed to the development of intestinal tumours develop tumours of increased size and number in response to an increase in fat intake (Wasan et al., 1997). This feature appears to be independent of total calorific intake. Rodents fed on high fat diets suffer inflammation and superficial lysis of epithelial cells in the colon. This may be caused by fatty acids and bile acids which irritate the epithelium. Large quantities of fat in the diet cause excretion of cholesterol and bile acids into the lumen of the gut. These are metabolised by bacteria in the intestine producing the irritating secondary bile acids, and possibly other carcinogenic polycyclic hydrocarbons as well (Reddy et al., 1981). Correlation between fat intake and development of cancer is less
extreme in animals lacking intestinal bacteria, hinting that these micro-organisms do indeed have a role in the tumourigenicity of fat (Reddy et al., 1975). Cell loss resulting from this irritation and lysis is compensated for by increased proliferation in the colonic epithelium. This stimulation of cell division may represent the mechanism by which fat increases the risk of colon cancer (Wargovich et al., 1984; Preston-Martin et al., 1990). Alcohol, another factor that increases the risk of colorectal cancer, has also been shown to induce proliferation of intestinal cells in the rat (Wu et al., 1987).

It may be that factors which effect the metabolism of bile acids and fibre can modify their influences on tumour formation. One hypothesis is that raised rates of bacterial fermentation of fibre cause a lowering of pH which reduces break down of bile acids (Thornton, 1981). High levels of calcium and fibre are thought to affect not only the metabolism of the intestinal bacteria but also the varieties, of bacteria inhabiting the gut (Cummings and Bingham, 1987). Calcium and some forms of fibre bind to bile acids and fatty acids and carry them out of the gut. Calcium does this by forming insoluble soaps, while fibre achieves this by physiochemical binding (Newmark et al., 1984).

As well as dietary factors to counteract the effect of fats there also exist foods which offer protection against more classic carcinogens. For example, certain families of vegetables contain small quantities of indoles which inactivate polycyclic aromatic carcinogens by inducing oxidases (Wattenberg and Loub, 1978). Many aspects of a diet probably act to promote or reduce the development of colorectal cancer. However determining which individual factors are acting at any one time is problematic due to the number of components in the diet.

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin and sulindac have been shown to protect against colorectal cancer (Kune et al., 1988; Rosenberg et al., 1991; Thun et al., 1991). Small doses of aspirin, taken regularly, reduce the incidence of colorectal cancer (Thun et al., 1991), while sulindac has been demonstrated to cause regression of polyps in the large bowel of patients with familial adenomatous polyposis (FAP) (Gonzaga et al., 1985; Labayle et al., 1991). The protective effect conferred by NSAIDs may result from inhibition of the synthesis of prostaglandins, which are associated with accelerated tumour progression (Lupulescu, 1978; Oshima et al., 1996). Alternatively, NSAIDs may have properties that enhance
apoptotic mechanisms in the colonic mucosa (Moser et al., 1996). Discontinuation of aspirin or sulindac treatment results in a return to a typical risk of colorectal cancer and, in FAP patients, a recurrence of polyps (Thun et al., 1991; Labayle et al., 1991).

Among the other factors known to increase risk of colorectal cancer are alcohol, total calorific intake, sedentary lifestyle, genetic factors (see section 1.5.), and gender (Klatsky et al., 1988, Graham et al., 1988, Slattery et al., 1988, Wynder and Shigematsu, 1967). Increased physical activity which aids peristalsis may reduce risk by increasing the rate of faecal passage through the gut. The fact that women are more likely to develop colorectal cancer than men might be due in part to smaller volumes of faeces and longer passage times through the intestine. However, declining incidence in post-menopausal women is suggestive of an hormonal association. A mechanism by which hormones influence susceptibility may be related to the fact that progesterone increases cholesterol levels in the blood and thereby decreases production of bile acid, while oestrogen has the opposite effect.

1.4. Aetiology of colorectal cancer.

1.4.1. Anatomy of the colorectum.

At an anatomical level the colorectum can be subdivided into nine different parts (figure 1.1.). It's microscopic structure reveals a mucosal surface characterised by invaginated crypts (crypts of Lieberkuhn) (figure 1.2.). The surface epithelium is predominantly composed of columnar cells, but also includes abundant goblet cells, particularly in the upper two thirds of the crypt. The lower third of the crypt, in which most cell regeneration and proliferation occurs, contains a majority of columnar cells with scattered enterochromaffin cells. Cells are produced by mitosis in this proliferative zone, and migrate up the sides of the crypt as they differentiate and mature, ultimately undergoing apoptosis and being shed into the lumen (Deschner, 1980).
The nine subdivisions of the colorectum.

1- Caecum
2- Appendix
3- Ascending colon (proximal or right colon)
4- Hepatic flexure
5- Transverse colon
6- Splenic flexure
7- Descending colon (distal or left colon)
8- Sigmoid colon
9- Rectum
Fig. 1.2. Histology of the colon.

The wall of the healthy colon and rectum is characterised by a layered structure.

Mucosa
(epithelium with crypts of lieberkuhn)

Muscularis mucosae

Submucosa

Circular muscularis propria

Longitudinal muscularis propria

Subserosa

Serosa
1.4.2. Progression from adenoma to carcinoma.

Histological evidence suggests that colorectal carcinomas develop from adenomas, progressing through the so called adenoma-carcinoma sequence. As a carcinoma develops it replaces the adenomatous tissue, however remnants of an adenoma can be found in up to one third of carcinomas examined, declining in frequency the more advanced the carcinoma (Hermanek and Gall, 1983). In this scheme the adenomatous polyp represents a discrete histological phase near the beginning of the pathway of colon carcinogenesis. The progression from benign adenoma to invasive carcinoma is thought to reflect an accumulation of mutations in oncogenes and tumour suppressor genes, leading to a break down in cell cycle control, hyperproliferation, and retarded or absent programmed cell death (apoptosis) (Vogelstein et al., 1988; Miyaki et al., 1990). Despite the fact that mutations disrupting these features of cellular control have been identified in many forms of cancer the specific alterations that are critical in the early stages of the progression to malignancy have yet to be defined in most forms of neoplasia. The existence of polyps as an easily recognised precancerous lesion has simplified this task in colorectal cancer.

1.4.3. Colorectal Polyps.

Colorectal polyps are discrete regions of hyperplastic epithelial and mesenchymal tissues. The proliferative compartment of the colonic crypts expand and shift to the mucosal surface forming lesions which project into the lumen. They show a large variation in size and exist as several different morphological types of which sessile polyps are the most common and semi-pedunculated the least (figure 1.3). The histopathological classification of adenomatous polyps falls into one of three categories depending on their microscopic architecture: tubular characterised by branching tubules; villous consisting of finger-like processes of lamina propria sheathed in epithelium; or tubulovillous which represents an intermediate form (figure 1.3.). The villous form of adenoma accounts for 10% of the total and has a malignancy rate of about 40%, tubular adenomas make up 75% but have only a 5% malignancy rate, and tubulovillous account for 15% of adenomas and have an
intermediate malignant potential (Muto et al., 1975). A large dysplastic adenoma which has severe atypia but has not penetrated the muscularis mucosae is described as carcinoma in situ, however after invasive growth through the muscularis mucosae has occurred it is termed an adenocarcinoma.
Fig. 1.3. Macroscopic appearance of colorectal polyps.

**Growth types**
- pedunculated
- semi-pedunculated
- sessile

**Surface structure**
- smooth
- papillary

**Histological varieties of adenoma**
- tubular
- tubulovillous
- Villous
1.4.4. Dukes staging.

A three stage system for classifying colorectal cancers, a modification of a system first proposed by Lockhart-Mummery in 1927, was suggested by Dukes in the nineteen thirties and bears his name (Dukes et al., 1932). Dukes stage A includes all colorectal carcinomas that have reached but not exceeded the muscularis propria, stage B covers all those which have penetrated the muscularis propria but have not invaded the lymph nodes, and stage C encompasses those which have spread to the lymphatic system (Dukes, 1932). The three different classifications (A-C) are also associated with a progressively worse prognosis. In 1967 a further alphabetised category, 'D' was introduced to represent cases of colorectal cancer incurable due to metastasis (Turnbull et al., 1967).

1.5. Inherited predisposition to colorectal cancer.

The majority of colorectal cancer cases are termed sporadic, caused by somatic mutation of critical genes in cells of the colonic epithelium. However as much as 19% of colorectal cancer may result from inherited predisposition to the disease (Cannon-Albright et al., 1988). Some of the best characterised genetic predispositions are discussed in the following section.

1.5.1. Familial adenomatous polyposis (FAP).

Familial adenomatous polyposis (FAP) is a dominantly inherited cancer predisposition syndrome with an incidence of 1 in 10,000. FAP shows almost complete penetrance and is thought to account for 0.5-1.0% of all colorectal cancer (Enker, 1978). In its classical form it is characterised by the development of at least 100 adenomatous polyps in the colon and rectum (figure 1.4.) (Lynch et al., 1985; Bulow et al., 1986). These premalignant lesions are usually evident by the second or third decade of life, and have a tendency to progress to carcinoma. Polyps greater than 2 cm in diameter frequently contain malignant cells. Although the probability of any individual polyp progressing to malignancy is small the sheer number of polyps present in the colon of an FAP patient causes their lifetime risk of colorectal cancer to
near 100% (Muto et al 1977). In the absence of prophylactic colectomy adenocarcinoma, arising from one or more adenomatous polyps, will inevitably develop causing death in FAP patients averaging forty years of age (Bussey, 1975). The underlying cause of FAP is mutation of the APC gene on chromosome 5q21-22 (Kinzler et al., 1992; Joslyn et al., 1991).

The number of polyps developed and the age of cancer onset is usually fairly consistent within a family, but often varies between different kindreds. Although the majority of patients fulfil the classical definition of the FAP phenotype there also exist both an attenuated form of the disease with much reduced numbers of polyps and a later age of cancer onset, and a severe form of FAP that causes the development of thousands of polyps and a correspondingly early onset of cancer.

FAP is also associated with a range of extracolonic manifestations including epidermoid cysts, osteomas (mainly of the head and jaw), fibromas, dental abnormalities (Gardner and Richards, 1953; Gardner, 1962), desmoid tumours (Lotfi et al., 1989), and hepatoblastomas (Li et al., 1987). In the majority of FAP patients disease is not entirely limited to the colon, however expression of extracolonic symptoms can vary a great deal between different families, and also to a lesser extent within a family (Nishisho et al., 1991; Groden et al., 1991; Varesco et al., 1993; Paul et al., 1993). Families which strongly express extracolonic manifestations fulfil the definition of Gardner’s syndrome.

The most frequently observed extracolonic manifestation of FAP is congenital hypertrophy of the retinal pigment epithelium (CHRPE) (Blair and Trempe, 1980; Lewis et al., 1984). Enlarged cells of the retinal pigment epithelium, sometimes multilayered and containing an increased number of melanin pigment granules, form the basis of these benign lesions (Parker et al., 1990; Traboulsi et al., 1990). The high prevalence of CHRPE, which occurs in 70-90% of FAP patients (Traboulsi et al., 1987; Baba et al., 1990; Burn et al., 1991; Iwama et al., 1990), has made it a useful predictive marker for the presymptomatic diagnosis of FAP (Burn et al., 1991; Morton et al., 1992). Although it has now been largely superseded in this regard by linkage and mutation analyses.

A number of environmental and epigenetic factors may influence the FAP phenotype. Hormones may have some role, a notion supported by the apparent stimulation of polyp development during puberty (Bulow et al., 1986), and the
increased risk of thyroid tumour development in female patients (Bell and Mazzaferri, 1993). Surgical trauma can also effect the development of extracolonic lesions, initiating the formation of desmoid tumours in a subset of FAP patients (Ramos et al., 1990). The site of mutation within the APC gene affects a variety of disease features, such as number of polyps, tendency to form desmoids, and CHRPE development (Nagase et al., 1992; Olschwang et al., 1993; Caspari et al., 1994). The expressivity of the FAP phenotype also seems to be affected by the genetic background, as evidenced by mouse models of the disease (Moser et al., 1992).
The colon of an FAP patient, surgically removed and opened up longitudinally to reveal the inner mucosa. In this enlarged image adenomatous polyps of varying size and dysplasia can be seen affecting the mucosal surface.
1.5.2. Hereditary non-polyposis colorectal cancer (HNPCC).

Another form of inherited predisposition to colorectal cancer which is characterised by dominant transmission and high penetrance is hereditary nonpolyposis colorectal cancer (HNPCC) (Fitzgibbons et al., 1987; Lynch et al., 1993). The incidence of HNPCC is higher than that of FAP, accounting for 4% to 15% of all colorectal cancer in industrialised nations (Lynch et al., 1985). Some authors have even speculated that as many as 1 in 200 individuals may be affected by the disorder (Papadopoulos et al., 1994). Although colon cancer is the most common form of malignancy in HNPCC families, approximately two fifths of patients develop neoplasia at other sites (Fitzgibbons et al., 1987; Mecklin and Jarvinen, 1991). The most common of these is endometrial cancer, although predisposition to neoplasia extends to a wide range of extracolonic tissues including stomach, small intestine, upper renal tract, and ovary (Cristofaro et al., 1987; Lynch et al., 1990; Lynch and Lynch, 1989). Members of HNPCC families are also at increased risk of developing multiple independent primary tumours, and of developing metachronous colorectal cancer (Lynch et al., 1977; Fitzgibbons et al., 1987). The presence of extracolonic cancers in some families but not others is used to divide HNPCC into two syndromes: Lynch I in which cancer is restricted to the colon, and Lynch II where there is a wider cancer susceptibility affecting a number of organs (Boland and Troncale, 1984; Lynch et al., 1985). Additionally a rare group of families that have features similar to Lynch II but also present with sebaceous gland tumours and skin cancers are said to have Muir-Torre syndrome (Cohen et al., 1991). Although it is possible to make clinical distinctions between these syndromes it has only recently been possible to separate some of them at a genetic level (Kolodner et al., 1995). A recent report suggests mutations that result in Lynch II may be caused by the production of mutant proteins that can interact with normal mismatch repair proteins, inactivating them in a dominant negative manner (Jager et al., 1997). A frequent hMLH1 founder mutation that is associated with a much reduced frequency of extracolonic tumours and is predicted to silence the mutant allele has now been reported (Jager et al., 1997).

The underlying genetic cause of HNPCC is mutation in one of several genes that function in DNA mismatch repair. The use of highly polymorphic microsatellite repeats, to look for loss of heterozygosity in the cancers of HNPCC patients, led to the
discovery that such sequences are unstable in a high proportion of tumours (Fischel et al., 1993; Leach et al., 1993). The instability was manifest as a high rate of mutation at microsatellite repeat loci, causing increases or decreases in the number of repeat units. This is probably due to slippage and consequent misalignment of the growing DNA strand during replication (Streisinger and Owen, 1985; Roberts et al., 1993). The repetitive nature of microsatellite repeat sequences leaves them particularly prone to slippage, however these errors are usually repaired with high efficiency. Tumours displaying allele sizes that are not found in the constitutional DNA of the patient are termed replication error positive (RER^+)(Leach et al., 1993).

The first gene to be implicated in HNPCC was hMSH2, the human homologue of the mutS gene of Escherichia coli (Fischel et al., 1993; Leach et al., 1993). The MutS protein is an essential component of the bacterial mismatch repair pathway, and facilitates the identification of mismatched nucleotides by binding to them (Su and Modrich, 1986). Other human homologues of bacterial genes that function in the well characterised MutHLS mismatch repair pathway (Modrich, 1989) were soon isolated. Several of these genes have now been shown to function in human mismatch repair and HNPCC including three homologues of bacterial mutL: hMLH1 (human MutL homologue 1); hPMS1 and hPMS2 (post meiotic segregation 1 and 2) (Papadopoulos et al., 1994; Bronner et al., 1994; Nicolaides et al., 1994).

Over 90% of HNPCC mutations can be attributed to mutation in hMSH2 or hMLH1. However, there are cases in which no mutation has been found in any of the known mismatch repair genes, suggesting that there are still more genes to be identified, or that alternative DNA repair pathways exist. In most cases it seems that the RER^+ phenotype is recessive at the cellular level, as HNPCC tumours have been detected with mutation in both alleles of a mismatch repair gene (Leach et al., 1993; Papadopoulos et al., 1994; Hemminki et al., 1994; Lu et al., 1996). This has been confirmed by cell hybridisation experiments that show that the RER^+ phenotype can be corrected by fusion with mismatch repair competent (RER-) cells (Lengauer et al., 1997).

The RER^+ phenotype, and mutations of mismatch repair genes have also been found in a significant proportion of sporadic colorectal cancers (15%), indicating that tumours without an inherited component can arise by the same mechanism (Liu et al., 1995). Furthermore, microsatellite instability has been detected in the extracolonic
tumours of HNPCC patients, and to a lesser extent in their sporadic equivalents. Such cancers include those of the breast, liver, stomach, pancreas, endometrium, and ovary (Han et al., 1993; Mironov et al., 1994).

The phenotype conferred by inactivation of a mismatch repair gene, not only increases the frequency of small deletions and insertions due to slippage at repetitive sequences, but also affects the frequency of single base substitutions in coding sequences (Eshleman et al., 1996; Bhattacharyya et al., 1995). Ultimately, it is the accumulation of mutations in tumour suppressor genes and oncogenes that causes cancer to develop, an increased mutation rate causes these defects to be acquired more rapidly. The APC gene, inactivation of which is thought to be essential for colorectal tumour formation, is mutated with equal prevalence in RER^+ and RER^- colorectal tumours (Huang et al., 1996). This suggests that both types of tumour undergo a similar combination of genetic alterations during progression to cancer. Furthermore, the APC mutations detected in RER^+ tumours were distinguished by an excess of frameshift mutations, characteristic of mismatch repair deficiency, suggesting that instability precedes APC mutation in RER^+ colorectal tumours (Huang et al., 1996). That microsatellite instability arises early in colorectal tumourigenesis is supported by evidence that the earliest adenoma precursor lesions, aberrant crypts, can display an RER^- phenotype (Augenlicht et al., 1996), although some evidence to the contrary has also been presented (Young et al., 1993).

### 1.5.3. Peutz-Jeghers syndrome.

Peutz-Jeghers syndrome, a dominant disorder first described by Peutz in 1921, is characterised by the development of pigmented spots, found mainly on the lips and buccal mucosa, and accompanied by intestinal polyposis. The polyps produced are less numerous than those found in FAP, numbered in dozens rather than hundreds, and are found throughout the gastrointestinal tract, most often in the small intestine (Utsunomiya et al., 1974). The large obstructive polyps formed are composed of an atypical arrangement of intestinal epithelium and smooth muscle in abnormal proportions, and are considered hamartomatous in nature (Bussey et al., 1975). Although there is no direct evidence that hamartomatous polyps can develop into malignant tumours, the polyps do reflect a propensity for neoplastic growth. In
addition to an increased risk of developing gastrointestinal malignancy Peutz-Jeghers patients are also prone to a variety of extra-intestinal tumours including testicular, uterine, and ovarian granulosa tumours (Spigelman et al., 1989; Ginns et al., 1996). It has been estimated that ultimately as many as 50% of Peutz-Jeghers patients may develop cancer by the age of sixty (Spigelman et al., 1989).

The gene responsible for Peutz-Jeghers syndrome has recently been localised to chromosome 19p, following comparative genomic hybridisation (CGH) and loss of heterozygosity studies (Hemminki et al., 1997). Linkage analysis has confirmed this localisation in 12 Peutz-Jeghers families and has provided no evidence of genetic heterogeneity (Hemminki et al., 1997).

1.5.4. Juvenile polyposis.

Juvenile polyposis is another autosomal dominant disorder characterised by hamartomatous polyps of the gastrointestinal tract. It shows complete penetrance, is unlinked to APC, and causes widespread polyposis before puberty (Petersen et al., 1990). The polyps developed are less in number than those found in FAP, and are histologically distinct from those of Peutz-Jeghers syndrome in that they lack the smooth muscle element and are found mainly in the colon (Bussey et al., 1975). Evidence suggests that patients with juvenile polyposis are at an increased risk of colorectal cancer, although malignancy is not thought to arise from the hamartomatous polyps (Cohen et al., 1995). Other features of juvenile polyposis include bleeding, anaemia, hypoproteinemia, and retarded development.

1.5.5. Turcot’s syndrome.

Turcot’s syndrome defines a disease in which colonic polyposis and tumours of the central nervous system are concurrent (Turcot et al., 1959). In comparison with the polyps developed by FAP patients those of Turcot’s syndrome are reported to appear earlier, be fewer in number (<100) and larger in size (Murday and Slack, 1988). Until recently the mode of inheritance was unclear, with both dominant and recessive patterns of inheritance reported (Lewis et al., 1983; Costa et al., 1987; Kumar et al., 1989). Hamilton et al., (1995) identified mutations in the APC gene in
Turcot’s syndrome families. A further 3/14 families had tumours which displayed microsatellite instability, due to mutation of hMLH1 in one family, and mutation of hPMS2 in another. Thus the association of polyposis and tumours of the central nervous system can result from mutation of the APC gene or of one of the genes underlying HNPCC. FAP patients are 92 times more likely to develop brain tumours than unaffected individuals (Hamilton et al., 1995) and consequently the coincident occurrence of brain tumours and polyposis is not unexpected.

1.5.6. Hereditary mixed polyposis syndrome (HMPS).

Hereditary mixed polyposis syndrome (HMPS) is a rare condition characterised by the development of characteristic polyps of the large bowel. The polyps formed are similar to juvenile polyps, but display histological differences and have a malignant potential.

1.6. Genetic alterations in colon cancer.

1.6.1 Chromosomal changes.

In colorectal cancer as in other cancers non-random loss, gain, or rearrangement of chromosomes has shed light on genes involved in tumourigenesis. Chromosomes 1p, 5q, 10q, 17p and 18q most frequently show structural rearrangements or deficiency in colon cancers (Solomon et al., 1987; Muleris et al., 1988; Vogelstein et al., 1988; Okamoto et al., 1988). The gene specifically lost on chromosome 17p has been identified as the tumour suppressor TP53, while loss of 18q appears to target the DCC gene and others, rearrangements of 5q are correlated with loss or disruption of the APC locus at 5q21-22. In one study 17 out of 18 colorectal tumours had deletions of 5q all involving APC (cited in Cohen et al., 1995). Increased copy number of chromosome 7, to which a number of proto-oncogenes map, is thought to be important in solid tumour carcinogenesis in general (Van Der Berghe 1987). Trisomy of this chromosome (Reichmann et al., 1985) and duplication of one parental chromosome 7 at the expense of the other (Rees et al., 1989) have been reported in colon cancers. Another chromosomal change often observed in colon
cancers is structural alteration of chromosome 1p, rearrangement of this chromosome is common in many forms of malignancy (Reichmann et al., 1984).

To investigate the proposed tumour suppressing properties of specific chromosomes, single normal human chromosomes have been introduced into the cells of a colon carcinoma cell line by microcell hybridization (Tanaka et al., 1991). Suppression of tumourigenicity by a single chromosome has been reported in several tumour cell lines including HeLa cells by the introduction of a fibroblast chromosome 11 (Saxson et al., 1986) and Wilms' tumour cells again by incorporation of chromosome 11 (Weissman et al., 1987). The introduction of chromosome 5 or chromosome 18 to colorectal cancer cells was found to cause a radical alteration of cellular morphology, and tumourigenicity in athymic nude mice was entirely suppressed (Tanaka et al., 1991). It is interesting to note that the acquisition of chromosome 5 returned the colon carcinoma cells to a morphology resembling epithelial cells, whereas incorporation of a normal chromosome 18 gave a different morphological change. This may reflect differing modes of action of the two tumour suppressors in normal cells. Another interesting but not unexpected observation was that the growth rate of the hybrid cells remained higher than normal, probably indicating that multiple growth promoting genetic changes had occurred during carcinogenesis, although the wide ranging biochemical effects of adding an entire chromosome to a cell should not be underestimated. Several tumour suppressor genes on various chromosomes (including 5q, 17p, and 18q) are believed to be inactivated simultaneously in advanced colon carcinomas. It seems likely that the provision of any of these areas of the genome would reduce certain aspects of the malignant phenotype in colon carcinoma cell lines. Not only are there the individual tumour suppressors to consider but also their interactions. The cells provided with normal copies of chromosome 5 by microcell hybridisation, for example, showed increased expression of the K-ras gene, and decreased expression of myc and TP53 genes.
1.6.2. Tumour suppressors in colorectal cancer.

1.6.2(a). The adenomatous polyposis coli gene (APC).

The APC gene is inactivated in most, if not all colorectal carcinomas, and fulfils a critical role as a gatekeeper at the beginning of the pathway to malignancy in the colon. Several gene products that interact with the APC protein, and may therefore be involved in colorectal tumourigenesis, have been identified. Molecular analysis of APC and associated genes is dealt with elsewhere in the text (chapter 4).

1.6.2(b). The TP53 gene (TP53).

It is likely that some critical regulatory features of cell growth and the cell cycle are common to all cell types. This probability has given hope that a biochemical pathway, which produces cancerous characteristics in all cell types when disrupted, will be discovered thereby shedding light on carcinogenesis regardless of biologic heterogeneity. Unlike APC, the tumour suppressive influence of which is restricted to a handful of tissues, the TP53 gene appears to be involved directly or indirectly in most human malignancies and may therefore represent an important gene in such a pathway (Hollstein et al., 1994). In the specific case of colorectal cancer mutations of TP53 have been identified in 75% of tumours examined (Baker et al., 1989).

The TP53 gene maps to chromosome 17p13 (Isobe et al., 1986; McBride et al., 1986) and is composed of 11 exons encoding a 2.8 kb mRNA transcript (Matlashewski et al., 1984; Bienz-Tadmour et al., 1984). Mutations of TP53 are found at particularly high frequencies in prostate, bladder, and lung cancers, as well as colorectal cancers, but are also found in a wide range of additional human malignancies (see Hollstein et al., 1994 for review), and in the germline of patients with Li-Fraumeni syndrome (LFS) (Srivastava et al., 1990; Malkin et al., 1990). LFS is a dominantly inherited cancer syndrome characterised by predisposition to a broad spectrum of tumours (Li and Fraumeni, 1969), including those of the breast, brain, lung, larynx, colon, as well as rhabdomyosarcomas, osteosarcomas, leukaemias, and adrenocortical carcinomas (Li et al., 1988).
The p53 protein exists as a tetramer and behaves as a multifunctional transcription factor orchestrating cellular responses to DNA damage (table 1.1.). In normal cells DNA damage activates p53 which in turn causes arrest of the cell cycle or apoptosis (see Levine, 1997 for review). This intervention prevents DNA replication from giving permanence to any DNA damage present, and presumably eliminates cells that have suffered excessive damage. Interruption of the cell cycle is incomplete in cells expressing mutant TP53 alleles (Kastan et al., 1991). The carboxyl terminus of the 53 kDa p53 protein is responsible for oligomerisation and also binds regions of damaged DNA, possibly targeting the attentions of proteins associated with DNA repair (Lee et al., 1995; Jayaraman et al., 1995). The cellular concentration of p53 is normally maintained at a low level by its relatively short half-life, and it is probable that in some cells p53 exists in a latent form. However, in response to several stressful situations the half-life of the protein increases and it is activated as a transcription factor. A variety of forms of DNA damage will elicit this response including the presence of DNA repair intermediates following exposure to ultraviolet light (Liu et al., 1996) and double strand breaks in DNA caused by γ-irradiation (Khanna and Lavin, 1993). The increase in p53 concentration is proportional to the amount of DNA damage. Ultimately the size of a tumour will be limited by the blood supply available, unless it can manufacture its own angiogenic factors. As a consequence tumourous cells frequently experience hypoxia. It is interesting therefore that hypoxia has also been shown to result in raised levels of activated p53 (Graeber et al., 1996). It has been suggested that these abnormally proliferating cells may be eliminated by a p53 dependent mechanism.
Table 1.1. Products of genes transcriptionally activated by p53 (modified from Levine 1996).

<table>
<thead>
<tr>
<th>Products of genes transcriptionally activated by p53</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>p21, WAF1, Cip1</td>
<td>Inhibits several cyclin-cyclin dependent kinases; bind cdk’s, cyclins, and PCNA; arrest cell cycle.</td>
<td>El-Diery et al., 1993; Harper et al., 1993</td>
</tr>
<tr>
<td>GADD45</td>
<td>Induced upon DNA damage; binds to PCNA and can arrest cell cycle; involved directly in DNA nucleotide excision repair.</td>
<td>Smith et al., 1994; Kastan et al., 1992</td>
</tr>
<tr>
<td>Cyclin G</td>
<td>A novel cyclin of unknown function.</td>
<td>Zauberman et al., 1995; Okamoto and Beach, 1994</td>
</tr>
<tr>
<td>Bax</td>
<td>A member of the Bcl2 family that promotes apoptosis; not induced by p53 in all cells.</td>
<td>Miyashita et al., 1994; Selvakumaran et al., 1994</td>
</tr>
<tr>
<td>IGF-BP3</td>
<td>The insulin-like growth factor binding protein 3; blocks signalling of a mitogenic growth factor.</td>
<td>Buckbinder et al., 1995</td>
</tr>
</tbody>
</table>
A large number of genes have been suggested to be activated by p53 (Ko and Prives, 1996), however those included in the table have all been shown to contain p53 dependent, cis-acting, DNA-responsive elements.

The central domain of p53 has the ability to bind specific DNA sequences, and is the region of p53 most frequently mutated during carcinogenesis. Mutations within this domain are usually missense rather than protein truncating and, if the carboxyl terminus is preserved, can create proteins that interact with normal p53 inactivating it in a dominant negative fashion (Eliyahu et al., 1988). Thus, unlike most other tumour suppressor genes elimination of both alleles of TP53 is not always necessary for elimination of tumour suppressor function.

Testing of large numbers of human genomic DNA clones have revealed several that will bind p53 (Kern et al., 1991), and has led to the identification of a consensus sequence necessary for efficient p53 binding (El-Diery et al., 1992). The expectation that genes adjacent to p53 binding sites may be activated by p53 has been shown to be valid. Confirmation was achieved by cotransfection of a TP53 expression vector and a plasmid carrying a p53 binding site upstream of a reporter gene, the result was a high degree of reporter activation in mammalian cells (Kern et al., 1992). Transcription stimulated by p53 has also been demonstrated by in vitro systems utilising purified p53 and nuclear extracts (Farmer et al., 1992). p53 can also have a negative effect on the transcription of some genes, achieved by sequence independent binding to TATA elements (Seto et al., 1992; Liu et al., 1993).

A mechanism by which TP53 influences aspects of the cell cycle, and possibly entry into apoptotic pathways, may involve a gene variously known as Cip1 or WAF1 (El-Diery et al., 1993; Harper et al., 1993). Cip1 has been shown to function in the control of the cell cycle and is transcriptionally activated by wild-type, but not mutant p53 (El-Diery et al., 1993). The gene encodes a 21 kDa protein that functions by interacting with and inhibiting cyclin dependent kinases (cdk) (Harper et al., 1993). When associated with cyclins, cdk4s have the ability to promote progression through the cell cycle. Through its influence on cdk-4 Cip1 interacts with a pathway central to progression through the cell cycle from G1 to S phase. The principal constituents of this pathway include p16 (a negative regulator of cyclin D1-Cdk4), cyclin D1, cdk4, and the retinoblastoma protein (RB) which is the major target of cyclin D1-Cdk4 for
cell cycle regulation. At least one of these components has been shown to be mutated in virtually every cancer investigated.

As well as preventing G1-S phase transition in cells with DNA damage p53 has also been implicated in a G2/M phase checkpoint. Treatment of cells expressing wild-type p53 with mitotic spindle inhibitors (e.g. nocodazole) results in arrest in G2. However in the absence of normal p53 this block fails leading to an increase in ploidy (Cross et al., 1995). Additional evidence also suggests that p53 regulates the number of centrosomes per cell (Fukasawa et al., 1996). Cultured cells from TP53\(^{-/-}\) (null) mice produce abnormal numbers of centrosomes, and initiate spindles with three or four poles after only a few doublings. This effect, not seen in normal cells in culture, is thought to contribute to the high aneuploidy rate seen in cultured TP53\(^{-/-}\) cells. Wild-type p53 also appears to inhibit the amplification of oncogenes observed in many tumours. How this is achieved is uncertain, although it has been hypothesised that p53 may be able to detect abnormal recombination intermediates and initiate apoptosis in affected cells. These features of p53 may partially explain the phenotype of genomic instability frequently observed in cells carrying a TP53 mutation.

The existence of a third cell cycle checkpoint mediated by p53 is suggested by investigation of the G0-G1-S phase transition. The Gas1 gene encodes a membrane protein which is only expressed in cells in G0, and acts to maintain cell cycle arrest. However, Gas1 can only achieve this effect in the presence of p53 (Del Sal et al., 1995).

A number of experiments have demonstrated that p53 can trigger apoptosis. DNA damage will cause an apoptotic response in normal thymocytes, but not in thymocytes from p53 deficient mice (Lowe et al., 1993). Apoptosis mediated by p53 can also occur in response to the expression of a viral or cellular oncogene (e.g. adenovirus E1A protein, myc gene product) or in the absence of certain tumour suppressor gene products such as RB (Wagner et al., 1994; Debbas and White, 1993). Thus cells that have unstable genomes due to DNA damage, activated oncogenes, or are in an abnormal environment, such as cells suffering hypoxia, are eliminated by programmed cell death in a p53 dependent fashion.

The precise mechanisms underlying the decision to commit to an apoptotic pathway are uncertain, however two p53 regulated genes, bax and IGF-BP3 could have a role (Miyashita and Reed, 1995; Buckbinder et al., 1995). Bax can bind Bcl-2
and the adenovirus E1B-19Kd protein, both of which are capable of blocking p53-dependent apoptosis when overexpressed. The binding of bax interferes with the ability of Bcl-2 and the E1B-19Kd protein to suppress apoptosis. Consequently p53 induced expression of bax may favour entry into an apoptotic pathway. p53 induced expression of the insulin-like growth factor-binding protein-3 (IGF-BP3) could also encourage apoptosis or reduce the mitogenic response of cells. IGF-BP3 binds to IGF, preventing its interaction with its receptor, and thereby blocks the IGF mitotic signalling pathway (Buckbinder et al., 1995).

1.6.2(c). The mutated in colon cancer gene (MCC).

Attempts to isolate the gene responsible for familial adenomatous polyposis coli (FAP) led to the identification of a number of genes including the causative APC gene and a second gene, MCC, somatically mutated in a subset of colon cancers (Kinzler et al., 1991; Nishisho et al., 1991). The MCC gene encodes a widely expressed protein consisting of 829 amino acids and including a conserved region with similarity to a G protein-m3 muscarinic acid acetylcholine receptor (mAchR). It has been hypothesised that MCC could be involved in suppression of cell signalling, exerting a tumour suppressive effect by subduing responses to growth factors. In support of this notion mAchR has been found to mediate stimulation of the phosphoinositide-specific phospholipase C via a G protein. Furthermore there is evidence that in the mouse wild-type MCC can negatively regulate the transition from the G1 to S phase following a change in phosphorylation, an activity lacking from mutant MCC (Matsumine et al., 1996). The MCC protein also contains a series of heptad repeats, which are predicted to form alpha helices. Such structures frequently have a role in protein-protein interactions and it was initially suggested that MCC might bind APC, another protein containing heptad repeats. Subsequent findings have not, however, provided any evidence supporting this possibility (Rubinfeld et al., 1993; Su et al., 1993; Su et al., 1995; Rubinfeld et al., 1996; Matsumine et al., 1996). Further estimations from the coding sequence of the gene suggest that MCC has globular domains at the end of the molecule which are connected by a series of alpha helical rods interrupted by short proline rich regions. The potential of MCC to form coiled-coils may allow it to act as a structural spacer, and therefore fulfil a role in the
organisation of the cytoplasm. Cytoplasmic re-organisation is a critical aspect of progression through certain phases of the cell cycle and may therefore influence tumourigenesis (Bohmer et al., 1996). The precise function of MCC remains to be elucidated.

Although somatic mutations of the MCC gene have been reported in colorectal carcinomas, and LOH has been detected in a number of malignancies (Morita et al., 1991; Boynton et al., 1992), no germline mutations have been identified thus far (Groden et al., 1991). Indeed a recent study calls into question the tumour suppressing classification of MCC, having found no evidence of mutation in 21 sporadic colorectal tumours with LOH for APC and MCC (Curtis et al., 1994).

1.6.2(d). The deleted in colon cancer gene (DCC).

Loss of heterozygosity on chromosome 18q has been reported to occur in over 70% of colorectal cancers hinting at the location of a third tumour suppressor gene (Vogelstein et al., 1988). This observation ultimately led to the cloning of the deleted in colon cancer gene (DCC) which maps to chromosome 18q21 (Fearon et al., 1990). The DCC gene encodes a 190 kDa transmembrane phosphoprotein that shares homology with cell adhesion molecules, and may fulfil a function in signal transduction by binding unknown ligands. In addition to deletion of DCC other types of somatic mutation have also been reported, 15 out of 17 colorectal cancer cell lines investigated by Fearon et al. (1990) displayed reduction or absence of DCC expression. As well as a tumour suppressor function in the colon DCC may influence tumour development in other tissues. Worsham et al. (1992) have reported that DCC is likely to be important in the development of most, if not all, squamous cell carcinomas. Mutation of cell adhesion molecules such as DCC is probably responsible for much of the disruption of cellular communication and cell-cell interactions observed in cancer. This may be particularly significant in epithelial cancers. A recent report indicates that the DCC protein is a receptor for netrins, chemoattractants that guide developing axons in the nervous system (Keino-Masu et al., 1996), although it is unclear whether this has any relevance to a function as a tumour suppressor. A recent study using homologous recombination to inactivate the
murine Dcc gene revealed that loss of the Dcc does cause defects in axonal projections, but provided no evidence for increased susceptibility to intestinal tumours (Fazeli et al., 1997).

1.6.2(e). Mad gene homologues.

Another gene from 18q21 that may act as a tumour suppressor in cells of the colonic epithelium is JV18-1. JV18-1 is one of a family of at least eight human genes related to the Drosophila gene Mad, a gene that is thought to be involved in the transduction of signals from transforming growth factor-β (TGF-β) (Riggins et al., 1996; Hahn et al., 1996). Human cancers frequently develop resistance to the action of TGF-β, which acts negatively on cell growth and division (Fynan and Reiss, 1993). Members of the Mad gene family are thought to function downstream of specific receptors with serine/threonine kinase activity that mediate the TGF-β signal (Savage et al., 1996; Hahn et al., 1996). Inactivation of such genes is expected to block the growth inhibitory signal of TGF-β, and they can therefore be considered tumour suppressors. Interestingly three other Mad related genes map to chromosomal regions previously known to display LOH in other human tumours. Not only is JV18-1 subject to deletion in the majority of colorectal tumours, as is DCC, it has also been shown to contain inactivating mutations within the coding sequence. Riggins et al. (1996) investigated a panel of 18 colorectal cancer lines displaying LOH at 18q21. In one cell-line a homozygous deletion of JV18-1 sequences, that did not extend to flanking markers situated 3 Mb either side of the gene, was identified. A second tumour was found to contain a truncated JV18-1 product resulting from a 42 bp intragenic deletion. These alterations were not present in the constitutional DNA of either patient.

Homozgyous deletion of a second Mad related gene also located at 18q21 and known as DPC4 (deleted in pancreatic carcinoma) has been found in 25 of 84 pancreatic tumours (Hahn et al., 1996). These deletions did not include the DCC locus. Additional inactivating mutations were discovered in 6 of 27 pancreatic tumours that did not have homozygous deletions of 18q21 (Hahn et al., 1996). The high levels of genetic instability observed in cancer cells makes it impossible to rule out the possibility that the mutations observed in these genes are unimportant in
carcinogenesis, but the finding of complete inactivation certainly makes \textit{JV18-1} and \textit{DPC4} good candidate tumour suppressor genes.

1.6.2(f). Protein Kinase C.

Protein kinase C (PKC) is a serine/threonine kinase that is a component of signal transduction pathways (Nishizuka, 1992). The endogenous activator of PKC is 1,2-diacylglycerol (DAG), however, tumour-promoting phorbol esters, and secondary bile acids can activate PKC in a similar manner hinting that it may have a role in tumourigenesis (Castagna \textit{et al.}, 1982). Activation of PKC can lead to a variety of significant cellular alterations, including changes in gene expression, morphological alterations, and suppression or stimulation of proliferation (Hug and Sarre, 1993).

The activity of PKC is decreased in human and rodent colorectal cancer and also in adenomas suggesting that reduction of PKC activity is an early event in colorectal tumourigenesis (Guillem \textit{et al.}, 1987; Baum \textit{et al.}, 1990; Kopp \textit{et al.}, 1991). Recent studies have demonstrated that decreased levels of PKC in colorectal cancer are associated with a reduction in the amount of a specific RNA isoform, PKC-beta (Levy \textit{et al.}, 1993), and that transfection of this isoform into cells of a colorectal cancer cell line inhibits growth and tumourigenicity (Choi \textit{et al.}, 1990).

1.6.3. Oncogenes in colon cancer.

1.6.3(a). Prostaglandin H Synthase-2.

A recent study has provided strong evidence that prostaglandin H synthase-2 (COX-2) has an important role in colorectal tumour formation (Oshima \textit{et al.}, 1996). Unlike COX-1, a housekeeping gene which is constitutively expressed in most tissues, COX-2 appears to be induced by growth factors, oncogenes, and tumour promoters (DeWitt and Smith, 1995; Herschman, 1994). COX-2 is not expressed in the normal colonic epithelium but is found at high levels in colorectal tumours (Sano \textit{et al.}, 1995; Kutchera \textit{et al.}, 1996).

Oshima and co-workers (1996) crossed mice heterozygous for an \textit{Apc} mutation with mice carrying a disrupted COX-2 gene. \textit{Apc}^{+/-} mice carrying two
normal COX-2 alleles developed an average of 652 intestinal adenomas after 10 weeks whereas those carrying two copies of the COX-2 null allele developed only 93, heterozygous animals developed an intermediate number of adenomas. Drugs used to specifically inhibit COX-2 also reduced the number of adenomas (Oshima et al., 1996). Earlier investigations using mice which carry a mutation at codon 850 of Apc (Apc\textsuperscript{Min}) have demonstrated that nonsteroidal anti-inflammatory drugs (NSAIDs), which function as COX inhibitors, can significantly reduce the incidence of intestinal cancer and number of polyps, as well as extending the lag time between exposure to chemical carcinogens and the development of colorectal cancer (Jacoby et al., 1996). A reduction in the number of polyps has also been observed in humans with FAP following treatment with the NSAID sulindac (Giardiello et al., 1993). Adenomatous polyps found in wild-type mice have been found to contain COX-2 protein indicating that sporadic and inherited tumours share a common pathway in this regard (Williams et al., 1996).

How expression of COX-2 promotes tumour formation is yet to be ascertained. COX-2 catalyses two reactions that convert arachidonic acid to prostaglandin H\textsubscript{2}. This intermediate usually undergoes further reaction to become one of a variety of terminal prostaglandins, but can also break down to form the mutagen malondialdehyde. Furthermore, a broad spectrum of compounds, including xenobiotics with which the colonic epithelium is in constant contact, can be oxidised by COX-2 to mutagens (Marnett, 1994). Other possibilities are that raised levels of COX-2 interferes with apoptosis and/or increases invasive potential. Induction of COX-2 in a rat intestinal cell line has been shown to prevent apoptosis, an effect that could be reversed by the introduction of a COX-2 inhibitor (Tsuji et al., 1995). An increase in invasiveness in cells constitutively expressing COX-2 has also been demonstrated (Tsuji et al. 1997). What ever the mechanism by which the induction COX-2 influences colorectal tumourigenesis it does not seem to be essential for tumour formation. The formation of tumours in COX-2\textsuperscript{−/−} mice illustrates that other pathways to malignancy that do not require COX-2 must exist (Oshima et al., 1996).
1.6.3(b). **K-ras.**

Activation of a handful of oncogenes have been reported in colorectal cancers, in particular the oncogene K-ras (Bos *et al.*, 1987). K-ras belongs to a family of closely related genes of which the oncogenes N-ras, and H-ras are also members (Valencia *et al.*, 1991). All three genes code for 21 kDa inner-membrane bound proteins which bind guanine nucleotides and are thought to be involved in inter-cellular signal transduction. Interference with the p21 ras product using antibodies has been shown to cause inhibition of DNA synthesis (Stacey *et al.*, 1988), while transfection studies have indicated that mutant forms of ras can induce neoplastic characteristics in the recipient cells (Barbacid, 1987). Colorectal cancers show a considerable bias in favour of mutation of the K-ras gene. These account for 90% of ras mutations compared with 10% in N-ras. Normal ras proteins are only active when they are in a GTP-bound state. Mutations of ras, which occur at three hot-spots situated at codons 12, 16, and 61 (Forrester *et al.*, 1987; Vogelstein *et al.*, 1988), produce intrinsically active p21 proteins that bind guanine triphosphate (GTP) but resist its hydrolysis to guanine diphosphate (GDP) (Haubruck and McCormick, 1991). Consequently ras-dependent mitogenic signalling pathways are continually stimulated leading to transformation. Overexpression of ras can produce similar results, as evidenced by increased ras expression detected in colorectal carcinomas and adenomas (Fearon and Vogelstein, 1990). It has been speculated that increased expression of ras might sometimes occur by hypomethylation of the 5' controlling regions of the gene, decreased methylation has been reported in adenocarcinomas (Fienberg and Vogelstein, 1983).

1.6.3(c). **C-myc.**

The oncogene c-myc encodes a 64 kDa nuclear phosphoprotein which functions in transcriptional regulation. C-myc is part of a large gene family which includes other genes with oncogenic potential such as N-myc and L-myc. Genes of the myc family were first identified by virtue of their homology to the transforming gene of retrovirus MC29, which causes tumour formation in chickens. The expression of c-myc fluctuates during the cell-cycle, rising with increasing mitogen stimulation, it is
thought that the c-myc protein is required for cell proliferation (Erisman et al., 1989). Overexpression of c-myc can induce tumours in animals and transform cultured cells (Dang, 1991).

As many as 75% of colorectal carcinomas have levels of c-myc RNA that are 3-40 times higher than in normal mucosa (Erisman et al., 1989; Guillem et al., 1990), and high levels of myc protein are seen throughout adenomatous crypts (Melham et al., 1992). In normal colonic epithelium expression of myc is restricted to the proliferative zone. Overexpression of N-myc and L-myc have also been reported in a large number of colorectal cancers (Finley et al., 1989; Melham et al., 1992). In almost all cases the myc gene product produced is normal in all respects, save for its increased cellular concentration.

1.6.3(d). C-src.

The cellular homologue of the Rous sarcoma virus transforming gene is the c-src gene, a membrane associated protein tyrosine kinase that has a role in mitogenic signal transduction. C-src has been shown to be activated in 70% of large colorectal adenomas, carcinomas, and colorectal cancer cell-lines (Bolen et al., 1987; Cartwright et al., 1990). The mechanism by which c-src activation occurs is not known, however post-translational interactions are thought to be involved (Bolen et al., 1987). Like K-ras and c-myc c-src is part of a gene family. Other src family members, which also function as tyrosine kinases, have been shown to be activated in a smaller proportion of colorectal cancer cell-lines (Rosen et al., 1989).

1.6.4. Colorectal cancer progression.

There is a significant amount of evidence both anecdotal and direct that allow us to deduce the order in which mutations most often accumulate during colorectal tumourigenesis. This sequence of events may not be the only route to the production of malignant cells, but presumably it is the most easily attained. The pathway is thought to involve multiple steps of mutation followed by limited clonal expansion that maintains the cell population available to undergo further critical mutation at a maximum. On average it may take many decades to acquire the mutations necessary
for cancer to form, however, they occur faster if the mutation rate is increased. Thus, exposure to mutagens/carcinogens or inheritance of defective DNA mismatch repair genes such as *hMSH2* and *hMLH1* can accelerate transition along the favoured tumourigenic pathway. Mutation in the genes which govern mismatch repair give rise to a mutator phenotype that is characterised by genomic instability, and influence the development of approximately 15% of sporadic colorectal cancer (Modrich, 1994). A correlation between methylation capacity and genomic instability in colorectal cancer cells has also been reported, and may represent an even more important mechanism underlying tumour development (Lengauer et al., 1997).

There is compelling evidence that complete inactivation of the *APC* gene on chromosome 5q21-22 is an early if not initiating event occurring during colorectal carcinogenesis (Powell et al., 1992). In contrast to loss of chromosome 18q and 17p loss of chromosome 5 alleles is even detected in early adenomas (Rees et al., 1989) including adenomas less than 0.5 cm in diameter (Vogelstein et al., 1988, Powell et al., 1992), indicating that *APC* allele loss occurs before malignant transformation is complete. The original loss of heterozygosity studies have recently been backed up by immunohistochemical analysis which has demonstrated total loss of normal *APC* product in 81% of colon cancer cell lines (Smith et al., 1993). Mutation of *APC* also appears to precede *ras* mutation. The proportion of tumours with a detected *APC* mutation remains constant through all stages of colorectal cancer progression (Goyette et al. 1992, Powell et al., 1992, Ichii et al., 1993). This is not so for mutations of *TP53* which increase in frequency as tumours become more advanced, and suggests that *APC* mutation is a critical early event in colorectal carcinogenesis.

Loss of APC gene product in colon cancer patients is reminiscent of the finding that all intestinal adenomas developed by mice carrying a germline mutation of the *Apc* gene undergo somatic loss of the normal chromosome (Luong et al., 1994). However, the mechanism of murine intestinal tumourigenesis may differ from that in the human, particularly as the mouse *Apc*, *Mcc*, and *Dcc* genes are syntenic, all situated on distal chromosome 18. This may well increase the importance of chromosomal loss in this case.

A number of loci that influence the probability of developing colorectal tumours are thought to exist in the mouse, and possibly in humans also. Evidence for
this comes from experiments on mice carrying a germline Apc mutation. Depending on the genetic background identical Apc mutations result in differing numbers of adenomas developed. Loci that modify the effects of germline Apc mutations are discussed further in chapter 4.

As APC mutation is one of the earliest, if not the first, genetic event leading to colon cancer it is probable that mutation of this gene is a rate-limiting step in the pathway.

The frequency of ras mutations are not influenced by presence or absence of 5q deletion, demonstrating that mutation of APC and ras are independent events (Vogelstein et al., 1989). 9% of small sporadic adenomas and 40%-50% of intermediate-stage adenomas show activation of ras (Goyette et al., 1992), this increases to 60% in large adenomas and also in carcinomas, suggesting that ras activation usually occurs early, but does not initiate colorectal tumourigenesis. Adenomatous cells which undergo activation of ras probably have increased growth potential and outgrow the original cell population of the adenoma. A study by Shibata et al. (1993) highlights this mechanism, 4 out of 7 adenomas examined contained discrete regions with K-ras mutation, whereas mutation was found throughout 7 out of 7 more advanced colorectal carcinomas.

Although ras mutation is an important event in the formation of some colorectal tumours it does not appear to be obligatory. This is illustrated by the colorectal tumours of patients with ulcerative colitis, only 4% of which have mutation of K-ras (Cohen et al., 1995). Presumably alternative genetic pathways for the progression of colorectal cancer also exist.

Another potentially rate limiting step, following inactivation of APC, is the induction of COX-2. In the mouse the COX-2$^{+/}$ genotype protects against intestinal adenomas suggesting that induction is usually required before polyps can form. However, all the adenomas investigated by Oshima et al. (1996) were found to have lost both copies of the Apc gene, but only adenomas over 2mm consistently expressed COX-2 (Oshima et al., 1996). Thus it appears that although induction of COX-2 is an early event in the mouse it is preceded by loss of the second Apc allele.

The oncogene c-myc shows increased expression in 60% of colorectal adenomas and 70% of adenocarcinomas, suggesting that like ras genetic alterations leading to the overexpression of myc tend to occur early in adenoma formation
(Erisman et al., 1985). Levels of c-myc expression increase linearly with adenoma size and show no correlation with villous histology, degree of dysplasia, or Dukes staging (Erisman et al., 1989; Guillem et al., 1990).

As with the other oncogenes discussed above activation of c-src appears to occur before malignant transformation is complete. However, activation is most pronounced in larger (>2 cm) more advanced colorectal adenomas and in carcinomas. Unlike the case of c-myc the degree of activation of c-src is correlated with villous histology (Cartwright et al., 1990). Villous adenomas more frequently contain malignant cells than adenomas of tubulovillous or tubular morphology, and levels of c-src may therefore have some prognostic significance.

Loss of heterozygosity at 18q21 (DCC) is observed in 70% of colorectal carcinomas and almost 50% of late adenomas (Vogelstein et al., 1988), indicating that inactivation of a tumour suppressor gene in this region is one of the later genetic alterations in the progression to cancer. In keeping with this observation an association between LOH for 18q and progression of colorectal cancer from intramucosal cancer to invasive carcinoma has been reported (Miyaki et al., 1990). Inactivation of DCC may therefore influence cell adhesion and the invasive potential of tumour cells. There is good evidence that DCC functions as a tumour suppressor gene in the colonic epithelium, however, it is yet possible that other loci on 18q (e.g. DPC4 or JV18-1) may prove to be more significant in this regard.

As discussed previously (section 1.6.2(b).) the TP53 tumour suppressor gene has been shown to be inactivated by a combination of allele loss and point mutation in a variety of human cancers including colon cancer (Nigro et al., 1989). Polymorphic markers in the vicinity of the TP53 gene on 17p13 show loss of heterozygosity in over 75% of colorectal carcinomas (Baker et al., 1989). Typically one TP53 allele acquires a point mutation which is believed to provide a growth advantage, and consequently allows clonal expansion to occur. This initial mutation may be able to function in a dominant negative fashion, interfering with the function of the wild-type allele (see chapter 4). Ultimately mutation (usually deletion) of the remaining wild-type allele occurs to leave the cell devoid of normal p53 protein (Baker et al., 1990). Early adenomas, which frequently display allele loss on chromosome 5q, rarely show loss of TP53 alleles (Vogelstein et al., 1988; Baker et al., 1990). Thus inactivation of TP53 appears to be a later event in colorectal tumourigenesis than inactivation of APC.
High levels of p53 nuclear protein and allelic loss on chromosome 17p have been shown to have prognostic significance, both are correlated with poor patient survival (Rodrigues et al., 1990; Kern et al., 1989).

**Part 3. The adenomatous polyposis coli (APC) gene and its protein product.**

1.7.1. Cloning of the APC gene.

The adenomatous polyposis coli (APC) gene responsible for FAP was localised following reports of patients with polyposis of the large bowel and interstitial deletions on the long arm of chromosome 5 (Herrera et al., 1986; Hockey et al., 1989; Rivera et al., 1990). Analysis of polymorphic markers on chromosome 5 yielded two markers (C11p11 and p227) that demonstrated close linkage to the APC locus (Bodmer et al., 1987; Leppert et al., 1987). These were subsequently mapped to 5q21-22 using somatic cell hybrids and by use of in situ hybridisation (Leppert et al., 1987; Stewart et al., 1987; Bodmer et al., 1987; Alitalo et al., 1987). Fortunately no evidence for genetic heterogeneity, which would have complicated the search for the locus on 5q, was discovered (Bodmer et al., 1987; Meera Khan et al., 1988; Nakamura et al., 1988; Dunlop et al., 1990). The identification of linked polymorphisms provided the first possibility of presymptomatic diagnosis for members of FAP families, and formed the foundations of a linkage map which would grow in resolution as further markers were revealed. Ultimately it became possible to limit the APC locus to a 4 cM region (Kinzel et al., 1991a), and to attempt isolation of the gene itself.

Research into the precise location of the APC gene was complemented by investigations into allele loss occurring during colorectal tumourigenesis. As discussed previously (section 1.6.1.) the cytogenetically visible aberrations most frequently noted in colorectal cancer involve loss of chromosomes 17p, 18q, and 5q, the sites of the TP53, DCC, and APC genes respectively (Muleris et al., 1985;
Solomon et al., 1987; Lothe et al., 1988). This prompted the initiation of loss of heterozygosity (LOH) studies in these regions, aimed at defining the genes targeted for deletion. Initial studies investigating polymorphic loci on chromosome 5q detected LOH in 20-40% of colorectal tumours (Solomon et al., 1987; Vogelstein et al., 1988; Law et al., 1988; Okamoto et al., 1988; Delattre et al., 1989).

The first markers employed for LOH studies were some distance from 5q21-22. When polymorphic loci closely flanking the APC locus became available even higher levels of LOH (54%) were detected (Ashton-Rickardt et al., 1989). This data suggested that LOH was centred in a 10-15 Mb region around the APC gene (Ashton-Rickardt et al., 1989), and thus the gene for FAP was also a promising candidate for the tumour suppressor gene deleted during the formation of sporadic colorectal tumours.

Having localised the APC gene to chromosome 5q21-22, a variety of different strategies were applied to the isolation of putative tumour suppressor genes from this region. Deletions and rearrangements were mapped and the regions affected cloned (Solomon et al., 1987; Vogelstein et al., 1988; Delattre et al., 1989; Ashton-Rickardt et al., 1989). Following a strategy that had proven successful in the cloning of the deleted in colon cancer (DCC) gene (Fearon et al., 1990) and the neurofibromatosis type 1 (NF1) gene (Viskochil et al., 1990), sequences mapping within deletions were subjected to cross-hybridisation revealing sequences conserved through evolution that frequently represent genes (Kinzler et al., 1991a). One group isolated genes from 6 contiguous stretches of sequence (contigs) spanning the APC locus that were contained within yeast artificial chromosomes (YACs) (Kinzler et al., 1991b). A second group analysed genes in phage clones, mapping within FAP patient deletions. These were identified by using a YAC subclone, known to lie in the deleted region, as a probe to select clones from a phage library (Joslyn et al., 1992). Both approaches revealed several genes including the APC gene (also called DP2.5) and the MCC gene (Kinzler et al., 1991; Joslyn et al., 1991).

Final proof that the gene causing FAP and deleted or inactivated in sporadic colorectal cancer had been found was provided by mutation analysis. Nonsense mutations and small deletions that result in a shift in the reading frame of transcription and consequently the introduction of a premature termination codon were found in the
constitutional DNA of FAP patients (Groden et al., 1991; Nishisho et al., 1991) and in sporadic colorectal cancers (Nishisho et al., 1991).

1.7.2. Expression of the APC gene.

The APC gene is widely expressed and exists as a large number of alternatively spliced transcripts. The cDNA sequence originally described for APC was investigated by Horii et al. (1992) and found to be expressed in 17 out of 20 tissues examined, including colon, stomach, pancreas, and thyroid. No such transcripts were identified in oesophagus or ileum, possibly explaining why FAP patients do not tend to develop adenomatous polyps in these gastrointestinal tissues.

The majority of alternatively spliced forms of APC appear to be ubiquitously expressed, although for some mRNA isoforms the study of tissue specificity is incomplete. Horri et al. (1992) found that APC has three possible transcription initiation sites and three untranslated exons at the extreme 5' end of the gene. They identified several mRNA transcripts expressed in a tissue specific manner. Alternative splicing of non-coding exons was shown to generate 5 different forms of transcript. Additionally a non-coding exon, expressed only in the brain, was detected. A subsequent study also identified three untranslated exons in this 5' region, but concluded that expression is ubiquitous rather than tissue specific (Thliveris et al., 1994). Horii et al. (1992) found no evidence for the existence of multiple control regions, however, Lambertz and Ballhausen (1993) identified an alternative 5' untranslated region (5'UTR), indicating that APC may have two distinct promoters. Furthermore, three additional AUG codons that may initiate transcription of truncated APC products were discovered. Another, well described gene with alternative 5'UTRs and two promoters is the lck (proto-oncogene) (Takadera et al., 1989).

The actual translated region of APC is also affected by alternative splicing. An alternative APC transcript lacking the first 303 nucleotides of exon 9 was discovered when the gene was originally identified (Groden et al., 1991). Since then mRNA isoforms have been reported which lack exon 7 (Oshima et al., 1993), lack exon 14 (Sulekova et al., 1995), skip exons 11-14 (Sulekova et al., 1995), or which include a previously unidentified exon (exon X or 10A) situated between exons 10 and 11 (Xia et al., 1995; Sulekova and Ballhausen, 1995). These alternative forms of
APC may have significant functional roles. Skipping of exon 14 creates a novel reading frame in exon 15 which terminates after 19 codons. This transcript is also found in murine cells suggesting a conserved function for the protein produced (Sulekova et al., 1995). The 54 nucleotide exon 10A is also found in the mouse, and contains heptad repeat motifs, such as those involved in APC dimerisation and other protein-protein interactions (Xia et al., 1995). Alternative splicing affecting exons 1-5 has also been reported (Thliveris et al., 1994; Samowitz et al., 1995; Kraus et al., 1996). This is important as these exons contain heptad repeats critical for APC dimerisation. Transcripts lacking these exons may be responsible for producing a monomeric form of APC, although the functional significance of such a protein is unknown.

Evidence for several mRNA transcripts that delete the 6.5 kb final exon of APC has been presented (Horii et al., 1992; Kraus et al., 1996). Polypeptide chains that include exon 3 but lack exons 1, 2, and the carboxyl terminus of exon 15 have been identified using Western blotting and exon specific antibodies (Kraus et al., 1996). Interestingly two types of transcript generated by connecting exon 13 of APC to a neighbouring gene, SRP19, have also been reported. The APC-APC and APC-SRP transcripts were found to be expressed in approximately equal proportions in most of the tissues examined (Horii et al., 1992).

It is conceivable that alternative splicing in the APC gene could have significant implications for the phenotype of FAP patients (see chapter 4). The skipping of exons that contain mutation may allow the production of some normal protein thereby reducing the severity of the disease. Differential splicing or promoter activity may also explain the tissue-specific features of FAP.

1.7.3. Structure and function of the APC protein.

Prior to the cloning of the APC gene facts concerning the structure and function of the protein that it encodes could only be guessed at. Microcell hybridisation allowing the introduction of a normal chromosome 5 to cells of a human colorectal cancer cell line had demonstrated correction of tumorigenicity by this chromosome in athymic nude mice, but provided little insight into the specific influence of the APC gene (Tanaka et al., 1991). Consequently the first information
on this subject was not obtained until the gene was isolated and sequenced (Kinzler et al., 1991; Nishisho et al., 1991). Analysis of the 8538 nucleotide cDNA sequence revealed no signal peptides, transmembrane regions, or nuclear targeting signals, suggesting a cytoplasmic localisation for the protein. Little sequence similarity to other proteins was found. However, localised APC sequences resembled myosins, intermediate filament proteins, and regions of the Drosophila armadillo protein (Kinzler et al., 1991). Whether these homologies had any real significance was not immediately apparent, and it was not until 1993 that further significant information on APC function was published.

1.7.4. Dimerisation of APC.

Immunoprecipitation experiments revealed that the APC protein exists as a homodimer (Su et al., 1993). Dimerisation is thought to be mediated by coiled-coil structures at the amino-terminus of the protein. A recurring seven amino acid motif (heptad repeat) composed of hydrophobic residues had been identified following analysis of the APC coding sequence (Kinzler et al., 1991; Groden et al., 1991). Such a motif is characteristic of proteins that form coiled coils (Cohen et al., 1986). Su and co-workers (1993) confirmed the existence of a homo-oligomerization domain, and employed in vitro transcription and translation of APC fragments to demonstrate that it was contained within the first 171 amino acids of APC. Later that year Joslyn et al. (1993) further refined the localisation of this domain by demonstrating that the first 55 residues of APC are sufficient for formation of a stable, helical dimer, as expected for a two-stranded parallel coiled coil.

The vast majority of APC mutations detected in FAP patients are predicted to generate proteins that retain the homo-oligomerisation domain (Nagase and Nakamura, 1993). As a consequence most mutant proteins are expected to associate with normal APC protein (Su et al., 1993). It is conceivable that these mutant proteins could interfere with the function of the normal protein in a dominant negative fashion, thereby conferring a similar effect as inactivation of both APC alleles (Herskowitz, 1987). A variety of mutant proteins that have a dominant negative mode of action have been described previously. These include mutants of the tumour suppressor gene TP53 that retain the C-terminus oligomerisation domain and can
therefore interact with, and disrupt the function of, wild-type p53 proteins (Kern et al., 1992).

The loss of heterozygosity for chromosome 5q alleles observed during colorectal carcinogenesis suggest that, like RBI in retinoblastoma, APC is recessive at the cellular level in respect to carcinoma formation, however it is less clear whether a single mutant APC allele is sufficient for adenoma formation. Whether the FAP phenotype is simply the consequence of underproduction of normal APC due to heterozygosity for an APC mutation, or whether a dominant negative mechanism inactivates the majority of APC gene products, has been a subject of debate.

Bodmer et al. (1987) suggested that there is a critical level of APC protein required to suppress excessive growth of the colonic epithelium. An individual with only one functional copy of the APC gene would not be able to produce sufficient quantities of the protein, and levels would frequently fall below the critical threshold. The resulting hyper-proliferation forming polyps would increase the likelihood of further mutation, including loss of the remaining APC allele. Vogelstein et al. (1988) proposed that inactivation of a single copy of APC, resulting in reduced gene expression, could lead to a loss of epithelial growth control. However, they suggested that other epigenetic events would be required for adenoma formation. A requirement of further mutation is supported by the observation that polyps do not usually appear until the second or third decade, and that regions of hyperplasia are discrete, not affecting the entire lining of the colorectum (Erbe, 1976).

Suggestions that mutant proteins interfere with the normal APC protein were pursued after several groups experienced difficulties in detecting mutation or loss of the wild-type allele in the adenomas of FAP patients (Solomon et al., 1987; Vogelstein et al., 1988; Law et al., 1988). This deviation from the expected ‘two hits’ model of tumour suppressor genes, as proposed by Knudson (1971), was taken as evidence that dominant negative interactions are a factor in APC inactivation. However, more recent data has led to the general acceptance that the majority, if not all colorectal tumours do carry mutations in both APC alleles (Ichii et al., 1992; Nagase and Nakamura, 1993).

To determine whether or not truncated APC proteins interfere with normal protein Oshima and colleagues (1995) constructed transgenic mice which contained
mutant Apc minigenes in addition to the two copies of Apc usually present. No predisposition to cancer was detected in these mice, arguing that no dominant negative interactions between mutant and normal Apc occur. Another consideration is that homozygosity for Apc<sup>Min</sup> mutation is lethal during early embryogenesis (Moser <i>et al.</i>, 1995), and consequently it seems unlikely that a dominant negative interaction, effectively eliminating APC function, could operate in all tissues or at all stages of development. However, there are significant differences between the progression to cancer in the murine and human systems (Luongo <i>et al.</i>, 1994).

The detection of LOH or two inactivating mutations in many adenomas, and the failure to detect any full length APC protein in 81% of colorectal cancer cell lines (Smith <i>et al.</i>, 1993), also argues against a dominant negative effect of mutant APC proteins, as it is suggestive of a need to silence the normal allele before progression to an adenomatous state can occur (Ichii <i>et al.</i>, 1992; Nagase and Nakamura, 1993).

1.7.5. Proteins interacting with APC.

Immunoprecipitation experiments were conducted to determine if any proteins associate with APC <i>in vivo</i>. The only protein clearly demonstrating association with APC was β-catenin (Rubinfeld <i>et al.</i>, 1993; Su <i>et al.</i>, 1993). There was also evidence that α-catenin might interact with APC (Su <i>et al.</i>, 1993), although it is now clear that this is an indirect association mediated by first binding to β-catenin. Initially the central region of the APC protein (codon 1014-1210) was identified as responsible for β-catenin binding (Su <i>et al.</i>, 1993). This region contains three 15 amino acid imperfect repeats any one of which can mediate this association. Later, Rubinfeld <i>et al.</i> (1995) identified additional low affinity β-catenin binding sites.

As well as binding to APC the catenins also associate with the cell adhesion molecule E-cadherin, a transmembrane protein that is a critical component of adherens junctions. Adherens junctions are essential for the maintenance of epithelial layers, mediating adhesion between cells, anchoring the cytoskeleton, and playing a role in contact inhibition, communicating that adjacent cells are present (Takeichi <i>et al.</i>, 1991). These functions all have potential significance for tumourigenesis, particularly during the later stages of invasion and metastasis. However, the interaction of APC with a protein that can influence the invasive and metastatic
potential of tumours, does not seem to entirely explain its tumour suppressing
capacity. Mutation of APC does not show any correlation with progression from
adenoma to invasive carcinoma, indeed mutation analysis has revealed ‘knock out’ of
both copies of the APC gene even in very small tumours displaying no invasive
characteristics (Nagase and Nakamura, 1993).

All studies to date indicate that β-catenin does not complex with E-cadherin
and APC simultaneously (Rubinfeld et al., 1993; Su et al., 1993). At first attempts to
eucidate whether both proteins interact with β-catenin in the same way were based on
comparison of the amino acid sequences of the two genes. Su et al. (1993) suggested
that the interactions must be different as the 15 amino acid repeats used by APC for β-
catenin binding are not present in E-cadherin. However, Rubinfeld et al. (1993)
identified an amino acid sequence (Ser-Leu-Ser-Ser-Leu) contained within the β-
catenin binding domain of E-cadherin and also present in a 20 amino acid repeat,
seven copies of which are interspersed within the central portion of APC (codon
1342-2075). Interestingly this region has been shown to be capable of
downregulating intracellular levels of β-catenin in tumours lacking APC activity
(Munemitsu et al., 1995). The most recent evidence available suggests that APC and
E-cadherin compete for binding to the same region of β-catenin, a highly conserved
40 amino acid repeat (Hulsken et al., 1994). Thus APC is likely to interact with
cytoplasmic β-catenin rather than that associated with adherens junctions.

The β-catenin repeats responsible for APC binding show 78% amino acid
identity with those originally described for armadillo the Drosophila homologue of β-
catenin (Peifer and Weischaus 1990). Armadillo is critical for normal development in
Drosophila, in which it regulates pattern formation of the embryonic cuticle (Peifer et
al., 1993). Armadillo, and several other segment polarity genes, encode parts of the
signal transduction pathway of wingless, a Drosophila cell-cell signalling protein
which acts to establish cell fate during embryogenesis. Investigation into the
homologous signalling pathway in vertebrates, known as Wnt, indicates that
cytoplasmic β-catenin (armadillo) has a direct role in signalling, independent of any
interaction with cadherins. Furthermore overexpression of cadherins results in
inhibition of the dorsal axis, presumably because excess E-cadherin molecules
sequester the cytoplasmic β-catenin required for signal transduction (Heasman et al., 1994).

Upon Wnt signal transduction β-catenin is stabilised and accumulates in the cytoplasm. It then associates with one of the high mobility group (HMG) box transcription factors, Tcf (Molenaar et al., 1996). The β-catenin-Tcf complex is thought to promote transcription of target genes, and this has been shown to be true in flies (van de Wetering et al., 1997). APC appears to be a negative regulator of this process. High levels of β-catenin cause APC to bind glycogen synthase kinase 3β (GSK3β) the human homologue of zeste white 3, another member of the wingless pathway (Rubinfeld et al., 1996). GSK3β has the effect of phosphorylating the central portion of APC, a process essential for efficient β-catenin binding (Rubinfeld et al., 1996). Experiments in frog embryos and cultured cells have demonstrated that interaction with APC results in the degradation of β-catenin molecules (Rubinfeld et al., 1996; Yost et al., 1996; Papkoff et al., 1996). Thus, high levels of β-catenin indirectly result in more efficient binding to APC which in turn causes a reduction in the level of free cytoplasmic β-catenin by initiating turnover. Colorectal cancer cells with no functional APC protein have high levels of uncomplexed β-catenin in their cytoplasm, and consequently the genes downstream of Wnt remain transcriptionally activated (Korinek et al., 1997; Morin et al., 1997). It is conceivable that expression of these genes confers certain neoplastic characteristics. Significantly, two colorectal cancer cell lines expressing only wild-type APC were found to carry β-catenin mutations (Morin et al., 1997) that affect residues previously implicated in the down-regulation of β-catenin (Yost et al., 1996). This suggests that certain β-catenin mutations may be able to substitute for APC mutation during colorectal tumourigenesis. That GSK3β is necessary for the negative regulation of β-catenin as well as APC has been demonstrated in Drosophila and Xenopus, in which overexpression of β-catenin results in a disturbance of cell fate identical to that caused by interference with GSK3β function (He et al., 1995; Peifer et al., 1994).

Recent investigations into other proteins associated with APC detected another Drosophila homologue: the disks large tumour suppressor protein (DLG) (Matsumine et al., 1996). Unlike interaction with β-catenin association with DLG requires the carboxyl terminal region of APC (amino acids 2475-2843) that is absent from all
mutant forms of the protein to have been reported. Double labelling immuno-electron microscopy indicates that APC and DLG are colocalised along the lateral plasma membrane (Matsumine et al., 1996). Whether or not DLG affects the tumour suppressing function of APC is not known. However, in Drosophila DLG is localised to the septate junctions of imaginal disk epithelia, and mutation causes loss of cell polarity and neoplastic proliferation (Woods et al., 1991).

Another binding site, that is deleted by all APC mutations reported so far, involves the 284 amino acids at the extreme carboxyl-terminus of the APC protein. These residues bind EB1, a highly conserved 30kDa protein of unknown function (Su et al., 1995). The consequences of association with EB1 can not be determined until more is known regarding the normal function of this protein, however the high degree of conservation and the total elimination of APC binding by mutations found in colorectal cancer hint at a significant role.

Immunohistochemical analysis of APC carried out by Smith and co-workers (1993) confirmed that the protein is localised in the cytoplasm, as expected for a protein with no obvious nuclear targeting signals or transmembrane regions. Furthermore, expression was more pronounced in the upper portions of the colonic crypts, suggesting increased APC expression with cell maturation. The subcellular localisation of APC was further investigated using fractionation experiments. These demonstrated the presence of APC in a 100,000g detergent insoluble fraction (Smith et al., 1993), and suggested that wild-type APC was complexed in the form of an insoluble aggregate or perhaps associated with the cytoskeleton. This latter hypothesis was supported by further investigations using immunofluorescent detection of the APC protein (Smith et al., 1994; Munemitsu et al., 1994). These experiments revealed association of wild-type, but not mutant APC with a filamentous network extending throughout the cytoplasm. The action of a microtubule depolymerising agent (nocodazole) and additional immunocytochemical experiments confirmed this network to be the microtubule cytoskeleton (Smith et al., 1994; Munemitsu et al., 1994). Additionally the APC protein was also shown to promote in vitro assembly of microtubules (Munemitsu et al., 1994). However, for unambiguous detection of APC, which usually accounts for less than 0.01% of total cellular protein, it was necessary to transiently overexpress transfected APC constructs, an approach
which could conceivably produce experimental artefacts. Expression of partial APC constructs allowed the putative microtubule binding site to be mapped to a protein fragment containing the 70 kDa carboxyl-terminal region of APC (Munemitsu et al., 1994). Much of this region is lost from the truncated APC proteins produced by colorectal cancer cells. Whether the elimination of APC binding to the microtubule cytoskeleton truly contributes to the development of neoplasia is not known. However, assembly, disassembly, and reorganisation of microtubules is an essential feature accompanying progression through the cell cycle. Drugs which stabilise the microtubule network (e.g. taxol) also block the cell cycle (Schiff et al., 1979). Additionally there is evidence that disassembly of the microtubule network may actually trigger DNA synthesis directly (Crossin, 1981).

1.7.6. The APC protein and apoptosis.

The homeostasis of an epithelial sheet, such as that lining the colon, is not only dependent on appropriate rates of cell division, but also on rates of genetically programmed autonomous cell death (apoptosis) (Williams et al., 1993; Kerr et al., 1972). Aberrant cell survival may allow mutations to accumulate leading to neoplastic transformation. In the colon cells displaying progressively more malignant characteristics demonstrate decreasing levels of apoptosis (Bedi et al., 1995). Resistance to anti-cancer drugs, many of which induce cell death, may also be acquired by cells that have escaped normal apoptotic mechanisms (Fisher et al., 1994).

In the colon stem cells at the base of each crypt proliferate giving rise to all other epithelial cells, as they mature they migrate up the sides of the crypt, undergo apoptosis, and are eventually shed into the lumen of the colon. The expression of certain genes varies with the maturity of the colonocytes. The bcl-2 proto-oncogene, that protects cells form apoptosis, is preferentially expressed in the proliferative region of the colonic crypt (Hockenbery et al., 1991). While TGF-β, a negative growth regulator is more highly expressed in non-proliferating cells at the top of the crypt (Avery et al., 1993). The APC protein is also more abundant in more mature cells in the colon, prompting speculation that it might be involved in cell maturation or apoptosis (Smith et al., 1993).
The finding that normal APC protein is absent from apoptotic cells in cultured colon cancer cell lines provided further evidence of a role for APC in cell survival. A 90 kDa protein, rather than the usual 300 kDa, was detected in apoptotic cells by monoclonal antibodies specific for the amino terminal of APC (Browne et al., 1994). Whether this is the consequence of a mechanism such as alternative splicing or due to post-translational modification is not yet clear. Association between apoptosis and proteolytic cleavage has been previously reported, for example poly(ADP-ribose) polymerase in the cells of a leukaemia cell line (Kaufmann 1993). Additionally the induction of apoptosis has been prevented in rat thymocytes by protease inhibitors (Bruno et al., 1992). An interesting possibility is that APC could regulate loss of cell adhesion, an important early characteristic of apoptosis, via it’s association with β-catenin.

Perhaps the most compelling evidence that APC influences cell survival comes from experiments conducted by Morin et al. (1996) in which wild-type APC was introduced to colon cancer cell lines that only express truncated forms of the protein. Expression of normal APC resulted in the immediate induction of apoptosis, and a reduction in cell growth. Earlier transfections of APC cDNA constructs into colon cancer cell lines succeeded in demonstrating an elimination of the tumourigenic phenotype, but could not be maintained (Groden et al., 1995; Westbrook et al., 1994). It is conceivable that the constructs were unstable because they induced apoptosis in cells successfully transfected.

A growing number of genes have been shown to have a function in pathways leading to apoptosis, including tumour suppressor genes such as TP53 and RB1 (Shaw et al., 1992; Morgenbesser et al., 1994). Wild-type TP53 expression, like that of APC, has been shown to bring about apoptosis in colon cancer cell lines (Shaw et al., 1992). However, the cell lines investigated by Morin et al. (1996) lacked functional p53 as well as wild-type APC suggesting that the two genes act in independent apoptotic pathways. Certain proto-oncogenes also have a function in apoptosis. For example, inhibition of apoptosis has been demonstrated in follicular lymphomas by bcl-2, and in chronic myeloid leukaemia by BCR-ABL (Korsmeyer, 1992; Bedi et al., 1994).

It has also been reported that overexpression of APC blocks progression of the cell cycle from G₀/G₁ to S phase in a mouse cell line (Baeg et al., 1995). However,
Morin et al. (1996) found no evidence of this phenomenon in human colon cancer cell lines. It may be that an accumulation of cells undergoing apoptosis, and therefore not synthesising DNA, could be mistaken for cells in G0/G1.

A protein as large as the 300 kDa APC protein is expected to have multiple functional domains. Considering that it exists in a number of alternatively spliced forms and is widely expressed, it is likely that APC performs a variety of different functions some of which may be tissue specific. Already several proteins that interact with APC have been identified, but it is still far from clear exactly which of these are important in colorectal tumourigenesis. In situations where a complex protein is under analysis additional functional information can sometimes be inferred from differences in phenotype that result from mutation in different areas of the protein. Genotype-phenotype correlation such as this is discussed in chapter 4.

**Part 4. Genome analysis.**

**1.8. Detection of DNA sequence mutations.**

Broadly speaking mutation analyses can be divided into two categories: those which are tailor made for the detection of a specific mutation that has already been characterised, and those which detect previously unknown mutations. This first set of techniques are generally used in a diagnostic context, and are usually employed to provide a rapid means of detecting common mutations. Southern blotting, with subsequent hybridisation of radioactively labelled allele specific oligonucleotide (ASO) probes is one such technique. Mutant sequences are detected by their ability to hybridise mutant sequence specific probes and their failure to hybridise normal specific probes. Applications of this technique have included the diagnosis of α1-antitripsin deficiency (Kidd et al., 1983), cystic fibrosis (Shuber et al., 1993), and the prenatal diagnosis of β thalassaemia (Pirastu et al., 1983).

The annealing of allele specific oligonucleotides is also the basis of the amplification refractory mutation system (ARMS). Although in this technique
oligonucleotides are not detected directly, but serve as primers for PCR. Amplification of the DNA indicates annealing of the primer and therefore presence of a specific mutant allele (Newton et al., 1989).

Another approach used for the detection of specific mutations is restriction fragment length polymorphism (RFLP) analyses. In the past this technique has been used to screen for uncharacterised mutations, however the rarity of the palindromic sequences recognised by individual restriction enzymes leads to a low mutation detection rate (Felley-Bosco et al., 1991; Palombo et al., 1992). As a consequence RFLP analyses are usually confined to diagnostic situations in which a small number of common mutations are responsible for the majority of disease. Specific restriction enzymes, that have a digestion site removed or added, can usually be chosen for the detection of such mutations (Chang and Kan, 1982; Orkin et al., 1982).

If the gene under analysis contains a heterogeneous spectrum of mutations, or if it is a candidate gene having no confirmed mutations at the time of analysis, then a general mutation screening method should be employed. The detection methods already discussed are inefficient at detecting unidentified mutations in a stretch of DNA.

In theory DNA sequencing should be the best way to proceed, as it has the capacity to reveal all sequence variants. However, protocols are slow and laborious, and consequently the constraints of finances and time have precluded it's wide application to mutation screening. Instead a variety of protocols that balance cost, length of procedure, and efficiency of mutation detection are now available. These, so called, 'scanning' strategies all have essentially the same purpose: the detection of uncharacterised mutations in a fragment of DNA. Scanning methodologies can be separated into two classes, those that rely on differences in the electrophoretic migration of mutant and normal DNA fragments, and those that depend on cleavage of mutant/wild-type heteroduplexes before analysis. The former category includes techniques such as single-strand conformation polymorphism (SSCP), denaturant gradient gel electrophoresis (DGGE), and heteroduplex analysis. These methods, which represent the most widely practised forms of mutation detection, have achieved popularity by combining simplicity with speed. However, while refinements of existing methods are under development, current protocols do not detect 100% of
mutations. Furthermore, the exact site of the mutation is not localised within the fragment of DNA assessed.

1.8.1. Heteroduplex analysis.

Heteroduplex analysis is the simplest of scanning methods. If a DNA sample is denatured and then allowed to reanneal much of the DNA reforms the original homoduplexes, however complementary strands from alternative alleles may also associate to form hybrid molecules. Heteroduplexes such as these are formed during amplification of DNA using the polymerase chain reaction. Heteroduplexes have an area of sequence mismatch somewhere along their length and this retards their rate of migration relative to homoduplex DNA during non-denaturing polyacrylamide gel electrophoresis (White et al., 1992). Thus, heteroduplex molecules can be resolved from homoduplexes indicating heterozygosity of the sample. Although inexpensive, rapid and simple heteroduplex analysis is of limited efficiency. Mutation detection rates are reported to be around 80% with deletions and insertions considerably easier to detect than single base substitutions (Glavac and Dean, 1995). Optimisation of electrophoretic conditions such as temperature, ionic strength of gel buffer, and gel matrix are critical (Keen et al., 1991; White et al., 1992).

Despite relatively low sensitivity heteroduplex analysis is sufficient for preliminary screening and for screening diseases generally caused by a small number of common detectable mutations. Heteroduplex analysis has found significant application to this type of screening in diseases such as β-thalassaemia (Xu et al., 1993; Wood et al., 1993) and cystic fibrosis (Chong et al., 1990).


SSCP is an assay which is capable of detecting single base pair substitutions, as well as small deletions and insertions, in fragments of DNA ranging in size from 100 to 500 base pairs (Orita et al., 1989a and b). Under nondenaturing conditions, single DNA strands take on sequence specific conformations which are stabilised by intrastrand interactions, of which the formation of hydrogen bonds are the most significant. DNA strands of different sequence usually adopt distinct conformations,
indeed a single base alteration can result in a quite radical change of conformation. Different conformations can then be separated by non-denaturing polyacrylamide gel electrophoresis provided that they have differing electrophoretic mobilities.

SSCP has become one of the most frequently used scanning strategies for mutation detection (see Hayashi 1992, for a review). A search of the scientific and medical literature reveals that, since January 1996, the application of SSCP was reported almost ten times more often than any other mutation detection technique. These citations include applications to both clinical diagnostics and pure research. There are several reasons for the popularity of SSCP: In its simplest form SSCP requires a minimal amount of equipment and the experimental procedure is uncomplicated making it relatively inexpensive; It can be performed without radioactive isotopes by using ethidium bromide (Hongyo et al., 1993) or highly sensitive silver staining to rapidly detect even small quantities of DNA (Ainsworth et al., 1990). SSCP has proven to be highly versatile. It has been applied to the detection of polymorphisms in human genes such as the dopamine D2 receptor gene (Bolos et al., 1990) and the APC gene (Gayther et al., 1995), and to the identification of mutations in many different genes including the cystic fibrosis gene (CFTR) (Dean et al., 1990), TP53 (Mashiyama et al., 1991), and in conditions such as phenylketonuria (Labrune et al., 1991).

1.8.3. Denaturant gradient gel electrophoresis (DGGE).

Like SSCP denaturant gradient gel electrophoresis (DGGE) employs electrophoresis of DNA fragments through a polyacrylamide gel, however, unlike SSCP the DNA molecules assessed are double rather than single stranded. As fragments migrate through the gel they are subject to a linear increase in denaturing conditions. This causes the double stranded fragment to denature or 'melt' in a series of distinct phases. A-T rich regions become single stranded first and are followed by progressively more G-C rich areas. Fragments of DNA, up to 1000bp in length, generally contain between two and five such melting domains, composed of blocks of sequence ranging from 25 to several hundred base pairs in size (Meyers et al., 1985a and b). As melting occurs the electrophoretic migration of the DNA fragment is retarded (Meyers et al., 1985a and b).
The concentration of denaturant at which dissociation is complete is equivalent to the melting temperature (Tm) of the fragment. Critically melting temperature is a sequence specific property. A mutant DNA fragment differing from the normal sequence by just one base can usually be resolved after electrophoresis by virtue of altered melting properties and consequently differing mobility. In addition to altered migration of DNA homoduplexes, heteroduplexes can also be identified and are a useful indicator of heterozygosity. Utilising physical characteristics of double stranded DNA DGGE has been successfully applied to the detection of single base substitutions and small deletions and insertions. (Fischer and Lerman, 1983; Meyers et al., 1985a; Mayers et al., 1985b; Meyers and Maniatis 1986)

On average over half of the melting domains in a fragment of DNA from 100-1000 bases length are of low melting point (Meyers et al., 1988). This led early estimates of the efficiency of DGGE to suggest a detection rate of over 50% for single base changes in fragments in this size range (Meyers et al., 1985a and b). However, current predictions of DGGE sensitivity approach 100% (Macek et al., 1997). The increase in effectiveness is due to several refinements of the original technique. Most important is the addition of a GC clamp, a high melting point sequence of approximately 40 guanine or cytosine residues which is usually attached to one of the oligonucleotide primers prior to PCR amplification (Sheffield et al., 1989). The GC clamp allows analysis of the highest melting point domains inaccessible to earlier protocols (Meyers et al., 1985a and b). A further advance came with computer programmes designed to model the melting behaviour of fragments of DNA. Such programmes allow denaturing conditions to be optimised for specific DNA sequences (Lerman and Silverstein, 1987). As well as increasing the efficiency of DGGE knowledge of the optimal denaturing temperature has allowed the development of constant denaturing gel electrophoresis (CDGE). This method involves polyacrylamide gel electrophoresis performed at a uniform concentration of denaturant previously determined as optimal by computer programme (Borresen et al., 1991).

DGGE has found wide application to the discovery of unidentified mutations in genes such as MEN2 (multiple endocrine neoplasia type 2) (Blank et al., 1996), β-hexosaminidase-A (Tay-Sachs disease) (Akli et al., 1992), CFTR (cystic fibrosis)
(Audrezet et al., 1992), and to the screening of known mutations in genes such as TP53 (Beck et al., 1993).

The methods of mutation analysis described above are among the most straight forward to perform. However, detection of a mutation with one of these techniques does not precisely define the mutation site, it generally provides localisation to a 200-400bp stretch of DNA which must then be sequenced in full. Cleavage methods of mutation detection have the significant advantage of localising a mutation to within a few base pairs of sequence even in fragments approaching 1.5kb in length.

1.8.4. Ribonuclease A cleavage analysis.

A heteroduplex molecule formed by the hybridisation of a DNA molecule to a RNA molecule of similar sequence can be enzymatically cleaved by Ribonuclease A (RNaseA) at any sights of mismatch. This forms the basis of RNaseA protection analysis, the oldest of the cleavage methods, first described by Myers et al. (1985c). RNA molecules of normal sequence can be made in vitro by transcribing DNA fragments that have been cloned into an expression vector (Green et al., 1983). Such RNA molecules can serve as probes if they are radioactively labelled and then allowed to hybridise to a single stranded DNA template under analysis. The DNA and RNA molecules remain associated by virtue of complementary base pairing. At any positions where the sequence of the test DNA differs from the normal RNA sequence there is a failure of base pairing and single stranded RNA, vulnerable to digestion by RNaseA, is left exposed. Cleavage of an imperfect hybrid with RNaseA results in the production of two (or more) smaller RNA fragments. The existence of digested molecules can be detected, after dissociation from the sample DNA, by electrophoretic resolution of RNA fragments of different size. Furthermore the size of the cleaved molecules provides an indication of where the mutation lies within the DNA fragment. A further application of this method includes the formation of RNA:RNA hybrids with subsequent detection of mismatches by RNaseA digestion. This popular technique allows the detection of mRNA variants (e.g. mutations in H-ras mRNA: Winter et al., 1985).
Like the other methods of mutation detection so far discussed the reported efficiency of RNaseA protection varies between different laboratories. Generally a detection rate of approximately 35% is suggested for the detection of single base substitutions, although the detection of small deletions and insertions appears to be more efficient as evidenced by genes prone to this sort of mutation such as the adenomatous polyposis coli gene \((APC)\) (Miyoshi \textit{et al.}, 1992a and 1992b; Nagase \textit{et al.}, 1992b). Analysis of both DNA strands increases the efficiency of RNaseA protection to around 65% (Myers \textit{et al.}, 1985c and 1988; Myers and Maniatis 1986), sufficient sensitivity for use in clinical screening of gene carriers in disorders such as familial hypertrophic cardiomyopathy (MacRae \textit{et al.}, 1994). The upper size limit of sequences that can be analysed in this fashion is restricted by technical difficulties in producing complete RNA fragments of over 1000bp.

1.8.5. Chemical mismatch cleavage analysis.

Chemical mismatch cleavage (CMC) accomplishes mutation detection in a similar manner to the RNaseA protection assay. The principal differences are that CMC involves the cleavage of DNA:DNA heteroduplexes rather than DNA:RNA hybrids and this is achieved by chemical reaction rather than enzymatic digestion (Cotton \textit{et al.}, 1988).

A DNA fragment of normal sequence and a similar fragment to be tested for mutation are radioactively labelled, denatured together, and then allowed to reanneal. Mismatches of base sequence in the heteroduplex molecules formed by this process can be detected by cleavage with specific chemicals. Firstly the mismatched bases are modified by treatment with hydroxylamine and osmium tetroxide, which modify cytosine and thymine respectively. The next step is to incubate the heteroduplex molecules with piperidine which results in specific cleavage at the site of modified bases (Cotton \textit{et al.}, 1988). Cleaved fragments can be identified by denaturing gel electrophoresis followed by autoradiography.

Investigations in which both strands of a DNA fragment have been analysed report the detection of 100% of mutations in fragments of up to 1200bp (Grompe \textit{et al.}, 1989; Condie \textit{et al.}, 1993). Thus CMC is the most sensitive scanning method of mutation screening currently available, and has been applied to the detection of
mutations in a growing number of genes. These include: the TP53 gene which has been analysed in breast tumours (Prosser et al., 1991); the ret proto-oncogene which has been assessed in patients with multiple endocrine neoplasia type 2A (Mulligan et al., 1993); and analysis of the hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene which has been carried out using a non-radioactive variant of CMC (Tsuboi et al., 1995).

The principal drawbacks of CMC are that the technique is rather cumbersome, and that the most widely practised forms of the method require a number of hazardous chemicals and radioactive labelling of the DNA. These factors hinder the application of CMC outside the research laboratory.

1.8.6. Enzyme mismatch cleavage (EMC).

A developing method of mutation detection which may bring the efficiency of CMC in reach of laboratories not dedicated to molecular genetics is enzyme mismatch cleavage analysis (EMC). The principle of EMC is analogous to that of CMC. however, in this technique cleavage of DNA mismatches is achieved by digesting the single stranded DNA at the mismatch rather than by chemical reaction (Youil et al., 1995).

Initial efforts at cleaving mismatched DNA duplexes with single strand specific nucleases had only limited success (Myers et al., 1985c), but the application of bacteriophage resolvases, whose function in vivo is to cleave branched DNA, appears to have overcome these difficulties. A detection rate of 94% in DNA fragments up to 1000bp in length has been reported (Marshall et al., 1995; Youil et al., 1995). The upper size limit of fragments allowing this level of detection remains to be established. Further experiments to determine the efficiency of the technique employed the resolvase T4 endonuclease VII, and successfully detected 81/81 single base changes situated within the mouse ß-globin promoter using a single set of conditions (Youil et al., 1996).

If an economical commercial supply of resolvase enzymes can be found and if EMC can be performed without purification of DNA fragments, then it should have the potential to displace SSCP and DGGE as the preferred method of mutation screening. Another possibility for the future of EMC is the use of DNA repair
enzymes which specifically identify and bind mismatched bases. The *Escherichia Coli* mismatch binding protein MutS has already been employed to create a sensitive non-radioactive assay (Wagner *et al.*, 1995).
Table 1.2. Efficiencies and limitations of different mutation detection methodologies.

<table>
<thead>
<tr>
<th>Method</th>
<th>DNA fragment size</th>
<th>Sensitivity</th>
<th>Localises site of mutation</th>
<th>Toxic chemicals</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSCP</td>
<td>250bp</td>
<td>80%</td>
<td>no</td>
<td>none</td>
</tr>
<tr>
<td>DGGE</td>
<td>600bp</td>
<td>95%</td>
<td>no</td>
<td>formamide</td>
</tr>
<tr>
<td>Heteroduplex analysis</td>
<td>300bp</td>
<td>80%</td>
<td>no</td>
<td>none</td>
</tr>
<tr>
<td>CMC</td>
<td>1700bp</td>
<td>98%</td>
<td>yes</td>
<td>usually OsO₄ + radioactivity</td>
</tr>
<tr>
<td>RNase A cleavage</td>
<td>1000bp</td>
<td>65%</td>
<td>yes</td>
<td>radioactivity</td>
</tr>
<tr>
<td>EMC</td>
<td>&gt;1000bp</td>
<td>94%</td>
<td>yes</td>
<td>currently radioactive</td>
</tr>
</tbody>
</table>

1.8.7. The protein truncation test (PTT).

In addition to the general mutation screening techniques described above are a class of assays designed to detect specific varieties of mutation. One such method is the protein truncation test (PTT) which is used to identify the presence of translation terminating mutations (Roest et al., 1993). PTT involves amplification of sample DNA using a modified primer that includes a bacteriophage T7 promoter, transcription initiation signals, and an in frame sequence from the gene under investigation. The amplified DNA fragment is added to an *in vitro* transcription and translation system and the polypeptides produced are run on an SDS-polyacrylamide gel. Protein truncating mutations are indicated by the presence of novel polypeptides migrating more rapidly than the wild-type polypeptide (Roest et al., 1993). PTT is appropriate for detecting mutation in genes with mutation spectra characterised by a
The preponderance of nonsense or frameshift mutations. So far tumour suppressor genes such as \textit{APC} (van der Luijt \textit{et al.}, 1994) and \textit{BRCA1} (Hogervorst \textit{et al.}, 1995) have received the most attention.

**Part 5. Genetic diagnosis.**

**1.9. Tracking the inheritance of mutant alleles.**

Individuals who are affected by a hereditary disease or who carry a mutant gene are at risk of transmitting a genetic disorder to their offspring. In the past such individuals were faced with the choice of starting a family, knowing that some of their children might suffer from disease, or alternatively not having their own children at all. With an understanding of the genetic basis of inherited disorders has come the possibility of screening for presymptomatic individuals and unaffected carriers of recessive disease. Knowledge of which family members have inherited a mutant allele provides an opportunity for more effective genetic counselling and clinical management (Vasen \textit{et al.}, 1996). Some individuals may learn that they do not carry the mutation that has caused disease in their family. This information may affect their future reproductive decisions. Furthermore, regular clinical investigation, such as that recommended for patients suffering from familial adenomatous polyposis (Vasen \textit{et al.}, 1990) and certain other diseases, can be discontinued or reduced in frequency. Conversely, patients who prove to be presymptomatic mutation carriers may be examined with greater regularity.

The discovery of linkage of a disease gene to a polymorphic site on the same chromosome usually precedes the cloning of the gene itself. Consequently the first genetic diagnoses are usually offered before the causative mutation or indeed the causative gene are identified, and employ linkage analysis. This utilises detection of an associated allele at a nearby locus rather than detection of the causative mutation itself. The closer the polymorphism to the gene the more useful it is for diagnosis, as it is less likely that the two sites will be separated by homologous exchange during meiosis. Polymorphic loci that comprise a large number of alleles, all present at
appreciable frequencies, are especially valuable because the probability of an individual being homozygous, and therefore uninformative is much reduced. For this reason hyper-variable microsatellite loci are of particular interest.

Once the gene responsible has actually been cloned and sequenced mutation analysis can be employed to detect the actual disease causing mutation. When this has been identified it is usually a straight forward task to search for the same mutation in family members of uncertain gene status. This sort of analysis is the only definitive way of proving that a patient is free of mutation. In addition to providing the potential for the most reliable diagnosis, knowledge of the precise mutation involved in a disease can also supply prognostic information. Information on the likely course of a disease such as age of onset and severity of symptoms can help refine treatment regimens.

1.9.1. Prenatal diagnosis.

Coupled with the development of techniques that allow sampling of human fetal cells during pregnancy, linkage analysis and direct mutation detection have introduced prenatal diagnosis as an option for families affected by an hereditary disorder.

1.9.2. Amniocentesis.

The first form of prenatal diagnosis developed was amniocentesis, a technique that involves sampling of fetal cells contained in amniotic fluid. For this purpose the amniotic cavity is pierced with a needle and approximately 20ml of fluid is removed. This procedure is normally carried out during the second trimester of pregnancy, between 15 and 18 weeks of gestation. Removal of amniotic fluid as a clinical procedure was first described by Prochownik in 1877 in the management of polyhydramnios. However, the application of the same technique in the context of prenatal diagnosis did not follow until 1953 when Beris employed amniocentesis to look for Rhesus isoimmunisation (Klinger et al., 1992). Methods providing visualisation of the sex chromatin, and therefore determination of foetal sex, allowed amniocentesis to be applied to the screening of X-linked disorders. Although this
approach was fairly cumbersome, failing to distinguish affected males from unaffected, it was successfully applied to disorders such as haemophilia and muscular dystrophy (Riis and Fuchs 1960; Serv and Margolis 1964). The detection of certain metabolic disorders soon followed and represented an important advance in amniocentesis (Jeffcoate et al., 1965; Nadler 1968; Dancis 1968), however it was not until amniocytes were successfully cultured that the technique found wide application (Steele and Breg 1966). Culture and subsequent karyotyping of amniocytes led to the diagnosis of chromosomal abnormalities such as trisomy 21 (Valenti et al., 1968), and amniocentesis is now used routinely for diagnosis of pregnancies at a high risk of aneuploidy due to effects such as maternal age.

Despite the success of amniocentesis a number of factors have sustained an interest in the development of alternative methods of fetal sampling and prenatal diagnosis. Amniocentesis presents a negligible risk to the mother, however miscarriage following the procedure is reported to be 0.5%-1.0% higher than normally encountered at the same stage of pregnancy (Taylor et al., 1986). Perhaps the most significant drawbacks of the technique are the length of time required for diagnosis, and the fact that amniocentesis before 16 weeks is technically difficult and is associated with a higher rate of fetal loss (Hanson et al., 1987). This means that the results of a diagnosis are not usually known before the eighteenth week, worsening the risk of medical complications and emotional trauma if a termination is required. These factors have prompted researchers to develop more rapid tests, such as direct analysis of chromosome number in uncultured amniocytes using fluorescent chromosome specific probes (Klinger et al., 1992) and rapid DNA tests for the same purpose (Pertl et al., 1994). However, even with these advances diagnosis is seldom possible before the seventeenth week of pregnancy.

1.9.3. Chorionic villus sampling (CVS).

A technique which allows sampling of foetal material earlier in pregnancy than amniocentesis is chorionic villus sampling (CVS). This method is usually carried out between the tenth and thirteenth week of gestation, during the first trimester. The acquisition of foetal trophoblasts by CVS was first reported in 1968, using an endoscopic biopsy (Mohr 1968). A vacuum was employed to draw chorionic
villi into a hole situated in the side of a transcervically inserted hysteroscope barrel. The villi were then cut free of the chorion using a knife. Current protocols for CVS use ultrasound guidance of a cervical catheter (Kazy 1982; Old 1982), or in some cases a transabdominal needle (Smidt-Jensen et al., 1984) and suction to aspirate villi. This has allowed successful diagnosis of a wide range of inherited diseases and also detection of chromosome abnormalities.

No unfavourable perinatal effects are associated with CVS (Williams et al., 1987), but the technique is not without some risks to the unborn fetus. The number of procedure related miscarriages is difficult to calculate because of the high rate of spontaneous abortion that naturally occurs during the first trimester (Boue et al., 1985). However, an increase over the background rate of pregnancy loss of 1% has been suggested (Rhoades et al., 1989). This figure is significantly increased if CVS is attempted at an earlier stage than normal, or if there is difficulty in positioning the catheter.

A further problem, apparently related to difficult or early CVS, was uncovered by Firth et al. (1991) who reported an increase in the incidence of severe limb abnormalities from 1 per 175,000 live births (Foster-Iskenius et al., 1989) to 1 in 66. A transabdominal CVS had been performed before the ninth week of pregnancy in each case. Further investigations by Burton et al. (1992) confirmed Firth’s observations and also implicated transcervical CVS as a potential problem. Major congenital abnormalities were detected in 3.3% of fetuses exposed to CVS between 9.5 and 11 weeks of gestation. It has been suggested that vascular insult is the chief cause of the impaired morphogenesis seen following some cases of CVS.

Chorionic villus sampling is based on the principle that the extra-embryonic tissues are representative of the developing fetus. However, it has emerged that in approximately 1% of pregnancies this is not entirely true (Silverman et al., 1992). Confined placental mosaicism, in which the placenta is karyotypically different from the embryo, may arise as a consequence of mitotic nondisjunction early in development. As only a minority of the cells comprising the human blastocyst go on to form the embryo proper it is feasible that a new cell line created by an error during cell division may only reside in extra-embryonic structures. Furthermore there is evidence that aneuploid cells may be preferentially allocated from the inner cell mass.
destined to form the fetus to the trophectoderm by an as yet unknown mechanism (James et al., 1995).

Despite refinements to foetal testing, producing quicker, safer, and less invasive procedures, prenatal diagnosis remains unacceptable to some prospective parents. The chief difficulty lies with the ultimate conclusion to an unfavourable diagnosis, that of termination of pregnancy. This poses ethical and religious problems for some individuals, while parents who are carriers of a genetic disease are also faced with the traumatic possibility of repeated abortion. A family segregating β-thalassaemia was reported by Penketh and McLaren (1987) in which only one normal child resulted from seven pregnancies, four of which ended in termination. Cases such as this have prompted research into alternative forms of diagnosis that avoid the need for termination.

1.9.4. Preimplantation genetic diagnosis (PGD).

The options for diagnosis of affected embryos before pregnancy ensues must, by necessity, focus on the detection of disease in gametes or preimplantation embryos. Protocols that involve analysis of the first polar body of the oocyte (Verlinsky et al., 1990; Verlinsky et al., 1992) are attractive as the issue of termination is completely circumvented. However, aneuploidy arising at meiosis II can not be detected by this method and the information obtained can be difficult to interpret because of homologous recombination. Furthermore polar body analysis provides no information on the genetic contribution from the father. Investigation of both the first and second polar bodies has also been achieved, and provides more reliable data on the genetic status of the embryo. However biopsy of both polar bodies is technically demanding and does not have the same ethical advantage that first polar body (preconception) diagnosis enjoys.

Analysis of trophectoderm cells from embryos at the blastocyst stage has also been reported. Biopsy at this stage allows the sampling of a larger number of cells than at other preimplantation stages, but unfortunately the survival of embryos to the blastocyst in culture is not sufficiently high at present for clinical application of this method. Because of these difficulties most centres offering preimplantation genetic
diagnosis (PGD) has focused on cleavage stage embryos produced by superovulation and in vitro fertilisation (IVF) procedures. Attempts to obtain preimplantation embryos by uterine lavage following natural fertilisation (Buster et al., 1985; Bacchus and Buselmaier, 1988) have not been successful thus far. Embryos generated using assisted conception techniques remain in culture until day three post-fertilisation, by which time they typically consist of 6-10 cells (blastomeres). For preimplantation diagnosis one or two blastomeres are usually removed and subjected to genetic analysis (figure 1.5.). To achieve this the embryo is first immobilised, by suction, against a glass pipette with a diameter too narrow for the embryo to pass into. It is important that the pipette is polished to avoid causing the embryonic cells to lyse. To gain access to the blastomeres a hole must be made in the zona pellucida that encapsulates the embryo. For this purpose a second, finer, pipette is used to pass a stream of acidified Tyrode’s solution over a localised area causing it to dissolve. It is then possible to draw blastomeres into a third highly polished pipette by pushing it through the hole in the zona pellucida and applying suction (Handyside et al., 1990).

At the cleavage stage all cells are totipotent and consequently the development of the embryo should not theoretically be impaired by this loss of material. This view is supported by metabolic studies showing that glucose and pyruvate uptake by biopsied embryos only decreases in proportion to the decrease in cell number, and that embryo survival in culture is unaffected by biopsy (Hardy et al., 1990). The clinical experience, although limited, also supports this notion (Soussis et al., 1996). It has even been suggested that drilling a hole in the zona pellucida may increase IVF pregnancy rates by aiding the hatching of the embryo (Gorden et al., 1988). The figures available suggest that the proportion of women becoming pregnant after IVF and preimplantation diagnosis (25% per cycle) is not significantly different from the proportion who achieve pregnancy after IVF alone (Harper, 1996).

Once a cell has been collected from an embryo there are two principal ways that it may be treated. It may be spread on a microscope slide and fixed, ready for subsequent cytogenetic analysis, or the cell may be lysed allowing PCR based analysis to be carried out on the DNA within. Preliminary experiments conducted on mice had even demonstrated the possibility of measuring enzyme levels, such as hypoxanthine phosphoribosyl transferase (HPRT) the enzyme which when deficient causes Lesch-Nyhan syndrome in humans (Monk et al., 1987). If the blastomere(s)
Fig. 1.5. Biopsy procedure for cleavage stage preimplantation embryos.

Biopsy of a human cleavage stage embryo three days after fertilisation. The embryo is held against a polished pipette by the application of a gentle suction (a). A second pipette is introduced and a stream of acidified Tyrode’s solution is passed through it and over a small region of the zona pellucida that encapsulates the embryo. This cause a hole to dissolve in the zona pellucida (b). Another polished pipette can then be pushed through the opening and cells removed from inside by gentle suction (c). Scanning electron micrograph of an embryo after biopsy (d).
biopsied are shown to be unaffected by a genetic disorder then it can be inferred that
the rest of the embryo is also free of the disease. Only unaffected embryos are
transferred back to the mother's uterus, and consequently any pregnancy resulting
from the procedure must be unaffected.

The first clinical application of preimplantation genetic diagnosis was for X-
linked disorders such as Duchenne muscular dystrophy (DMD) and retinitis
pigmentosa. For this purpose the sex of the embryos was determined by PCR using
primers specific for DNA sequences on the Y chromosome. A blastomere giving
amplification was indicative of a male embryo at a high (50%) risk of developing
disease (Handyside et al., 1989; Handyside et al., 1990). Several normal girls were
born following PCR sexing, however the possibility of a misdiagnosis, resulting from
amplification failure, remained a concern. In all eight women underwent a total of
thirteen cycles of IVF treatment, with five of the patients becoming pregnant.
Chorionic villus sampling and subsequent chromosome studies revealed two sets of
female twins and three singletons, two female but critically one male (Handyside and
Delhanty, 1993).

Current PCR sexing protocols use one set of primers to amplify related
sequences present on both the X- and Y-chromosome. The sequences amplified are
identical at the site of primer annealing, but differ internally being of different size or
containing a restriction site only present on one of the two sex chromosomes,
examples include the homologous ZFX and ZFY (Chong et al., 1993) and the
amelogenin gene (Nakagome et al., 1991). As all cells sampled should have at least
one X chromosome an X-specific product should always be produced. Absence of X-
product is indicative of a failed PCR, and consequently any data on Y-specific
amplification from the same reaction should not be trusted. Despite this improvement
contamination with exogenous DNA coupled with simultaneous amplification failure
of the biopsied cell could still produce an erroneous diagnosis, and consequently a
new strategy for approaching X-linked disease was desirable. This coincided with the
increasing use of fluorescent in situ hybridisation (FISH) for the detection of specific
chromosomes (Pinkel et al., 1986). The hybridisation of X- or Y-chromosome
specific repetitive DNA probes to the nuclei of human blastomeres was successfully
accomplished in 1991 (Griffin et al., 1991). However, the method was limited by the
possibility of hybridisation failure which could theoretically lead to misdiagnosis.
Dual FISH using both X- and Y-chromosome probes, detected in different colours, was reported a year later (Griffin et al., 1992). The use of two probes in tandem provides a useful in built control that excludes cells with abnormal chromosome complements or failed hybridisation. For example, a male (XY) cell in which the Y-chromosome specific probe failed to hybridise to the nucleus would appear to be derived from a Turner’s syndrome (X0) embryo and would not therefore be transferred. By September 1995 49 patients had undergone preimplantation sexing using FISH in the course of 70 IVF cycles, resulting in a total of 11 normal girls born and no misdiagnoses (Harper, 1996).

Since the initial application to sexing FISH has also been applied to autosomal chromosome anomalies. Chromosome copy number has been assessed in patients predisposed to the production of aneuploid gametes. Such patients include carriers of balanced translocations and those in which an aneuploid cell lineage is restricted to the germinal tissue (Conn et al., 1997).

Although FISH has superseded PCR for the preimplantation analysis of sex, the specific diagnosis of single-gene defects remains dependent on DNA amplification. Cystic fibrosis, a disease which represents the most common autosomal recessive disorder amongst people of Caucasian origin, was the first single gene disorder to be approached in preimplantation embryos. Cystic fibrosis is caused by a heterogeneous group of mutations in the cystic fibrosis transmembrane regulator gene (CFTR). However, the three base pair deletion ΔF508 accounts for the majority (approximately 75%) of cystic fibrosis alleles in the UK population (McIntosh et al., 1989). Because of the prevalence of ΔF508 a PGD strategy was designed for couples in which both partners are carriers of ΔF508. Following embryo biopsy a ‘nested’ PCR protocol was applied to provide specific amplification of the CFTR gene, and heteroduplex analysis was employed for mutation detection. Of the three couples initially treated two had unaffected embryos available for transfer back to the mother. In each case two embryos were returned, leading in one case to pregnancy and the subsequent birth of an unaffected baby girl (Handyside et al., 1992). Heteroduplex formation has since been applied to PGD for Tay Sachs disease, a severe neurodegenerative disorder resulting from deficiency of the lysosomal hydrolase β-hexosaminidase-A (Gibbons et al., 1995). The target of heteroduplex analysis in this case was the common 4 bp insertion in exon 11 of the β-hexosaminidase-A gene.
which accounts for approximately 80% of Tay Sachs alleles in the Ashkenazi Jewish population (Peleg et al., 1994).

A different mutation detection system was used for the preimplantation diagnosis of Lesch-Nyhan syndrome, an X-linked disorder caused by mutation in the hypoxanthine phosphoribosyl transferase (HPRT) gene. Disease causing mutations in HPRT tend to be unique to individual families and consequently it is difficult to develop a single strategy for all families segregating a mutation. Despite this problem two couples have received preimplantation diagnosis for this disorder, and a healthy girl has been delivered (Hughes et al., unpublished). In these cases the mutations were detected in PCR amplified fragments by enzymatic digestion using restriction endonucleases specific to the mutation sites, followed by electrophoresis to reveal cleaved DNA fragments.

Since these early successes diseases with an autosomal dominant mode of inheritance have also been approached by PCR and preimplantation genetic diagnosis. These include Marfan syndrome a disease of the connective tissue caused by inheritance of a defective fibrillin gene (Harton et al., 1996), and familial adenomatous polyposis (FAP) a disorder predisposing to colorectal neoplasia (undertaken during this study). Embryos affected with Marfan syndrome were identified by virtue of a linked dinucleotide repeat polymorphism. This protocol has been successful in the single treatment cycle attempted so far, leading to the birth of a healthy male. The preimplantation diagnosis of FAP is discussed fully in chapter four of this thesis. Protocols for PGD of a variety of other diseases have also been reported, including myotonic dystrophy and β-thalassaemia (El Hashemite et al., 1996; Ray et al., 1996; Sermon et al., 1997).
Chapter 2

Materials and Methods
Materials and Methods

2.1. Materials.

2.1.1. Chemicals.

The majority of chemicals used in this study were supplied by BDH Chemicals, Pool, Dorset and were of AnalaR grade. This includes all general laboratory reagents such as ethanol, and chemicals used for silver staining and the preparation of buffers. Additional chemicals were supplied by the following companies:

Sigma Chemical company: ethidium bromide, standard grade agarose, Tween 20 detergent (polyoxyethylene sorbitan monolaurate), phosphate buffered saline (PBS) tablets.

Severn Biotech Ltd: 19:1 acrylamide/bis-acrylamide (6% solution).

Biorad: 37.5:1 acrylamide/ bis-acrylamide (30% solution), ammonium persulphate (AMPS), NNN’N’tetramethylenediamine (TEMED).

Pharmacia: dextran sulphate, deoxynucleoside triphosphates (dATP, dGTP, dCTP, dTTP).

Amersham: $[^{35}S]$methionine, [$\alpha-^{33}P$]dideoxynucleosides.

2.1.2. Enzymes.

The restriction endonucleases (Acc I, Apo I, Msp I, Taq I, Mae I) used for mutation detection were obtained from Gibco BRL or Promega. Enzymes were supplied at a variety of concentrations and came with an optimised reaction buffer and in some cases with bovine serum albumin (BSA). The Exonuclease I (10U/µl) and alkaline phosphatase (2U/µl) used in the preparation of PCR products for sequencing were supplied by Amersham. Desiccated Proteinase K (Arthobacter leteus) was obtained from Boeringer Mannheim and made to a final concentration of 50 µg/ml,
while desiccated Lyticase was supplied by Sigma Chemical Company and dissolved to form a solution of concentration 25U/μl. DNA polymerase was obtained from a variety of sources. The thermostable Thermus aquaticus (Tag) DNA polymerase was supplied by H.T. Biotechnologies at a concentration of 5U/μl and was used for general PCR. A modified thermostable DNA polymerase (Thermo Sequenase) was supplied by Amersham and used for cycle sequencing. DNA polymerase was also supplied as a component of the Boehringer Mannheim nick translation kit and the Gibco BRL Bio nick kit. These kits also included DNAse I. The TnT kit used for the protein truncation test contained T7 RNA polymerase (Promega). All enzymes were stored at -20°C with the exception of Lyticase which was stored at 4°C and T7 RNA polymerase which was stored at -70°C.

2.1.3. Nucleic acids.

DNA size standards (100 bp ladder, 1 kb ladder) and Cot1 DNA (1μg/μl) were supplied by Gibco BRL and stored at -20°C. Oligonucleotide primers for PCR were obtained from Oswel DNA services and were supplied as purified working solutions of approximately 50pM/μl concentration (see section 2A.9.), again storage was at -20°C. Salmon/herring sperm DNA was supplied by Sigma Chemical Company at a concentration of 10mg/ml and was kept at 4°C.

2.1.4. Patient and tumour material.

The majority of blood samples from patients with familial adenomatous polyposis and their relatives were provided by the Polyposis Registry, St. Marks Hospital, London, and the Northern Polyposis Registry, University of Newcastle upon Tyne. A smaller number of samples were also sent by Mr. D. Finnis of the Salisbury Health Authority, by the Hadassah University Hospital, Israel, and by Dr. I Borg, University of Malta.
2.1.4. Definition of severe/average phenotype.

Severity of phenotype in families with histologically confirmed FAP was judged on the basis of age of polyp development, number of polyps, and age of onset of neoplastic disease. A patient undergoing regular screening was considered to have an average phenotype if polyps appeared between 15 and 20 years of age (Murday and Slack, 1989). The phenotype was classified as severe if diagnosis could be made before 12 years of age or if advanced neoplastic disease occurred before the age of 30. Because individuals with FAP resulting from fresh mutation have not undergone regular screening the classifications of severe and average phenotype differ for this subset of patients. In this case a patient was classed as severe if they presented with symptoms during their teens or with overt cancer during their twenties, and were classed as average if presentation was not until after 30 years of age. Table 2.1. lists members of FAP kindreds and patients with fresh APC mutation that have been investigated using mutation analysis during this study. In addition to these individuals DNA samples derived from 50 normal individuals from a variety of geographic areas and ethnic origins were used as controls, and also to establish the incidence of any non-disease variants/polymorphisms detected during mutation analysis.

DNA samples from desmoid tumours and corresponding normal tissue were obtained from St. Marks Hospital, London, and from the Instituto Nazionale Tumori, Milan, Italy. Desmoids classified as sporadic were derived from patients with no colonic symptoms or family history of FAP.
Table 2.1. Patients investigated for *APC* mutation during this study.

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Individuals in bold face type are FAP patients or presymptomatic APC mutation carriers, those in normal type are unaffected family members.
2.1.5. Cell culture media.

Cell culture flasks (50ml, 200ml, 500ml) were supplied by Nunclon™. RPMI 1640 medium, non-essential amino acids, phytohaemagglutinin (PHA), and the antibiotics glutamine, penicillin, and streptomycin were supplied by Gibco. Iscoves modified DMEM medium was obtained from Imperial, while foetal calf serum was purchased from GlobePharm Ltd. Storage was at -20°C except for RPMI, Iscoves, and non-essential amino acids which were stored at 4°C.

2.1.6. Solutions and buffers.

All solutions and buffers were made as described in 2A.3. using distilled deionised water. To increase shelf life solutions were sterilised by autoclaving at 15lbs psi 121°C for 30 minutes. Unless otherwise stated storage was at room temperature (15-25°C).

2.1.7. Yeast media.

Yeast artificial chromosomes used during fluorescent in situ hybridisation experiments were derived from contig 3 described by Kinzler et al. (1991), and were provided by Professor Phillip Hedge, ICI. Casamino acids, Bacto-agar, and Bacto-yeast nitrogen base (minus amino acids) required as components of S.D. medium were supplied by Difco, while β-mercaptoethanol, ampicillin, adenine, and tyrosine were all provided by Sigma Chemical Company. Glycerol, sucrose and glucose were supplied by BDH.

Short term yeast cultures maintained for up to four months were grown from a single colony streaked onto S.D. agar in a 5cm petri dish, incubated at 30°C for 48 hours, and then stored at 4°C. For long term storage yeast strains grown in S.D. medium were mixed with sterile glycerol (30% final concentration) and stored in 1ml Nunc™ cryotubes at -70°C.
2.1.8. FISH materials and solutions.

Solutions and buffers used for fluorescent *in situ* hybridisation (FISH) were as described in 2A.5.. Nick translation reagents were supplied in kit form by Boehringer Mannheim and Gibco BRL. Sigma Chemical Company supplied the mouse anti-digoxin (MaD), rhodamine conjugated rabbit anti-mouse (RaM), and rhodamine conjugated goat anti-rabbit (GaR) used in the immunocytochemical detection of digoxigenin labelled probes. Detection of biotinylated probes required fluorescein isothiocyanate (FITC) conjugated avidin, and biotinylated anti-avidin from Vector. All components of the immunocytochemical detection were stored at -20°C. Propidium iodide and diamidophenylindole (DAPI) counterstains were supplied by Sigma Chemical Company and stored at 4°C, the anti-fade mounting medium was supplied by Vector Labs.

2.1.9. Reagents for the protein truncation test.

The *in vitro* transcription and translation (TnT) kit was supplied by Promega, as was the RNase inhibitor. The radio-labelled amino acid, $^{35}$S-methionine (cell labelling grade), was obtained from Amersham. All these reagents were stored at -70°C. Diethyl pyrocarbonate (DEPC) was supplied by BDH and stored at 4°C. Other solutions and buffers were prepared as described in section 2A.4..

2.2. Standard DNA Protocols.

2.2.1(a). DNA extraction from blood samples.

DNA was extracted from patient blood samples according to the protocol of Laird *et al.* (1991). This technique is relatively rapid requiring few centrifugation steps and avoiding the need for organic solvent extractions. The yield of DNA is said to exceed that usually obtained by phenol/chloroform extraction followed by ethanol precipitation.
Patient blood samples (approximately 5ml) collected in EDTA tubes were centrifuged at 6,000g for 5 minutes. The supernatant was then removed and the cell pellet washed in 10ml of 0.9% sodium chloride. This preceded another 5 minute centrifugation at 6,000g after which the supernatant was discarded and 0.5ml of LCL lysis buffer (2A.3(a).) was added to the cell sample. The pellet was then resuspended and incubated for 2 hours at 37°C with occasional agitation. Following this digestion step one volume of isopropanol was added to the lysate and the centrifuge tube inverted until precipitation was complete. The DNA was then recovered by lifting the precipitate from the solution using a sterile inoculating loop. The precipitate was washed in 70% ethanol at room temperature and then allowed to air dry. Finally the DNA was transferred to a micro-centrifuge tube containing an appropriate volume of 1 X TE (2A.3(c).) in which the DNA was dissolved.

2.2.1(b). DNA extraction from lymphoblastoid cell lines.

The protocol described above was also used for the extraction of DNA from lymphoblastoid cell lines. Similar volumes of reagents were used for DNA extraction from approximately 50 X 10^6 cells.

2.2.1(c). Single cell isolation.

Single cells were isolated from blood samples or from lymphoid lines. Lymphoblastoid cell samples were centrifuged at 6,000g for 5 minutes to produce a cell pellet, while blood lymphocytes were obtained by centrifugation through Ficoll-paque according to the manufacturer's protocol. The supernatant was then discarded and the pellet resuspended in 2-3ml of sterile PBS containing 10mg/ml bovine serum albumin (BSA). Approximately 50μl of this concentrated cell suspension was then pipetted onto a 5cm petri dish in a laminar flow cabinet. The use of the cabinet was restricted to single cell isolation and no PCR products or extracted DNA samples were allowed inside. Approximately ten 30μl droplets of PBS (10mg/ml BSA) were also pipetted onto the petri dish and a thin layer of mineral oil added such that all droplets were submerged and therefore protected from evaporation. To isolate single cells 0.5μl of cell suspension was transferred to a neighbouring droplet of PBS using a p2
Gilsen pipette, reserved for this purpose alone, while viewing under an inverted microscope (Olympus). This step was usually repeated five or six times to progressively dilute the concentration of cells to a level at which the isolation of a single cell became possible. Single cells isolated in approximately 2μl of PBS were pipetted into a previously unused droplet of PBS to double check that only a single cell had been sampled. Once this was confirmed they were transferred, again in a 2μl volume, into individual microcentrifuge tubes ready for cell lysis and PCR.

2.2.1(d). DNA extraction from single cells.

Isolated single cells were lysed to make their DNA accessible to PCR reagents. This was achieved by adding 5μl of single cell lysis buffer (2A.3(b).) to the isolated cell, layering with 50μl of mineral oil, and heating to 65°C for 10 minutes. Single cells treated in this way could be stored at -70°C for up to two months.

2.2.2. The polymerase chain reaction (PCR).

To provide sufficient DNA for mutation analysis and sequencing it was necessary to amplify samples using the polymerase chain reaction as described below.

2.2.2(a). Oligonucleotides.

Oligonucleotides used as primers for PCR are detailed in full in 2A.9. along with respective optimal annealing temperatures. Approximate melting temperatures were initially estimated using the following formula:

\[ Tm = 69.3 + (GC \times 0.41) - (650 / L) \]

Where ‘GC’ is the percentage nucleotides that are either guanine or cytosine, and L is the number of nucleotides in the primer.

Optimal annealing temperatures were determined by experimentation with temperatures either side of this original estimate. All oligonucleotides were supplied
2.2.2(b). Amplification reactions.

Polymerase chain reactions were set up on ice in a laminar flow cabinet using dedicated Gilsen pipettes and sterile pipette tips. The final concentration or volume of reagents were as follows 25pM of each primer, 0.2mM of each 2’-deoxynucleoside 5’-triphosphate (dATP, dGTP, dCTP, dTTP), 5μl of 10 X enzyme reaction buffer (HT Biotechnologies), and 0.5 units of Taq DNA polymerase (HT Biotechnologies), in a total volume of 50μl. Reaction mixtures were added to 250ng-500μg of genomic DNA in a microcentrifuge tube and were overlayed with 50μl of mineral oil (Sigma). Thermal cycling was carried out using an Omnigene PCR machine (Hybaid).

Amplification of DNA fragments from APC, hMSH2, and microsatellite loci for SSCP analysis was achieved using the following set of conditions:

1- 4.50 minutes at 94°C (denaturation)
2- 0.50 minutes at 94°C (denaturation)
3- 0.75 minutes at the appropriate temperature* (annealing)
4- 0.75 minutes at 72°C (elongation)
5- 10.0 minutes at 72°C (elongation)

*See section 2A.9.

Steps one and five only took place once while steps 2-4 were repeated 35 times. Amplification of large APC fragments for PTT involved essentially the same programme the only difference being an increase in the duration of step four to 1.5 minutes.
2.2.2(c). PCR from single cells.

The single cell PCR protocol was similar to standard PCR, however precautions against contamination were considerably more rigorous. These included setting up the PCR in a laminar downflow cabinet in a room with restricted access, the wearing of a clean gown and gloves, filtration of all reagents using 0.22μm filters (Millipore), and the use of filter pipette tips to guard against aerosols. The incidence of contamination was assessed regularly using numerous control blanks (>50), containing PCR reaction mixture but no DNA. Any reagents shown to cause contamination were immediately discarded, even if amplification was only detected in a small proportion of blanks.

2.2.2(d). Primer extension preamplification (PEP).

As well as amplification of specific loci from single cells whole genome amplification (WGA) was also employed. This generated sufficient DNA from a single cell to allow multiple subsequent PCRs. Thus it was possible to amplify two APC fragments, one containing a mutation and one containing a polymorphism without performing multiplex-PCR. The WGA protocol used was primer extension preamplification (PEP). 60μl reaction mixtures contained a lysed cell in 5μl of single cell lysis buffer, 5μl of neutralising buffer (2A.8(d).), 6μl of potassium free PCR buffer (2A.8(b).), 15 base oligonucleotide primers of random sequence at a concentration of 33.3mM, 0.1mM of each deoxynucleotide (dATP, dGTP, dCTP, dTTP), and 5 units of Taq polymerase. Thermal cycling involved 50 cycles of the following: a one minute denaturation step at 92°C; two minutes annealing at 37°C; a temperature ramp of one degree every 10 seconds to 55°C; and finally the temperature was maintained at 55°C for four minutes. PEP products were stored at 4°C for up to one month.

2.2.2(e). Nesting.

Amplification of the APC gene in single cells followed a nested PCR strategy to improve the specificity of the reaction and to reduce the risk of contamination
caused by previously amplified DNA fragments. For the outer amplification 45μl of reaction mixture was added to either single cells suspended in 5μl of single cell lysis buffer or to 5μl of PEP product. The reaction mixture for single cells consisted of 5μl of neutralising buffer, 5μl of 10 X potassium free buffer, 1mM of each deoxynucleoside triphosphate (dATP, dGTP, dCTP, dTTP), 4μM of both forward and reverse primers (2A.9(d).), and 1 unit of Taq polymerase, in a final volume of 45μl.

For amplification of PEP products neutralising buffer and potassium free PCR buffer were omitted in favour of 5μl of SC-PCR buffer (2A.8(C).). Mixtures were overlaid with mineral oil and amplification performed according to the following cycling parameters:

<table>
<thead>
<tr>
<th>Codon 1678 polymorphism detection</th>
<th>Codon 764 mutation detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer amplification</td>
<td>Outer amplification</td>
</tr>
<tr>
<td>96°C    0.75 min</td>
<td>96°C    0.75 min</td>
</tr>
<tr>
<td>61°C    0.75 min X 28</td>
<td>55°C    0.75 min X 28</td>
</tr>
<tr>
<td>72°C    1.50 min</td>
<td>72°C    1.50 min</td>
</tr>
</tbody>
</table>

After completion of the outer amplification 2μl of PCR product was transferred to a fresh microcentrifuge tube and subjected to further amplification. This involved the addition of 48μl of reaction mixture containing 1 X SC-PCR buffer, 1mM of each deoxynucleoside, 4μM forward and reverse primers situated internal to those used for the first reaction, 1.5mM MgCl₂, and 5 units of Taq polymerase. The thermal cycling conditions are listed below:
APC codon 1678 polymorphism detection

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>96°C</td>
<td>0.75 min</td>
<td>Outer amplification</td>
</tr>
<tr>
<td>56°C</td>
<td>0.75 min</td>
<td>X 32</td>
</tr>
<tr>
<td>72°C</td>
<td>1.50 min</td>
<td></td>
</tr>
</tbody>
</table>

APC codon 764 mutation detection

Outer amplification

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>96°C</td>
<td>0.75 min</td>
<td>Outer amplification</td>
</tr>
<tr>
<td>65°C</td>
<td>0.75 min</td>
<td>X 32</td>
</tr>
<tr>
<td>72°C</td>
<td>1.50 min</td>
<td></td>
</tr>
</tbody>
</table>

2.2.3. Agarose gel electrophoresis

Electrophoresis using 2% agarose gels was used to gauge the efficiency and specificity of amplification products after PCR and also for the analysis of products digested by restriction enzymes. Gels were prepared by heating 1g of agarose in 50ml of 1X TBE until all the agarose had been dissolved. The molten agarose was then poured into minigel moulds (8mm x 100mm, Cambridge Electrophoresis) with 16-well gel-slot-formers and left to set at room temperature. Gels were then immersed in 50ml of 1X TBE containing 1μg/ml ethidium bromide. Finally 5-10μl of each sample was mixed with a one tenth volume of loading buffer and loaded into well-slots. To allow estimation of the size of DNA fragments after restriction digestion DNA size standards were loaded along side digestion products. Depending on the expected size of digested fragments either 100bp or 1kb ladders were used, diluted one in ten in sterile water and mixed with a 1/10th volume of loading buffer. Electrophoresis was performed at 50V for 30 minutes after which gels were viewed under ultra violet trans-illumination.

2.2.4. Restriction digestion.

Digestion of PCR amplified DNA using restriction endonucleases was performed under a variety of conditions as detailed below. All reactions involved addition of 5 units of enzyme to a mixture containing 2μl of 10X reaction buffer (supplied by the manufacturer), 5μl of PCR product, and 12μl of water. Digestions were incubated for 3 hours at the following optimal temperatures, recommended by the manufacturer: Msp I, Mae I and Acc I 37°C, Apo I 50°C, Taq I 65°C.
2.2.5(a). Single strand conformation polymorphism (SSCP)

Both SSCP patterns and heteroduplex bands were visualised using the Phastsystem™ (Pharmacia) a rapid automated system for protein and DNA analysis which utilises pre-formed polyacrylamide gels (PhastGels™). 1µl of each PCR amplified sample was denatured by heating at 99°C for 10 minutes in the presence of 1.5µl of formamide. Denatured samples were immediately placed on ice and then loaded on to non-denaturing 20% polyacrylamide gels using sample applicators supplied by Pharmacia. These facilitate the transfer of approximately 0.3µl of sample to the gel surface. Prior to loading samples gels were usually pre-run according to the following conditions: 400V, 10mA, 2W for 10Vh at 4°C, 10°C, or 15°C. However in the case of APC fragments used for single cell analysis heteroduplexes could only be visualised if the pre-run was extended to 50Vh. PhastGels™ employ solid buffer strips situated at either end of the gel rather than total immersion in buffer. Each buffer strip is in contact with the surface of the gel and one of the two electrodes. In all cases native buffer strips (Pharmacia) were used for SSCP and heteroduplex analysis. The electrophoresis programme used was: 400V, 5mA, 2W, for 2Vh at 4°C, 10°C, or 15°C usually followed by 400V, 10mA, 2W for 250Vh at the same temperature, although the smaller APC fragments generated during single cell analysis only required electrophoresis for 116Vh.

2.2.5(b). Silver staining of PhastGels.

DNA was detected by silver staining carried out automatically in the development chamber of the Phastsystem™. The staining process involved nine different solutions all of which were made fresh on the day of use. Seventy five millilitres of solution was required for each of the 16 steps that constituted the procedure.
Table 2.2. Silver staining of polyacrylamide gels using the Phastsystem.

<table>
<thead>
<tr>
<th>Step number</th>
<th>Solution</th>
<th>Temperature (°C)</th>
<th>Time (minutes)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20% trichloroacetic acid</td>
<td>20</td>
<td>5</td>
<td>Fixitive</td>
</tr>
<tr>
<td>2</td>
<td>50% ethanol, 10% glacial acetic acid</td>
<td>50</td>
<td>2</td>
<td>Wash</td>
</tr>
<tr>
<td>3</td>
<td>10% ethanol, 5% glacial acetic acid</td>
<td>50</td>
<td>2</td>
<td>Wash</td>
</tr>
<tr>
<td>4</td>
<td>10% ethanol, 5% glacial acetic acid</td>
<td>50</td>
<td>4</td>
<td>Wash</td>
</tr>
<tr>
<td>5</td>
<td>5% gluteraldehyde</td>
<td>50</td>
<td>6</td>
<td>Sensitisation</td>
</tr>
<tr>
<td>6</td>
<td>10% ethanol, 5% glacial acetic acid</td>
<td>50</td>
<td>3</td>
<td>Wash</td>
</tr>
<tr>
<td>7</td>
<td>10% ethanol, 5% glacial acetic acid</td>
<td>50</td>
<td>5</td>
<td>Wash</td>
</tr>
<tr>
<td>8</td>
<td>distilled water</td>
<td>50</td>
<td>2</td>
<td>Wash</td>
</tr>
<tr>
<td>9</td>
<td>distilled water</td>
<td>50</td>
<td>2</td>
<td>Wash</td>
</tr>
<tr>
<td>10</td>
<td>0.4% silver nitrate</td>
<td>40</td>
<td>10</td>
<td>Staining</td>
</tr>
<tr>
<td>11</td>
<td>distilled water</td>
<td>30</td>
<td>0.5</td>
<td>Wash</td>
</tr>
<tr>
<td>12</td>
<td>distilled water</td>
<td>30</td>
<td>0.5</td>
<td>Wash</td>
</tr>
<tr>
<td>13</td>
<td>2.5% sodium carbonate, 0.02% formaldehyde</td>
<td>30</td>
<td>1</td>
<td>Developer</td>
</tr>
<tr>
<td>14</td>
<td>2.5% sodium carbonate, 0.02% formaldehyde</td>
<td>30</td>
<td>10</td>
<td>Developer</td>
</tr>
<tr>
<td>15</td>
<td>3.7% tris-HCl, 2.5% sodium thiosulphate</td>
<td>30</td>
<td>2</td>
<td>Background reduction</td>
</tr>
<tr>
<td>16</td>
<td>10% glycerol</td>
<td>50</td>
<td>5</td>
<td>Preservative</td>
</tr>
</tbody>
</table>

Ethanol, acetic acid, and other chemicals were of AnalR grade. All solutions used were made with distilled deionised water.

Gels stained according to this protocol slowly faded over a period of 6-12 months and consequently it was advisable to photograph significant results soon after completion of the experiment.
2.2.6(a). Protein truncation test (PTT).

To generate polypeptides from a defined region of the APC gene it was first necessary to amplify the region using PCR. The forward primer used for this purpose was as described by Van der Luijt et al. (1994) and included T7 phage promoter and Kozak consensus sequences situated in the same transcriptional reading frame as an initiation codon and 26 bp of APC coding sequence. The reverse primer was 15J (antisense) as described by Groden et al. (1991), thus allowing amplification of APC codons 1034-1706. APC gene fragments generated in this way were added to an in vitro transcription and translation (TnT) kit (Promega). In vitro transcription and translation reactions used pipette tips and microcentrifuge tubes that had been soaked in sterile deionised water containing 0.1% diethyl pyrocarbonate (DEPC) at 37°C for at least 12 hours to inactivate RNase. This DEPC treatment was followed by several rinses in sterile deionised water and autoclaving at 15 lbs psi 121°C for 30 minutes. Reactions were set up on ice behind a perspex shield to block β-radiation, and in a safety cabinet. The reaction mixture contained 2µl of water (0.1% DEPC), 0.5µl of TnT reaction buffer, 0.25µl of RNase inhibitor, 0.25µl of amino acid mixture lacking methionine, 6.5µl of rabbit reticulocyte lysate (thawed rapidly from -70°C), 0.5µl of 35S-methionine (370MBq/ml), and 0.25µl of T7 RNA polymerase. With the exception of the radioactive methionine and the DEPC treated water all these ingredients were components of the TnT kit (Promega). The resulting mixture was vortexed and then 10µl removed and added to 2µl of TnT PCR product in a fresh microcentrifuge tube. This preceded a 90 minute incubation at 30°C, after which reactions were stored at -20°C or used immediately. TnT products were assessed on 10%-20% polyacrylamide gradient gels as describe below.

2.2.6(b). Polyacrylamide gradient gel.

Three acrylamide gel mixtures were made up as indicated in the following table:
Table 2.3. Composition of polyacrylamide gels used for PTT.

<table>
<thead>
<tr>
<th></th>
<th>10% acrylamide mix</th>
<th>20% acrylamide mix</th>
<th>Stacking gel mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>distilled deionised water</td>
<td>6.8ml</td>
<td>-</td>
<td>7.5ml</td>
</tr>
<tr>
<td>glycerol</td>
<td>0.9ml</td>
<td>2.7ml</td>
<td>-</td>
</tr>
<tr>
<td>30% acrylamide/bis 37.5:1</td>
<td>5.6ml</td>
<td>11.2ml</td>
<td>1.3ml</td>
</tr>
<tr>
<td>1.87M Tris pH8.8</td>
<td>3.3ml</td>
<td>3.3ml</td>
<td>-</td>
</tr>
<tr>
<td>1M Tris pH6.8</td>
<td>-</td>
<td>-</td>
<td>1.3ml</td>
</tr>
<tr>
<td>10% sodium dodecyl sulphate (SDS)</td>
<td>200μl</td>
<td>200μl</td>
<td>100μl</td>
</tr>
<tr>
<td>NNN'N' tetramethylene diamide (TEMED)</td>
<td>15μl</td>
<td>15μl</td>
<td>10μl</td>
</tr>
<tr>
<td>25% ammonium persulphate (AMPS)</td>
<td>15μl</td>
<td>5μl</td>
<td>40μl</td>
</tr>
</tbody>
</table>

The 10% and 20% polyacrylamide mixtures were made up first. NNN'N' tetramethylene diamide (TEMED) and ammonium persulphate (AMPS), responsible for initiating and catalysing polymerisation, were the last components added. A gradient gel was produced by controlled mixing of the 10% and 20% solutions, using a Masterflex® Economy Drive pump. Gel mixture of progressively lower acrylamide concentration was pumped between two glass electrophoresis plates (Hoefer, 160mm x 195mm) that had previously been cleaned with distilled water and absolute ethanol. The plates were held in an upright position and separated by three plastic spacers (1.5mm thickness), one at either side and one at the bottom of the apparatus. These served to maintain even separation of the plates and to prevent gel mixture from escaping. The gradient gel was allowed to set for 30 minutes before the stacking gel mixture was made and poured on top of the gradient gel. An electrophoresis comb was then inserted and remained in place until the stacking gel had set (approximately 30 minutes), thus creating wells into which samples could be applied. Next the comb and the spacer at the bottom of the gel were removed and the gel immersed in 1000ml of SDS running buffer (2A.4(b)).

Once the gel had been prepared 3μl of each sample was mixed with 12μl of sample buffer (2A.4(d)) in a microcentrifuge tube. The mixture was heated to 100°C for 3 minutes and then loaded into the wells previously formed by the comb. A
protein size standard (Rainbow Ladder™, Amersham) was run along side PTT products to allow an estimation of molecular mass. For this purpose 3μl of water was added to 7μl of size standard and 10μl of SDS sample buffer. Electrophoresis then proceeded at 200V, 100mA for 3 hours.

After electrophoresis had been completed the stacking gel was removed and the gradient gel fixed in 30% methanol / 30% acetic acid for 30 minutes, this preceded 12 hours of soaking in 10% methanol / 5% glycerol. Finally the gel was dried for 2 hours at 80°C using a Biorad gel dryer. Autoradiography involved exposure of Kodak Biomax MR film placed in contact with dried gels for a period of 24 to 72 hours.

2.2.7(a). Preparation of PCR amplified template for DNA sequencing.

Prior to DNA sequencing it was necessary to eliminate excess primers and nucleotides from PCR mixtures. This was achieved by digestion using exonuclease I and alkaline phosphatase. 10 units of exonuclease I and 2 units of alkaline phosphatase were added to 5μl of PCR product and the resulting mixture incubated at 37°C for 15 minutes. A 15 minute incubation at 80°C was employed to inactivate both enzymes.

2.2.7(b). Sequencing reactions.

Sequencing of DNA fragments, treated as described above, followed a protocol based on the chain termination method described by Sanger et al. (1977). A mixture consisting of 1μl of treated PCR product, 0.5-2.5pmol of primer, 2μl of Thermo Sequenase reaction buffer (Amersham), and 8 units of Thermo Sequenase polymerase in a final volume of 20μl was prepared on ice. 4.5μl of this mixture was then dispensed into each of four microcentrifuge tubes labelled G, A, T, or C. To this mixture 2μl of dGTP termination mix (7.5mM dATP, dCTP, dGTP, dTTP) was added followed by 0.5μl of the appropriate ^31P labelled dideoxynucleoside- either ddGTP, ddATP, ddCTP, or ddTTP. The mixtures were agitated, pulse centrifuged for 5 seconds, and then overlaid with 50μl of mineral oil, before being subjected to 55 cycles of the following: 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute.
After reactions had been completed 4μl of stop solution (Amersham) was added and the tubes stored at -20°C for up to one week.

2.2.7(c). Polyacrylamide gel electrophoresis for sequencing.

Sequencing reactions were analysed after electrophoresis on 21 x 50cm polyacrylamide gels of 0.4mm thickness using an apparatus supplied by Bio-rad. Glass sequencing plates were thoroughly cleaned with deionised distilled water and absolute ethanol. One of the two plates (the top plate) was then treated with Gel-Slick™ (AT Biochem) according to the manufacturers instructions, this was necessary to prevent the gel sticking to the top plate. The polyacrylamide gel mixture was 50ml in volume and consisted of 7M urea, 6% acrylamide, 1 X TBE. To initiate polymerisation 60μl of TEMED (NNN\textsubscript{4}tetramethylenediamide) and 60μl of 25% w/v ammonium persulphate (no more than two weeks old) were added. The gel mixture was then syringed between the two plates and given at least one hour to polymerise at room temperature. To provide an even, straight edge, at the top of the gel the reverse side of a 48 well sharks-tooth comb, 0.4mm thick, (Bio-rad) was inserted while the gel mix was still liquid. After polymerisation was complete the comb was reversed and the teeth inserted into the gel to a depth of 1mm such that wells, into which sequencing products could be loaded, were formed. After immersion in 1 X TBE running buffer the polyacrylamide gel was pre-run for 30-60 minutes at 45 W, allowing the gel to reach a temperature of 45-50°C optimal for electrophoresis. Following the pre-run samples were denatured at 80°C for 3 minutes and 2.5μl of each sample was loaded between the teeth of the sharks-tooth comb. Electrophoresis then proceeded at 2,000 V, 45 W, for 90 minutes after which samples were denatured and loaded for a second time. Gels were run for a further 2.5 hours and then dried as described below.
2.2.8. Drying of polyacrylamide gels and radioactive signal detection.

After electrophoresis was complete the apparatus was disassembled and the sequencing plates carefully separated. The gel, still remaining affixed to the lower glass plate, was blotted onto 3mm filter paper (Whatman), and the exposed face of the gel was evenly covered with Saran film (Dow Chemical Company). The gel was then transferred to an oven at 80°C until dry (45-90 minutes). Autoradiographic detection of DNA fragments separated by electrophoresis involved exposure of Kodak X-OmatR film for 12-72 hours at room temperature.

2.3. Tissue culture and cytogenetic preparation.

2.3.1. Blood culture.

To obtain extended chromosomes for analysis by FISH blood was collected in heparinized tubes and then cultured as follows: 1ml of whole blood was added to a 50ml culture flask containing 17ml of Iscoves modified Dulbecco’s medium, 2ml GPS (glutamine 200mM, penicillin 300mg/ml, and streptomycin 500mg/ml), 10% foetal calf serum (heat inactivated), and 200μl of the mitotic stimulant phytohaemaglutinin (PHA). Culture flasks were then placed in an incubator at 37°C for least 72 hours, with occasional agitation. After this time 100μl of 30mg/ml thymidine was added to block the cell cycle in early prophase. The block was released 18 hours later by the addition of 100μl of 0.227mg/ml 2-deoxycytidine, by this time there should have been an accumulation of cells at the same stage of early mitosis. The cells which had been blocked at prophase reach metaphase approximately 4 hours and 40 minutes later. After this amount of time had elapsed 100μl (10μg/ml) colcemid was added. Colcemid disrupts spindle formation preventing cells from continuing along the cell cycle and dividing, the result is a high proportion of cells trapped in metaphase. Harvesting usually commenced about 20 minutes later.
2.3.2. Culture of lymphoid cell lines.

Lymphoblastoid cells in foetal calf serum (FCS) plus 5% dimethyl sulfoxide (DMSO) were stored in cryotubes under liquid nitrogen (-196°C). When required the cell samples were thawed at 37°C added to 15ml of RPMI medium in a 25ml tissue culture flask and placed in a 5% CO₂ incubator at 37°C. It was necessary to renew the medium every 2-3 days by carefully decanting off approximately 10ml of starved medium and adding a similar volume of fresh medium. For the analysis of cytogenetically visible deletions chromosome preparations were required. These were obtained using essentially the same method as described for blood culture, but with volumes adjusted such that concentrations of thymidine, 2-deoxycytidine, and colcemid remain unchanged.

For analysis of interphase nuclei cells in G₀/G₁ of the cell cycle were required. To provide these cells LCL cultures were starved for 14 days before harvest, rather than being fed every 2-3 days.

2.3.3. Harvest of cultured cells.

To collect cultured cells for cytogenetic analysis cell suspensions were spun for 5 minutes at 6,000g. The supernatant was then removed leaving only 1ml of medium in which the pellet was resuspended. Next half strength KCl (0.075 M) at 37°C was added, dropwise initially (approximately 100µl volumes), then filling the tube and mixing carefully. Hypotonic swelling was allowed to continue for 20 minutes at room temperature after which the cells were spun down and the supernatant removed as before. Once again the cells were resuspended in 1ml of residual fluid. Following resuspension 5-10ml of fresh fixative mixture (3 parts methanol, 1 part glacial acetic acid) was added dropwise with constant agitation. The fixation step was repeated another three or four times, until the cell pellet lost all brown coloration. Cell suspensions were stored in fixative at -20°C until required.
2.3.4. Preparation of slides for cytogenetic analysis.

Before making slides fixed cell suspensions were centrifuged at 6,000g for 5 minutes. The supernatant was removed and the cell pellet resuspended in fresh fixative leaving the density of cells at an appropriate level. Slides to be used for in situ hybridization were cleaned in methanol/0.5% concentrated HCl, and dried using a lint free cloth immediately before dropping approximately 100μl of cell suspension onto the slide. Next the slides were dried and then flooded with fixative. After the slides had been dried a second time they were flooded with 70% acetic acid and dried once more. This step strips away cytoplasm which can impair the penetration of the probe used for FISH. Finally the slides were dehydrated by three successive 5 minute washes in 70% ethanol, 90% ethanol, and absolute ethanol. They were then allowed to air dry before examination under phase contrast microscopy (Polyvar). Slides to be used for chromosomal analysis were considered suitable if they were found to have a sufficient number of well spread metaphases, and no cytoplasm. Slides were either used immediately or stored at -20°C for up to 6 months, although in some cases they were aged at room temperature for one week to improve chromosomal morphology.

2.4. Preparation of probes for FISH.

2.4.1. Culture of yeast.

To obtain sufficient yeast artificial chromosome (YAC) DNA for FISH single yeast colonies grown on short term agar plates were transferred into 2ml of S.D. medium (2A.6(a)) contained in Sterilin tubes. Alternatively inoculation was achieved using 100μl of cell suspension from glycerol stocks stored at -70°C. Cultures were incubated at 30°C with constant agitation for 12-24 hours. This was followed by the addition of a further 8ml of S.D. medium and culturing for 12-24 hours more.
2.4.2. Extraction of yeast (YAC) DNA.

Cells grown as described above were pelleted by centrifugation at 3,000g for 10 minutes and the supernatant discarded. The pellet was resuspended in 0.5ml of yeast resuspension buffer (YRB) (2A.6(d).) to which 25 units of Lyticase (1μl of 25U/μl stock solution) had been added. The mixture was incubated at 37°C until 80-90% of cells had formed spheroblasts (at least 30 minutes). The percentage of spheroblasts was determined by diluting 1μl of the mixture in 20μl of sterile water and viewing on a microscope slide under phase contrast microscopy (Reichert Polyvar microscope). Once the correct proportion of spheroblasts was obtained the mixture was centrifuged at 6,000g for one minute, the supernatant decanted off, and the cell pellet resuspended in 0.5ml of 50mM Tris-HCl/20mM EDTA, pH 7.4, and 50μl of 10% sodium dodecyl sulphate (SDS). The mixture was then transferred to a 1.5ml microcentrifuge tube and incubated at 65°C for 30 minutes. Next 200μl of 5M potassium acetate was added, and after one hour incubation on ice the mixture was centrifuged at 10,000g for 5 minutes. The supernatant was transferred to a fresh 1.5ml microcentrifuge tube and an equal volume of isopropanol was mixed in. DNA was precipitated at room temperature for 5 minutes after which centrifugation at 10,000g was carried out for 10 seconds. The supernatant was removed and the pellet air dried before being dissolved in 300μl of 1 X TE, and incubated at 37°C for one hour with 2μl of 10mg/ml pancreatic RNase. 30μl of 3M sodium acetate was added next, followed by an equal volume of phenol. Vigorous shaking or vortexing was employed to ensure complete mixing of the two layers which were then separated again by centrifugation at 3,000g for 5 minutes. The aqueous (upper) layer was then removed and subjected to a second phenol extraction followed by a single extraction with chloroform, all volumes and centrifugation steps were identical. Finally DNA was precipitated by the addition of two and a half volumes of absolute ethanol and incubation at -70°C for 30 minutes. DNA was recovered by centrifugation at 10,000g for 30 minutes, and was air dried, resuspended in 100μl of 1 X TE, and then stored at -20°C until required.
2.4.3. Bacterial culture.

Culture of bacteria took place in 'cosmid broth' (2A.7(a).) at 37°C over a period of 24 hours. The precise volume of broth inoculated ranged from 10ml to 250ml, depending on how much cosmid DNA was required. To this was added 0.2mg of ampicillin per millilitre of broth.

2.4.4. Extraction of bacterial (cosmid) DNA.

DNA from bacteriophage lambda vectors was obtained after bacterial culture as described above. 10ml of culture was centrifuged at 5,000g for 10 minutes and the supernatant discarded. The cell pellet was resuspended in 200μl of bacterial lysis buffer (2A.7(b).) and the mixture incubated at room temperature for 10 minutes. This preceded two 5 minute incubations on ice, the first after the addition of 400μl of AD buffer (2A.7(c).) and the second after 300μl of 3M sodium acetate (pH 5.2) had been added. Each step required thorough mixing. Next the mixture was transferred to a 1.5ml microcentrifuge tube and spun for 5 minutes at 10,000g. The clear supernatant was then collected into a new microcentrifuge tube containing 600μl of isopropanol and the mixture incubated at -70°C for 10 minutes or at -20°C for approximately 16 hours. Precipitated DNA was recovered by centrifugation at 20,000g for 5 minutes, after which the supernatant was removed and the pellet air dried. Finally the DNA pellet was dissolved in 200μl of 0.3M sodium acetate (pH 6.5).

A number of measurers were employed to purify the DNA sample. Firstly an equal volume of equilibrated phenol (Gibco BRL) was added and the mixture vortexed. Organic and aqueous layers were separated by centrifugation at 5,000g for 5 minutes, after which the aqueous layer was transferred into a fresh tube. DNA precipitation was then accomplished by addition of 200μl of isopropanol and incubation at -70°C for 10 minutes. The precipitate produced was spun at 20,000g for 5 minutes and the resultant supernatant discarded. The pellet was dried and then resuspended in 200μl of TE (2A.3(c).). Next RNA was removed from the solution by incubation for 15 minutes at 37°C after the addition of 10μl of 1mg/ml RNase. The DNA solution was further purified by phenol-chloroform extraction. For this purpose an equal volume of phenol was added to the solution and the two phases mixed.
together vigorously. Again centrifugation at 5,000g for 5 minutes caused formation of
discrete organic (phenol) and aqueous layers. The DNA containing aqueous layer was
collected into a fresh microcentrifuge tube and the extraction repeated this time using
an equal volume of chloroform rather than phenol. Finally DNA was precipitated
once more, this was achieved by the addition of a 1/20\textsuperscript{th} volume of 4M NaCl and 2.5
volumes of absolute ethanol, followed by a 10 minute incubation at -70°C. The
precipitated DNA was recovered by centrifugation, dried, and resuspended in 50\textmu l of
TE. Storage was at 4°C for up to 6 months.

The above method could be scaled up to accommodate any volume of bacterial
culture up to 250ml.

2.4.5. Fluorometry.

The concentration of extracted probe DNA was measured using a Hoefer
Scientific Instruments TKO 100 fluorometer according to the manufacturers
instructions.

2.4.6. Labelling of DNA probes.

DNA probes for fluorescence \textit{in situ} hybridisation (FISH) were labelled by
nick translation using kits obtained from Gibco BRL (biotin labelling) or Boehringer
Mannheim (digoxigenin labelling). Typically 1\mu g of DNA was added to components
of the kit and labelled during a 1 hour incubation at 16°C. The labelled probe was
then eluted through a sephadex column (Nick\textsuperscript{TM}- Pharmacia), equilibrated with 1 X
TNE (2A.5(k).), to remove unincorporated nucleotides. Probes were collected in a
final volume of 400\textmu l and stored at 4°C.

2.4.7. Preparation of probes for FISH.

To prevent repetitive sequences in the YAC and cosmid probes from
hybridising during FISH Cot-1 DNA (50 x probe concentration) was added to the
purified probe (usually 200ng). 2\mu l of 10mg/ml salmon sperm DNA and one tenth
volume 3M sodium acetate were also added to the probe mixture. Finally two and a
half volumes of absolute ethanol (97.7-100%) were added and the mixture incubated at -70°C for 1 hour to precipitate the DNA. Next the mixture was subjected to 30 minutes centrifugation at 10,000g and the supernatant discarded. Finally the DNA pellet was freeze dried for 20 minutes before being resuspended in 10μl of hybridisation mixture (2A.5(c)). In the case of dual FISH two different YAC probes were prepared as described above and both were resuspended in the same 10μl of hybridisation mixture.

2.5. Fluorescent *in situ* hybridisation (FISH).

2.5.1. Prehybridisation treatment of probes for FISH.

Biotin and/or digoxigenin labelled probes held in hybridisation mixture were denatured at 75°C for 5 minutes and then allowed to cool to 37°C. Probes were maintained at this temperature for at least 30 minutes, to allow annealing between repetitive sequences within the probe and the unlabelled competitor DNA, before being applied to denatured chromosome spreads.

2.5.2. Prehybridisation treatment of metaphase chromosomes and interphase nuclei.

Unless otherwise stated slides were always washed and treated in 50ml coplin jars with solutions of similar volume. Slides produced as described above were washed in PBS for 5 minutes at room temperature, and then dehydrated through an alcohol series (70%, 90%, 100% ethanol for 5 minutes each). Next they were removed from the coplin jar and allowed to dry. Endogenous RNA, which may impair hybridisation of the probe, was removed by treatment with RNase, a 10mg/ml RNaseA stock was diluted 1 in 100 with 2 X SSC and 100μl was added to slides. Coverslips were applied and slides incubated for 1 hour at 37°C in a humidified chamber. After removal of the coverslips slides were transferred back into a coplin jar and the RNase was removed with two washes in 2 X SSC at room temperature, each
lasting 5 minutes. Slides were then washed in proteinase K buffer (2A.5(g.) at 37°C for 5 minutes, before a 7 minute treatment again at 37°C with proteinase K (50 ng/ml in proteinase K buffer). Following immersion in magnesium chloride buffer (2A.5(d).) for 30 seconds the slides were fixed with paraformaldehyde buffer (2A.5(e).) for 10 minutes at room temperature. After this step the slides were washed in PBS, sent through an alcohol series as before, and then removed from the coplin jar and left to dry.

Denaturation of the slides was achieved by applying 100μl of 70% deionised formamide/2 X SSC under a coverslip and heating the slides in an oven at 75°C for 5 minutes. Immediately after denaturation the coverslips were removed and the slides transferred to a coplin jar that had been stored in a -70°C freezer. The coplin jar was then filled with 70% ethanol chilled to -20°C, completely submerging the slides. This preceded passage through another alcohol series (70%, 90%, 100% ethanol, 5 minutes each) and drying. Finally the probe, prepared and denatured as previously described (section 2.5.1), was added to the slides under a coverslip, and the coverslip sealed with rubber solution. Hybridisation of the probe proceeded over 24 hours, during which the slides were kept in a humidified chamber at 37°C.

2.5.3. Single colour FISH: post hybridisation washing and signal detection.

All single colour FISH experiments were carried out using biotinylated probes according to the following protocol:

After hybridisation the coverslips were gently removed and the slides were given three 5 minute washes in 50% formamide/2 X SSC at 45°C. These were followed by another three washes this time in 2 X SSC at the same temperature. Higher stringencies can be achieved at higher temperatures and at higher formamide concentrations, but were unnecessary during this study. Next the slides were placed in SSCT (2A.5(I).) for 5 minutes before being incubated at room temperature for 20 minutes in SSCM (2A.5(h).) to reduce background fluorescence. Another 20 minute incubation at room temperature this time in 100μl of SSCM containing 0.5μl of avidin-fluorescein isothiocyanate (avidin-FITC) under a coverslip followed next. Avidin and biotin will spontaneously form strong noncovalent bonds, thus the probe is indirectly detected by the fluorochrome FITC that is conjugated to avidin.
Unbound avidin-FITC was washed away with three 5 minute SSCT washes conducted at room temperature. The signal provided by the bound avidin-FITC was then amplified by a 2 step procedure:

First 100μl of SSCM containing 1μl of biotinylated anti-avidin antibody was applied to each slide under a coverslip. Slides were then incubated for 20 minutes at room temperature allowing the biotinylated anti-avidin to associate with the bound avidin-FITC. Surplus antibody was removed with three 5 minute washes in SSCT.

The second step involved another 20 minute incubation in 100μl of SSCM containing 0.5μl avidin-FITC, again this was performed at room temperature and under a coverslip. This was followed by one 5 minute wash in SSCT and two washes in PBS all at room temperature.

If desired signals could be further amplified by repetition of these two steps, although this was rarely necessary. The slides were dehydrated by 5 minute washes in 70%, 90%, and 100% ethanol, and air dried. Finally slides were mounted in anti-fade medium II (2A.5(b)) containing propidium iodide and diamidophenylindole (DAPI) to counterstain the chromosomes and nuclei, and to give an R-banding pattern.

2.5.4. Dual Colour FISH: post hybridisation washing and signal detection.

Following simultaneous hybridisation of biotinylated and digoxigenin labelled probes, post hybridisation washes identical to those used in single colour FISH were undertaken. However after the initial 20 minute incubation in SSCM the probe detection steps were different and proceeded as follows:

Firstly 100μl of TNB (2A.5(m)) containing 0.5μl of avidin-FITC and 0.1μl of anti-digoxigenin antibody (produced in mice) was applied under a coverslip and the slides incubated for 20 minutes at 37°C in a humidity chamber. This preceded the removal of coverslips and three 5 minute washes in TNT (2A.5(l)), at room temperature. The next step involved a further 20 minute incubation at 37°C, again in a humidity chamber. On this occasion 1μl of biotinylated anti-avidin and 0.1μl of rabbit anti-mouse antibody conjugated with tetramethylrhodamine isothiocyanate (TRITC) were contained in 100μl of TNB applied to each slide. Again this was followed by three 5 minute washes in TNT. The final detection step involved another incubation at 37°C, in a humidity chamber. During the incubation slides were
exposed to 100μl of TNB with 0.1μl of TRITC labelled goat anti-rabbit and 0.5μl of avidin-FITC. Components of this detection step that had not associated with the probe after 20 minutes were removed with one 5 minute wash in TNT and two 5 minute washes in PBS, all conducted at room temperature. Finally slides were mounted in the same way as for single colour FISH, the only difference being the exclusion of propidium iodide from the mountant medium (anti-fade medium I).

2.5.5. Visualisation of nuclei, chromosomes, and signals.

The fluorescence microscope used was a Nikon optiphot with an Omega dual band-pass filter for simultaneous FITC and TRITC detection and a Nikon UV 2a filter for detection of DAPI fluorescence. An MRC 600 confocal laser microscope (Biorad) was used to capture images, while signal separation in cell nuclei was calculated using MRC 500/600 software.
## Appendix to Materials and Methods

### 2A.1. DNA probes used for FISH.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Probe</th>
<th>Location</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>YAC</td>
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<td>5q21-22</td>
<td>APC</td>
</tr>
<tr>
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</tr>
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<tr>
<td>COSMID</td>
<td>ym64</td>
<td>5q21-22</td>
<td>3' of APC</td>
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<td>3' APC</td>
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<td>COSMID</td>
<td>ym75</td>
<td>5q21-22</td>
<td>central APC</td>
</tr>
</tbody>
</table>

Reference:
- Kinzler et al., 1991
- Mandl et al., 1996
- HGPM
- Dr M. Fox, UCL
2A.2. Cell culture and media.

2A.2(a). Iscoves medium.

For culture of 1ml of blood: 17ml of Iscoves medium; 2ml GPS (glutamine, penicillin, and streptomycin); 2ml fetal calf serum; 200μl of phytohaemaglutinin (PHA).

2A.2(b). RPMI medium.

For 100ml: 76ml sterile distilled water; 9ml 10 X RPMI; 10ml fetal calf serum; 1ml GPS (glutamine, penicillin, and streptomycin); 1ml non-essential amino acids; 3ml 5.3% sodium hydrogen carbonate (NaHCO₃); 0.5-1.5ml NaOH. Stored at 4°C.


All solutions were made using distilled deionised water. Generally solutions were sterilised by autoclaving at 121°C, 15 lbs psi, for 30 minutes, and were stored at room temperature (15-25°C).

2A.3(a). LCL lysis buffer.

0.1M Tris-HCl; 5mM EDTA; 0.2% SDS; 0.2M NaCl. After autoclaving Proteinase K was added to a final concentration of 10mg/ml.

2A.3(b). Single cell lysis buffer.

0.2M KOH; 50mM DTT. Stored at -20°C.

2A.3(c). TE.

10mM Tris-HCl, pH8.0; 0.1M EDTA pH8.0. Sterilised by autoclaving.

2A.4(b). TBE.

90mM Tris-HCl, pH 8; 90mM boric acid; 2mM EDTA- made up as a 10 X concentrate.

2A.4(c). SDS running buffer.

0.025M Tris; 0.192M glycine; 0.1% SDS.

2A.4(d). Loading buffer for agarose gels (10X).

40% sucrose; 0.025% w/v bromophenol blue; 0.025% w/v xylene cyanol.

2A.4(e). Sample buffer for SDS gels.

0.06M Tris-HCl, pH 6.8; 2% w/v SDS; 10% v/v glycerol; 5% v/v 2-$\beta$-mercaptoethanol; 0.025% w/v bromophenol blue.

2A.5. Fluorescence in situ hybridisation solutions.

2A.5(a). Saline sodium citrate (SSC)

150mM NaCl; 15mM sodium citrate, pH 7.0 (adjusted with NaOH)- made up as a 20 X concentrate.
2A.5(b). Anti-fade medium

(I) 9 parts 2% 1,4-diaza-bicyclo-(2,2,2)-octane (DABCO); 1 part 0.2 M Tris-HCl, pH 7.5, 0.02% NaN3; 0.5μg/ml 4,6-diamidino phenylindole (DAPI). Stored at -20°C and protected from light.

(II) As above with the addition of 1μg/ml propidium iodide

2A.5(c). Hybridisation mix.

50% deionised formamide; 20% w/v dextran sulphate; 2 X SSC; 0.1mM EDTA, pH 8; 0.2mM Tris-HCl, pH 7.6.

2A.5(d). Magnesium Chloride (MgCl₂) buffer.

1 X PBS; 1% MgCl₂.

2A.5(e). Paraformaldehyde buffer.

1% Paraformaldehyde (Fluka); 1% w/v MgCl₂.


0.01M phosphate buffer; 0.0027M potassium chloride; 0.137M sodium chloride, pH 7.4.

2A.5(g). Proteinase K buffer.

20mM Tris-HCl, pH 7.5; 2mM calcium chloride (CaCl₂).

2A.5(h). SSCM.

4 X SSC with 5% Marvel™ non-fat dried milk.
2A.5(i). SSCT.

4 X SSC with 0.05% Tween 20.

2A.5(j). TN.

0.1mM Tris-HCl, pH7.5; 0.15M NaCl. Made as a 10 X stock solution.

2A.5(k). TNE

0.2M NaCl; 10mM Tris-HCl, pH8.0; 1mM EDTA.

2A.5(l). TNT.

As TN but with the addition of 0.1% Tween 20 detergent.

2A.5(m). TNB.

As TN but with the addition of 0.5% blocking agent (Boeringer Mannheim), dissolved after incubation for three hours at 60°C, filtered through 1mm filter paper (Whatman) and stored at -20°C.

2A.6. Solutions for yeast culture and DNA extraction.

2A.6(a). S.D. medium.

7g/l Bacto yeast nitrogen base (without amino acids); 20g/l glucose; 55mg/l adenine and tyrosine. After sterilisation by autoclaving 56ml/l of 20% filter sterilised casamino acids was added. The medium was stored at 4°C.
2A.6(b). S.D. agar.

10g/l bacto-agar added to S.D. medium and autoclaved. Stored at 4°C until used.

2A.6(c). YLB (Yeast lysis buffer).

100mM EDTA; 10mM Tris-HCl, pH 7.5; 1% lithium dodecyl sulphate.

2A.6(d). YRB (Yeast Resuspension Buffer).

1.2M sorbitol; 10mM Tris-HCl, pH 7.5; 20mM EDTA. Sterilised by autoclaving. Immediately prior to use 14 mM β-mercaptoethanol was added.

2A.7. Solutions for bacterial culture and DNA extraction.

2A.7(a). Cosmid broth.

1.2% w/v tryptone, 2.4% w/v yeast extract, 0.5% v/v glycerol, 0.072M K₂HPO₄, and 0.028M KH₂PO₄.

2A.7(b). Bacterial lysis buffer.

50mM glucose, 10mM EDTA, 25mM Tris, pH 8.

2A.7(c). AD buffer.

0.2M NaOH, 1% SDS.

2A.8. Buffers for the polymerase chain reaction.
2A.8(a). PCR buffer (HT Biotechnologies).

500mM Tris-HCl, pH 9.0; 500mM KCl; 70mM MgCl$_2$; 160mM (NH$_4$)$_2$SO$_4$.
Obtained as a 10 X concentrate and stored at -20°C.

2A.8(b). Potassium free PCR buffer.

2.5mM MgCl$_2$; gelatine 0.1mg/ml; 10mM TrisHCl, pH 8.3.
Made as a 10 X concentrated stock, and stored at -20°C.

2A.8(c). SC-PCR buffer.

50mM KCl; 10mM Tris HCl, pH 8.3. Made as a 10 X concentrate, stored at -20°C.

2A.8(d). Neutralising buffer.

900mM Tris HCl, pH 8.3; 300mM KCl; 200mM HCl.

2A.9(a). Primers for the amplification of the APC gene (Ando et al., 1993):

Exon 6  
(F) CAAGGATCCTGAGCTTTTAAGTGGTAG  51°C  
CTGAAGCTTTTTCGAGAATAACTACCTA (R)

Exon 8  
(F) CATGATGTTATCTGTATTTACC  51°C  
CTTAGCAAAGTAGTACGCGG (R)

Exon 14  
(F) CAACTCTAATTAGATGACCCA  51°C  
GAGAGTATGAAATTCTGTACCTT (R)

2A.9(b). Primers for the amplification of the APC gene (Groden et al., 1991):

Exon 11  
(F) GATGATGTGTTTTTCTTTTCTTTGC  53°C  
CTGAGCTATCTTAAGAAATACATG (R)

Exon 15 A  
(F) GTTACTGCATAACACATTGTGAC  52°C  
GCTTTTTGTTTTCTTAACATGAAG (R)

Exon 15 B  
(F) AGTACAAGGATGCAATATTATG  52°C  
ACTTCTATCTTTTCAGAACGAG (R)

Exon 15 C  
(F) ATTTGAATACACTACAGTGTTACCC  49°C  
CTTGTATTCTAATTTCAGCATAAGG (R)
2A.9(c). Primers for PTT analysis of the APC gene (van der Luijt et al., 1994).

Exon 15 E - 15 J 63°C

(PTT)

(F)GGATCCTAATACGACTCACTATAGGAAACAGACCACCACATGCTTAATTATTTATTTATTTATTTATGCTA

Reverse primer is APC 15J (Goden et al., 1991).
2A.9(d). Primers for preimplantation genetic diagnosis of FAP.

<table>
<thead>
<tr>
<th>Codon 764 mutation (outer)</th>
<th>(F) GGAATCTCATGGCAAATAGGCCT TGGTGAAAGGACAGTCATGTTGC (R)</th>
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<tr>
<th>Codon 764 mutation (inner)</th>
<th>(F) TGTCTCCTGGCTCAAGCTTGCCA TACGATGAGATGCCTTGGAAGCTT (R)</th>
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<tr>
<th>Codon 1678 (outer) polymorphism</th>
<th>(F) CCCAGACTGCTTCAAATAATTACC GAGCCTCATCTGTACTTCTGC (R)</th>
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<tr>
<th>Codon 1678 (inner) polymorphism</th>
<th>(F) AGCTGCCTGTGTACAAACTTCT AGGAATGGGTATCTCGTTTTTCA (R)</th>
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2A.9(e). Primers for \textit{hMSH2}.

Exon 12

(F) ACAGGCTATGTAGAACCAATGC \hspace{1cm} 57^\circ C

AAAAACAAAACCCTACCC (R)

R. Sud (personal communication)

Exon 13

(F) CGCGATTAATCATCAGTG \hspace{1cm} 58^\circ C

GGACAGAGACATACTTCTATC (R)

Fischel \textit{et al.}, 1993
2.9(f). Primers for the analysis of LOH and replication errors at microsatellite loci:

<table>
<thead>
<tr>
<th>Locus</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Annealing Temperature</th>
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<tr>
<td>D2S119</td>
<td>(F) CTTGGGGAACAGAGGTTCATT</td>
<td>GAGAATCCCTCAATTTCTTTGGA (R)</td>
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<td></td>
<td>Genome Database (GDB, Baltimore)</td>
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<tr>
<td>D2S123</td>
<td>(F) AAACAGGATGCTGCGTTTA</td>
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<td></td>
<td>Leach et al., 1993</td>
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<tr>
<td>D2S391</td>
<td>(F) ATGGAGCCAGTAGGTTACAGC</td>
<td>GGTGAGAGGGTGATAGGGAA (R)</td>
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<td>D4S175</td>
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<td>Mills et al., 1992</td>
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<tr>
<td>D5S346 (APC)(F)</td>
<td>ACTCACTCTAGTGATAAACTGGG</td>
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<tr>
<td>D9S156</td>
<td>(F) ATCACTTTITAACACTGGCGG</td>
<td>AGATGGTGTTGAATAGAGGG (R)</td>
<td>56°C</td>
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<tr>
<td></td>
<td>Nature, June 1994</td>
<td></td>
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</tbody>
</table>
D14S50  (F) AACACCCCTAAATTCACCACT  58°C
   ATGATTCCACCAGATGGCAG (R)

D15S129  (F) ACAGCACTTTGCTCTTTTCATC  64°C
   TGTCTGTGCCCTCCTCCCT (R)
Richard et al., 1993.

TP53 (CA)  (F) AGGGATACTATTCAGGGCGAGGTG  63°C
   ACTGCCACTCCTTGCCCCCATT (R)
Jones and Nakamura, 1992.

D18S61  (F) AATTTCTAAGAGGACTCCCAAACT  58°C
   ATATTTTGAACCTCAGGAGCA (R)
Jen et al., 1994.

DCC  (F) TCCCTCTAGAAATTGTGTG  52°C
   TGACTTATACCTCATTGGAG (R)

D19S49  (F) ACTCATGAAGGTGACAGTTCC  58°C
   GTGTTGGACCTATTGACAT (R)
Weber et al., 1990.

D22S351  (F) GTCAAGGGCAGGTAAGGGTTGA  60°C
   CTCCTGCCCCTCGAAAGTCAT (R)
Sainz et al., 1993.

All primers are read in the 5' to 3' direction. The (F) primer in each pair is the forward (sense) primer and lies 5' of the DNA fragment to be amplified; the (R) primer is the reverse (anti-sense) primer and lies 3' of the DNA fragment to be amplified.
Chapter 3

Results
Results

3.1. Analysis of the APC gene in FAP patients.

Previous studies had indicated that approximately two thirds of all germline APC mutations are situated within the 5’ half of exon 15 of the APC gene (see Nagase and Nakamura, 1993 for review). Additional sites that contain a disproportionate number of mutations for their size have also been reported in exons 6, 8, and 14 (Ando et al., 1993). For this reason it was decided to direct mutation detection efforts to these regions of the gene. In all 60% (over 5,000 bp) of the APC coding region was assessed. Consensus sequences for splicing which flank exons 6, 8, 11, and 14 were also investigated as well as the splice acceptor sequence of exon 15. The 5’ half of exon 15 (codons 652-1701) was assessed in 10 overlapping fragments 300 bp-400 bp in length, and designated 15A, 15B, 15C,…15J. These fragments and the fragment generated for the investigation of exon 11 were amplified by the polymerase chain reaction (PCR) using primers described by Groden et al. (1991), while the other exons were amplified using oligonucleotides designed by Ando et al. (1993) (see section 2A.9.).

A wide variety of methods have been applied to the detection of APC mutations. The initial approach used for the purpose of this investigation was a combined single strand confirmation analysis (SSCA) and heteroduplex analysis. Both analyses were conducted simultaneously using the Pharmacia Phastsystem™. These established forms of mutation analysis are well suited to the detection of small deletions and insertions such as are frequently reported in the APC gene (Nagase and Nakamura, 1993). As it became clear that the vast majority of APC mutations result in protein truncation a rapid protein truncation test (PTT) was also adopted to provide a quick screen of mutation hotspots in exon 15 (van der Luijt et al., 1994). Restriction endonuclease digestion was employed for the rapid detection of common mutations in exons 6, 8, and 14 (Ando et al., 1993).
To determine whether DNA variants detected by the above techniques were responsible for FAP DNA sequencing was undertaken to characterise the nature of the mutation, providing information on whether it would be likely to disrupt protein function. Furthermore, if other affected individuals from the same family were available for testing then they too were checked for the alteration.

During the course of this study a total of 52 unrelated patients with histologically confirmed FAP were investigated for mutation of the APC gene. 28 cases were familial, segregation of a mutant APC allele being evident in at least two generations, while a further 24 patients had no previous family history of FAP, and were presumed to have the disease as a consequence of fresh mutation. Where possible the paternity of such individuals had been tested by Dr Michele Rees, Luiza Bowles and Katia Tsioupra, using highly polymorphic DNA probes.

Mutation analysis revealed germline mutation in 35 out of 52* (67%) patients with FAP. The majority of mutations (85%) were frameshift caused by insertion or deletion of nucleotides, all of which produced new stop codons immediately downstream. Such mutations are predicted to cause premature truncation of the APC protein. Small deletions, ranging from 2 bp-5 bp, formed the most common class of frameshift, accounting for 65% of mutations detected. Insertions of 1 bp or 2 bp were responsible for a further 20% of APC mutations. Almost all of the frameshift mutations occurred at tandem repeated nucleotide sequences, although in many cases the repeat motif was imperfect. The remaining 15% of mutations were the consequence of single nucleotide substitutions. Four of the five substitutions identified were nonsense mutations, introducing a new stop codon, and presumably truncating the APC protein. The only substitution not to introduce a stop codon alters a highly conserved nucleotide of the exon 8 splice donor sequence, which may have similar consequences for the APC protein as nonsense mutation. Three of the substitutions were cytosine to thymine transitions, the remaining alterations were a guanine to adenine transition and a cytosine to guanine transversion.

Mutations were found in all exons of APC surveyed with the exception of exon 11. However the distribution of mutations was not even, and showed a marked clustering between codon 1060 and codon 1465 (15E-15H). Mutations in this 1.2 kb stretch of DNA account for 71% of the variants detected during this study. In all 25/52 (48%) FAP patients investigated had mutation in this region. Two distinct
hotspots for mutation were evident: a 5 bp deletion at codon 1309 (15G) was seen in 19% of patients; and another deletion of 5 bp was identified at codon 1061 (15E) in 10% of patients. Three other sites recorded more than one mutation. Nonsense mutation at codon 213 (exon 6) was observed twice (4% of patients), while a single base insertion at codon 764 (15B) and a 2 bp deletion at codon 1465 (15H) were each detected three times (6% of patients).

*14 of the 52 unrelated FAP patients were investigated by S. Gayther and a single patient by R. Sud.
Family 82

Mutation revealed by restriction endonuclease digestion of APC exon 6 using Acc I.

Key:
Squares denote males
Circles denote females
Diamond shapes denote individuals of unknown sex
Filled in squares or circles represent patients with confirmed FAP.

DNA sequence variant revealed by restriction endonuclease digestion using AccI. Mutant DNA sequence is not digested by this enzyme.
Sequence analysis of *APC* exon 6 in patient 82 III 3.

Codon 232 nonsense mutation:
CGA to TGA

Sequence analysis of 82 III 3 demonstrating that the sequence variant detected by restriction endonuclease digestion is due to a single base substitution. An identical mutation was detected in affected members of family 523.
Family KAL

Single strand conformation polymorphism (SSCP) and heteroduplex analysis of *APC* exon 15G.

DNA sequence variant in *APC* exon 15 revealed by altered mobility of single stranded DNA and heteroduplex formation.
Sequence analysis of *APC* exon 15G in patient KAL II 3 and parallel analysis of normal control DNA.

**Codon 1309 deletion:**

AGAAA

Sequence analysis of KAL II 3 and a control individual demonstrating that the SSCP and heteroduplex variant identified in *APC* exon 15G is due to a 5 bp deletion (ΔAAAAGA). An identical mutation was detected in nine other FAP patients/families (see table 3.1.).
Family 456

Single strand conformation polymorphism (SSCP) and heteroduplex analysis of APC exon 14.

DNA sequence variant in APC exon 14 revealed by altered mobility of single stranded DNA and heteroduplex formation.
Figure 3.6.

Sequence analysis of *APC* exon 14 in patient 456 IV 1 and parallel analysis of normal control DNA.

<table>
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<th>Normal</th>
<th>456 IV 1</th>
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<td>G A</td>
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<tr>
<td>A</td>
<td>A</td>
<td>T C</td>
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</table>

Codon 636 insertion: GC

Sequence analysis of 456 IV 1 and a control individual demonstrating that the SSCP and heteroduplex variant identified in *APC* exon 14 is due to a 2 bp insertion (GC).
Figure 3.7.

Family 391

Single strand conformation polymorphism (SSCP) and heteroduplex analysis of *APC* exon 15E.

DNA sequence variant in *APC* exon 15E revealed by heteroduplex formation.
Sequence analysis of $APC$ exon 15E in patient 391 II 5 and parallel analysis of normal control DNA.

Codon 1061 deletion:
ACAAAA

Sequence analysis of 391 II 5 and a control individual demonstrating that the heteroduplex variant identified in $APC$ exon 15E is due to a 5 bp deletion (ΔACAAAA). An identical mutation was detected in five other FAP patients/families (see table 3.1.).
Family DAV

Single strand conformation polymorphism (SSCP) and heteroduplex analysis of APC exon 8.

DNA sequence variant in APC exon 8 revealed by altered migration of single stranded DNA.
Figure 3.10.

Sequence analysis of APC exon 8 in patient DAV IV 5 and parallel analysis of normal control DNA.

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<td>C</td>
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</table>

Splice donor mutation:
AAG/gta to AAG/ata

Sequence analysis of DAV IV 5 and a control individual demonstrating that the SSCP variant identified in APC exon 8 is due to a single base change in the exon 8 splice donor sequence.
Family COO

Single strand conformation polymorphism (SSCP) and heteroduplex analysis of $APC$ exon 14.

DNA sequence variant in $APC$ exon 14 revealed by heteroduplex formation.
Family BLO

Single strand conformation polymorphism (SSCP) and heteroduplex analysis of APC exon 15H.

DNA sequence variant revealed by heteroduplex formation.
Figure 3.13.

Sequence analysis of *APC* exon 15H in patient BLO IV 3 and parallel analysis of normal control DNA.

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BLO IV 3

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<td>T</td>
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Codon 1465 deletion:
AG

Sequence analysis of BLO IV 3 and a control individual demonstrating that the heteroduplex formation detected in *APC* exon 15H is due to a deletion of 2 bp (ΔAG). An identical mutation was detected in three other FAP patients/families (see table 3.1.).
SSCP, heteroduplex, and sequence analysis of \textit{APC} exon 15E performed on DNA from patient 75 III 4 and normal control DNA.

**Codon 1085 deletion:**

\texttt{AACA}

DNA sequence variant in \textit{APC} exon 15E revealed by altered mobility of single stranded DNA and heteroduplex formation. Sequence analysis of new mutation patient 75 III 4 demonstrated that this variant is due to a deletion of 4 bp (\texttt{ΔAACA}).
Protein truncation test analysis of *APC* exon 15E-15J performed on DNA from patient 409 I 1, and patient 558 III 3.

**Figure 3.15.**

Truncating mutation at *APC* codon 1357 and at codon 1556:

DNA sequence variants in *APC* revealed by the production of truncated polypeptides after an *in vitro* transcription and translation test. Sequence analysis of new mutation patient 558 III 3 demonstrated that this variant is due to a nonsense mutation at codon 1357. A single base insertion at codon 1556 was identified in patient 409 I 1.
Figure 3.16.

Sequence analysis of APC exon 15G performed on DNA from patient 558 III 3 and normal control DNA.

<table>
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<td>A</td>
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<td>T</td>
<td>T</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
</tr>
</tbody>
</table>

Codon 1357 nonsense mutation:

Sequence analysis of 558 III 3 and a control individual demonstrating that the truncated protein detected by PTT is due to a C to G transversion at codon 1357 that creates a stop codon.
Figure 3.17.

Sequence analysis of *APC* exon 151 performed on DNA from patient 409 I 1 and normal control DNA.

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</table>

Codon 1556 insertion:

Sequence analysis of 409 I 1 and a control individual demonstrating that the truncated protein detected by PTT is due to a single adenine insertion at codon 1556.
Protein truncation test analysis of *APC* exon 15E-15J performed on DNA from patient 531 IV 1.

Truncating mutation detected in *APC* 15E-15J:

DNA sequence variant in new mutation patient 531 IV 1 revealed by the production of a truncated polypeptide after *in vitro* transcription and translation. The migration rate of the truncated polypeptide suggests that the mutation is situated within amplicon 15F of the *APC* gene.
3.2. Correlation between FAP phenotype and site of \textit{APC} mutation.

Comparison of patients for whom mutations had been detected revealed an influence of mutation site on severity of phenotype. Patients with no family history of FAP were considered severely affected if they presented with symptoms in their teens or with colorectal cancer in their twenties. Families were classed as severe if at-risk patients undergoing regular screening developed polyps before the age of 12 or advanced neoplastic disease before reaching thirty years of age.

Sixteen patients in this study had a severe phenotype, characterised by early onset of polyps or cancer, or the development of thousands rather than hundreds of polyps. Twenty three patients had an average or mild phenotype, while a further 13 could not be categorised because there was insufficient clinical information. Mutations of the \textit{APC} gene were detected in 87.5\% (14/16) of patients classed as severely affected. In 13 out of 14 cases the mutations were clustered between nucleotides 3927 and 4392, a region encompassing the frequently mutated codon 1309. Only one family with a severe phenotype, consisting of early onset of malignancy and multiple primary cancers had mutation before codon 1309. The mutation in this family, family HUD, is a single base insertion at codon 764 of the \textit{APC} gene. However, previous investigation had indicated that affected family members carry a second germline alteration, a large genomic rearrangement in the vicinity of the \textit{APC} gene (discussed in chapter 4), which may influence the phenotype in this case. Two other families with an identical mutation at codon 764 but no genomic rearrangement have been found, and shown to have an average phenotype. No patients with an average phenotype had mutations situated between nucleotides 3927 and 4392. The association between severity of symptoms and site of mutation was highly statistically significant [significant at the 0.001\% level, $\chi^2$ test].

Interestingly, 67\% of new mutation cases had mutation within the ‘severe’ region compared with only 12\% of familial cases. Consequently the phenotype of new mutation patients tended to be more severe than that of patients with a prior family history of FAP [significant at the 0.01\% level, $\chi^2$ test]. Clustering of mutation was particularly evident at codon 1309, 8/24 (33\%) new mutation cases having
mutation at this site compared to 2/28 (7%) cases resulting from inherited \textit{APC} mutation.
Table 3.1. Phenotypic data and results of mutation analysis.

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<thead>
<tr>
<th>Family / Patient</th>
<th>Family / New mutation</th>
<th>Mutation</th>
<th>Polyph Number</th>
<th>Age of cancer / diagnosis</th>
<th>CHRPE</th>
<th>Desmoid tumours</th>
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<tr>
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<td>surgery around 40</td>
<td>-</td>
<td>average</td>
<td>Wide spectrum of cancers</td>
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<td>&gt;2000</td>
<td>cancer @ 17</td>
<td>+</td>
<td>severe</td>
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<tr>
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<td>&gt;100</td>
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<td>average</td>
<td></td>
<td>Epidermoid cysts</td>
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</tr>
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<td>CHA F</td>
<td></td>
<td>1</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Atypical FAP</td>
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</tr>
<tr>
<td>COO F</td>
<td>deletion CAGA 625</td>
<td>cancer @ 38</td>
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<tr>
<td>DAV F</td>
<td>splice site G to A exon 8</td>
<td>reported as average</td>
<td>surgery @ 19</td>
<td>-</td>
<td>average</td>
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</tr>
<tr>
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<td>Family / New mutation</td>
<td>Mutation</td>
<td>Polyp Number</td>
<td>Age of cancer / diagnosis</td>
<td>CHRPE</td>
<td>Desmoid tumours</td>
<td>Phenotype</td>
<td>Comments</td>
</tr>
<tr>
<td>-----------------</td>
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<tr>
<td>DOU F</td>
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<td></td>
<td></td>
<td>presented @ 7</td>
<td>-</td>
<td>+</td>
<td>severe</td>
<td>Osteomata</td>
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<td>GIB F</td>
<td></td>
<td></td>
<td></td>
<td>surgery @ 67</td>
<td>-</td>
<td>+</td>
<td>mild</td>
<td>Epidermoid cysts</td>
</tr>
<tr>
<td>GRU F</td>
<td></td>
<td></td>
<td></td>
<td>surgery @ 32</td>
<td>+</td>
<td>average</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIT NM</td>
<td>deletion ACAAA 1061</td>
<td></td>
<td></td>
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<td>HOA F</td>
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<td></td>
</tr>
<tr>
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<td>insertion T 764</td>
<td>&gt;2000</td>
<td>cancer B @ 21</td>
<td>+</td>
<td></td>
<td>severe</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HUM F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
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</tr>
<tr>
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<td>deletion AAAGA 1309</td>
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<td>presented @ 19</td>
<td>+</td>
<td></td>
<td>severe</td>
<td>Lipomas, ovarian cysts</td>
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<tr>
<td>KEE NM</td>
<td>transition C to T 1368</td>
<td>&gt;2000</td>
<td>presented @ 15</td>
<td>+</td>
<td></td>
<td>severe</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAW F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+/-</td>
<td></td>
<td>Osteomas</td>
<td></td>
</tr>
<tr>
<td>MAR F</td>
<td>insertion T 764</td>
<td>&lt;500</td>
<td>cancer @ 50</td>
<td>+</td>
<td></td>
<td>average</td>
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</tr>
<tr>
<td>NUT NM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QUE NM</td>
<td>deletion AAAGA 1309</td>
<td></td>
<td>surgery @ 12</td>
<td>+</td>
<td></td>
<td>severe</td>
<td>Many epidermoid cysts</td>
<td></td>
</tr>
<tr>
<td>THU NM</td>
<td>deletion AAAGA 1309</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>TOL NM</td>
<td></td>
<td>&lt;500</td>
<td>presented @ 29</td>
<td>+</td>
<td></td>
<td>average</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Information on the genotype and phenotype of 52 unrelated FAP patients after mutation analysis of the APC gene.

*14 Unrelated FAP patients were investigated by S. Gayther and a single patient by R. Sud.

### 3.3. Detection of polymorphisms within the APC gene.

During the course of mutation analysis several intragenic *APC* polymorphisms were identified. Two common polymorphisms were detected in exon 15 of *APC*. One polymorphism was due to a G to A substitution at codon 1493 (15I). A previously reported alteration which does not cause any change in amino acid at this position (Powell *et al.*, 1992; Nagase *et al.*, 1992b). The variant codon 1493 sequences could be detected by SSCP at 4°C and at 15°C, however, they were considerably easier to distinguish if electrophoresis was conducted at 10°C. A total of 73 unrelated individuals, of varying geographic and ethnic origin, were tested for the 15I polymorphism and the following gene frequencies were established: 0.58 for the ‘G’ allele and 0.42 for the ‘A’ allele. Heterozygotes, ‘G’ allele homozygotes, and ‘A’ allele homozygotes accounted for 53.4%, 31.5% and 15.1% respectively, as expected for a population in Hardy-Weinberg equilibrium. Similar frequencies were observed in 45 FAP patients also assessed for the 15I polymorphism.
The second polymorphism detected in exon 15 was situated at codon 1678 (15J) and was of equal frequency. Like the 15I polymorphism this alteration, a substitution of adenine for guanine, was not associated with a change in amino acid, and had been previously described (Nagase et al., 1992b). Visualisation of the 15J variants was best achieved by SSCP at 15°C. A total of 87 unrelated individuals, 14 with FAP, were analysed for both 15I and 15J polymorphisms. In every case inheritance of the ‘G’ allele at codon 1493 was accompanied by inheritance of the ‘A’ allele at codon 1678. This indicates that alleles of the two polymorphisms are in complete linkage disequilibrium. A further 50 of these individuals were also typed by K. Tsiouprra for the polymorphism that occurs at codon 1960 (FB54D, 15N) (Cottrell and Bodmer, 1992), to determine if this too displays evidence of linkage disequilibrium. Only two samples out of 50 demonstrated any evidence of recombination between FB54D and 15I/15J.

In addition to the common polymorphisms discussed above four rare variants, not associated with a disease phenotype, were discovered (Table 3.2.). Each variant is the consequence of a single base substitution, all resulting in a change of predicted amino acid. That the variants are asymptomatic was indicated by the discovery of more significant alterations, such as frameshift mutations, in affected carriers of the variant, or by the discovery unaffected family members carrying the variant.
Polymorphism in \textit{APC} 151 (codon 1493):

Guanine to adenine substitution at codon 1493 detected by SSCP at 10°C. Allele frequencies of this silent polymorphism are: 0.58 'G' and 0.42 'A'.
Figure 3.20.

Family KAL:

Linkage Analysis in the Vicinity of \textit{APC}

Linkage analysis in the vicinity of the APC gene.

Linkage analysis in family KAL demonstrating that \textit{APC} mutation occurred in the germline of individual KAL I 1 giving rise to this families FAP. Patient KAL II 4 was the first to carry this new mutation.
Family 391

Single strand conformation polymorphism (SSCP) analysis of $APC$ exon 15C.

DNA sequence variant in $APC$ exon 15C revealed by altered migration of single stranded DNA during SSCP analysis. This rare variant segregates with the disease and is the consequence of a C to T transition at codon 870. Although this does result in a change of amino acid (proline to serine) a 5 bp deletion at codon 1061 also detected in this family is the more likely cause of FAP.
Table 3.2. Rare variants detected in APC exon 15.

<table>
<thead>
<tr>
<th>FAP family</th>
<th>Amplicon</th>
<th>Codon / nucleotide</th>
<th>Sequence change</th>
<th>Amino acid change</th>
<th>Allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>391</td>
<td>15C</td>
<td>870 / ÇCA to Proline</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2610 TCA</td>
<td>Serine</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>552</td>
<td>15E</td>
<td>1083 / GAT to Aspartic</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3249 GAG</td>
<td>to Glutamine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*</td>
<td>564</td>
<td>15E</td>
<td>1129 / TTG to Leucine</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3386 TCG</td>
<td>Serine</td>
<td></td>
</tr>
<tr>
<td>* and</td>
<td>15G</td>
<td>564</td>
<td>1317 / GAA to Glutamine</td>
<td>&lt;0.0125</td>
<td></td>
</tr>
<tr>
<td>BLE*</td>
<td>3949</td>
<td>CAA Glysine</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

100 chromosomes were tested for each variant with the exception of the codon 1317 substitution which was tested with 160 chromosomes.

*Identified by S. Gayther.

3.4. Further analysis of FAP patients with unexpected phenotypes.

Although there is a strong correlation between site of mutation within the APC gene and severity of symptoms, two families had somewhat unexpected phenotypes. Patients from family HUD have a mutation at codon 764 of the APC gene, a region usually associated with average phenotype. Surprisingly patients from this family are severely affected with large numbers of polyps (>2000) and an early age of onset. The results of further investigations on this family are detailed in section 3.4.1.-3.3.3. below.

The second family with atypical features, family 82, displays a range of extracolonic malignancies in addition to average FAP symptoms. This family has a mutation in exon 6 of the APC gene which can account for the features typical of FAP, but cannot explain all of the cancers observed. Several types of malignancy seen in family 82 are characteristic of the cancer predisposition syndrome hereditary
nonpolyposis colorectal cancer (HNPCC), hinting that this disorder may explain some aspects of this family’s phenotype. The localisation and subsequent cloning of the hMSH2 gene (Fischel et al., 1993; Leach et al., 1993), responsible for almost 50% of HNPCC cases, provided a target for analyses to test this hypothesis.

3.4.1. Linkage analysis in family 82.

Initial experiments were aimed at establishing whether or not a common haplotype in the vicinity of hMSH2 (chromosome 2p21) was shared by patients who developed extracolonic malignancy. Polymorphic DNA sequences flanking and within the hMSH2 gene were assessed by PCR-SSCP. Three of the sequences assessed (D2S391, D2S119 and D2S123) were at highly polymorphic dinucleotide repeat loci, while a fourth sequence contained a bi-allelic polymorphism within the gene itself (Hall et al., 1994). Analysis of eighteen family members demonstrated the existence of seven different haplotypes, and a single recombination event occurring between D2S119 and D2S391. A DNA sample was available from four of the five individuals who developed extracolonic cancer. Two haplotypes in the vicinity of hMSH2, H3 and H4, were each shared by three of these patients. However, both H3 and H4 were also present in family members with no extracolonic malignancy.
Figure 3.22. Analysis of $(CA)_n$ repeat locus D2S391.

Appearance of the D2S391 polymorphism used to assess linkage to the hMSH2 gene. Fragments containing differing numbers of (CA) repeats produce bands with distinct migration rates during SSCP.
Figure 3.23.

Family 82: 
Linkage Analysis in the Vicinity of *hMSH2*

<table>
<thead>
<tr>
<th>III</th>
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<th>6</th>
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<th>2</th>
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<td>1</td>
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<td>3</td>
<td>1</td>
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<td>1</td>
<td>1</td>
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</tr>
<tr>
<td>D2S123</td>
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<td>2</td>
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<table>
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<table>
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<th>4</th>
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<td>1</td>
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</tbody>
</table>

82 III 1 Ovarian cancer
82 III 2 Pleomorphic adenoma of salivary gland
     Adenocarcinoma of the uterus
     Basal cell papilloma under chin
82 III 3 Pancreatic cancer
82 IV 4 Papillary adenocarcinoma of the thyroid
82 IV 6 High grade lymphoma

- Polyposis
- Colorectal cancer
- Cyst or desmoid
- Extra-colonic cancer

Haplotype H3
Haplotype H4
3.4.2. Mutation analysis of the hMSH2 gene.

Although the haplotype data was not entirely conclusive it provided sufficient incentive for further investigation of the hMSH2 gene in this family. Mutation of hMSH2 was analysed by PCR-SSCP and heteroduplex analysis of the most highly conserved region of the gene, exons 12 and 13. An identical method had successfully identified a wide variety of mutations in the APC gene, however no mutations were detected in the hMSH2 gene in this family.

During the course of this investigation a bi-allelic polymorphism affecting the exon 13 splice acceptor sequence was identified. Not only were heteroduplexes formed in heterozygous samples, but it was also clear that the two alternative homoduplexes migrate at different rates. Analysis of this variant in an additional 40 individuals revealed the allele frequencies to be 0.91 (fast migrating homoduplex) and 0.09 (slow migrating homoduplex). Published allele frequencies of this alteration, a C to T transition 6 bp upstream of position 2006 (exon 13), are 0.87 and 0.13 respectively (Hall et al., 1994).
Figure 3.24. Polymorphism detected in *hMSH2* during SSCP and heteroduplex analysis.

SSCP and heteroduplex analysis of *hMSH2* exon 13 revealing polymorphism in the splice acceptor site 6 bp upstream of position 2006.
3.4.3. Analysis of mismatch repair competence and loss of heterozygosity in tumours from family 82.

A further attempt to determine whether a mismatch repair gene underlying HNPCC was involved in the phenotype of family 82 focused on analysis of microsatellite loci. Mutation in genes involved in DNA mismatch repair, such as \textit{PMS1}, \textit{PMS2}, \textit{hMLH1}, and \textit{hMSH2} cause increased mutability at microsatellite loci throughout the genome (Fischel et al., 1993; Leach et al., 1993; Papadopoulos et al., 1994; Bronner et al., 1994; Nicolaides et al., 1994). Parallel analysis of microsatellite loci in patient's constitutional DNA and in the DNA of their tumours was carried out using PCR-SSCP. A paraffin imbedded section was available from the endometrial carcinoma developed by patient 82 III 2, and fresh samples of pancreatic cancer along with corresponding lung metastasis were obtained immediately following the death of patient 82 III 3. A total of 13 dinucleotide repeat loci, situated on 10 separate chromosomes were investigated. Analysis of 12 unrelated individuals revealed a large amount of variability in the SSCP band patterns observed, as expected for these highly polymorphic DNA sequences. Furthermore, analysis of four positive controls demonstrated that the method is capable of detecting both replication errors and loss of heterozygosity (LOH). Despite indications that the technique is reliable no differences were noted between tumour and constitutional DNA samples from individual patients.

These results indicate that the tumours investigated are RER' and therefore provide no evidence for the influence of a defective mismatch repair gene in the phenotype of this family. Analysis of heterozygous microsatellite loci in the vicinity of the tumour suppressor genes \textit{p53}, \textit{hMSH2}, \textit{APC}, \textit{MCC} and \textit{DCC} revealed no allele loss in the tumours investigated. A bi-allelic polymorphism within the \textit{APC} gene was also shown to retain heterozygosity in all informative samples. Although it is not possible to rule out involvement of these genes in tumourigenesis the results suggest that the series of genetic events leading to tumour formation did not involve their inactivation by a mechanism of allelic deletion.
Figure 3.25. Positive controls for the detection of loss of heterozygosity and replication error phenotype.

Matched normal and tumour DNA samples from patients with sporadic colorectal cancer. Loss of heterozygosity detected at D2S123 and replication errors identified at the D18S61 locus. These samples were provided by R. Sud and served as positive controls for LOH and microsatellite instability studies.
Table 3.3. Comparison of polymorphic loci in patient and tumour DNA.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Patient 82 III 2 Constitutional DNA</th>
<th>Patient 82 III 2 endometrial cancer</th>
<th>Patient 82 III 3 Constitutional DNA</th>
<th>Patient 82 III 3 Pancreatic cancer</th>
<th>Patient 82 III 3 Lung metastasis</th>
</tr>
</thead>
<tbody>
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<td>D2S119</td>
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<td>Uninformative</td>
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<td></td>
</tr>
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<td>D2S391</td>
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<td>Heterozygous</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D2S123</td>
<td>Heterozygous</td>
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<td>Heterozygous</td>
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</tr>
<tr>
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<td>Uninformative</td>
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<td>Uninformative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D5S346 (APC)</td>
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<td>Uninformative</td>
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</tr>
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<td>D9S156</td>
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<td>Heterozygous</td>
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<tr>
<td>D14S50</td>
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<td>Heterozygous</td>
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</tr>
<tr>
<td>D15S129</td>
<td>Heterozygous</td>
<td></td>
<td>Heterozygous</td>
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<td></td>
</tr>
<tr>
<td>17 (TP53)</td>
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<td></td>
<td>Heterozygous</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D18S61</td>
<td>Heterozygous</td>
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<td>Heterozygous</td>
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</tr>
<tr>
<td>DCC (AT)</td>
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<td>Heterozygous</td>
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</tr>
<tr>
<td>APC 15J (intragenic)</td>
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<td>Heterozygous</td>
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</tr>
</tbody>
</table>

Heterozygosity was maintained at all informative loci assessed. No new alleles indicative of replication errors were detected.

NB patient 82 III 2 and 82 III 3 are monozygotic twins.
3.4.4. Analysis of chromosomal rearrangement in family HUD.

Pulsed field gel electrophoresis (PFGE) had previously shown affected members of family HUD to contain a genomic rearrangement in the vicinity of the APC gene (Gayther et al., 1993). This finding was of particular interest because affected members of family HUD exhibit a severe phenotype despite having a mutation in a region of APC usually associated with average disease symptoms. To further investigate the alteration, thought to be an inversion of 150 kb, fluorescent in situ hybridisation (FISH) was employed. A set of five yeast artificial chromosome (YAC) clones (Kinzler et al., 1991), from a contig spanning the APC-MCC region of chromosome 5q were selected as probes for FISH. These clones form part of a nine YAC contig spanning 2,000 kb that was used for the original identification of the APC gene (contig 3 described by Kinzler et al., 1991). Recent long range physical mapping has indicated that the order of these YACs relative to centromere and telomere are the reverse of that originally suggested (Ward et al., 1993). The correct order of the YACs is illustrated below (Figure 3.26).

3.4.5. Interphase FISH performed on normal lymphoblastoid cells.

Prior to investigation of patient material a set of pilot experiments were conducted on normal cells, to establish the hybridisation behaviour of the five probes. Each YAC was hybridised to normal metaphase chromosomes and was shown to map to the APC locus on chromosome 5q21-22. None of the YACs were chimeric, although YAC 19AA9 did display weak hybridisation with chromosome 19 due to homologous sequences on this chromosome. A minimum of 20 metaphase spreads were analysed for each YAC probe.

Earlier studies had indicated a correlation between genomic distance and observed separation of FISH signals in interphase nuclei (Trask et al., 1991; Lawrence et al., 1992). However, the range of distances for which this relationship holds true was uncertain, and it was suspected that variations in experimental procedure might influence results. In this study combinations of different pairs of YAC probes were hybridised simultaneously to the interphase nuclei of normal cells (see figure 3.27.).
The probes were differentially labelled and were detected in different colours, either red or green. The separation between the different probes was measured with the assistance of computer software (MRC 500/600, Biorad), after capture of images using a laser scanning confocal microscope. The results indicated a linear correlation between separation observed in interphase nuclei and actual genomic distance, and confirmed that in interphase nuclei probes separated by as little as 44 kb could be resolved. Although the relationship between estimated genomic separation and measured distance in interphase is apparently linear it is somewhat skewed by the excess of overlapping signals recorded for closely situated YACs. Clearly as probes become closer and closer together the proportion of overlapping signals will increase, reaching 100% at the limit of resolution for interphase FISH. Linearity is likely to be lost at an increasing rate as this limit is approached. The exclusion of overlapping signals from the data set provides a more linear relationship (figure 3.28.).
Figure 3.26. Combinations of YACs used to detect chromosomal rearrangement in family HUD.

Five combinations of YACs were assessed during the pilot experiment. Digoxigenin labelled YACs were detected using a series of antibodies (see section 2.5.4.) conjugated to the fluorochrome rhodamine (red). Biotin labelled YACs were detected with avidin-fluorescein isothiocyanate (avidin-FITC) (green).
Figure 3.27. Examples of double FISH performed on normal interphase nuclei.

a) Combination of YACs 9HA11 (red) and 19AA9 (green) hybridised to normal lymphoblastoid cell nuclei. These probes are separated by approximately 50 kb.

b) Combination of YACs 19AA9 (red) and 37HG4 (green) hybridised to normal lymphoblastoid cell nuclei. These probes are separated by approximately 300 kb.
Table 3.4. Separation of contiguous YACs encompassing the *APC* region determined by fluorescence *in situ* hybridisation (FISH) on normal nuclei.

<table>
<thead>
<tr>
<th>YAC combination</th>
<th>Physical separation of YACs (kb)</th>
<th>Total number of signals</th>
<th>Unresolved signals (%)</th>
<th>Mean separation (microns)</th>
<th>Adjusted mean separation (microns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9HA11 + 19AA9</td>
<td>50kb</td>
<td>44</td>
<td>50%</td>
<td>0.214</td>
<td>0.418</td>
</tr>
<tr>
<td>9HA11 + 24ED6</td>
<td>180kb</td>
<td>39</td>
<td>28%</td>
<td>0.404</td>
<td>0.562</td>
</tr>
<tr>
<td>19AA9 + 37HG4</td>
<td>300kb</td>
<td>42</td>
<td>20%</td>
<td>0.596</td>
<td>0.736</td>
</tr>
<tr>
<td>9HA11 + 37HG4</td>
<td>530kb</td>
<td>38</td>
<td>8%</td>
<td>0.778</td>
<td>0.844</td>
</tr>
<tr>
<td>9HA11 + 39GG3</td>
<td>630kb</td>
<td>35</td>
<td>14%</td>
<td>0.766</td>
<td>0.893</td>
</tr>
</tbody>
</table>

1- Estimated physical separation of various combinations of YACs (Kinzler *et al.*, 1991a).

2- Unresolved signals are scored as those in which red and green signals were no longer distinct due to extensive overlap.

3- Mean separation refers to the average distance between signals measured after image capture. Overlapping signals are scored as zero microns separation.

4- The adjusted mean separation is the average measured distance between signals excluding those that could not be resolved.
Figure 3.28. Linear relationship between physical separation of YACs plotted against observed separation of FISH signals.

Observed signal separation (microns)

Data set inclusive of unresolved signals

Data set excluding unresolved signals
Figure 3.29. Non-linear relationship between the proportion of signals overlapping and the physical separation of probes.

3.4.6. Interphase FISH performed on patient material.

The preliminary dual-FISH experiments were repeated on lymphoblastoid cell lines from affected members of family HUD and on normal controls. Any measurements of signal separation deviating from that predicted from actual genomic distance would be indicative of rearrangement. Furthermore, if any of the YAC hybridisation signals appeared to be split this would indicate the existence of a rearrangement breakpoint within the YAC.

Pilot experiments on normal nuclei revealed that YAC probes sometimes gave two signals per chromosome rather than the expected single signal. This phenomenon was due to post-replicative cells in G₂ of the cell cycle, with one signal localising to each chromatid. To eliminate these confusing results patient and normal lymphoblastoid cell lines were starved for fourteen days causing arrest in G₀/G₁ phase of the cell cycle and therefore preventing DNA replication from taking place. Pairs of YACs were selected such that a variety of genomic separations could be assessed across the APC-MCC region. The reporter molecules with which YACs were labelled
and the combinations of YAC investigated were as indicated in figure 3.26. These YAC combinations were considered most informative for analysis of the rearrangement in family HUD.

The influence of sample preparation, hypotonic swelling of cells, spreading of cells onto a slide, and the effects of aspects of the FISH procedure such as denaturation, on the nature of chromatin packaging and organisation are unknown. For this reason normal cells were prepared and subjected to FISH in tandem with patient material. These normal cells served as a reference, indicating the results that should be obtained in the absence of a rearrangement, and circumvented the risk that small differences in experimental conditions might affect results. Indeed comparison of experimental controls and results from the earlier pilot experiment (also conducted on normal material) do display some differences despite using identical methodology. Importantly the linear relationship between signal separation and genomic distance was maintained in both experiments.

Results from the dual-FISH experiment conducted on patient material, alongside results from the relevant normal controls, are presented in table 3.5. Because the patient is heterozygous for the rearrangement the average separations given include interphase measurements taken on both mutant and normal chromosomes. There was no way of distinguishing between the two chromosomes and thus the inclusion of the normal chromosome in the average was unavoidable. Average measurements from three out of four YAC combinations were comparable between normal controls and patient nuclei. Only the combination of YACs 19AA9 and 37HG4 on patient nuclei gave measurements significantly different from normal controls. The most obvious difference was seen in the number of signals scored as overlapping: 40% in patient nuclei compared to only 18.5% in normal nuclei, suggesting that 19AA9 and 37HG4 are situated closer together in this patient. Indeed nuclei in which both pairs of signals were clearly separated were never observed in patient nuclei despite being common in normal controls. The mean separation of signals in patient nuclei was 0.44 microns compared to 0.58 microns in the normal control. Statistical analysis (standard t-test) revealed this to be a significant difference (P<0.01). This is particularly striking given that 50% of measurements made on patient nuclei were conducted on a normal chromosome. The data suggests that the two YACs had been moved closer by deletion of the intervening region, or by an
inversion bringing one clone nearer to the other. This latter hypothesis is in accord with the PFGE data.

An inversion shifting the MCC gene (19AA9) closer to APC (37HG4) is also supported by the remaining dual-FISH results which indicate that APC is not moved relative to YAC 9HA11. Thus, it is MCC that is involved in the inversion and not APC. The telomeric break point of the inversion appears to lie in between MCC and YAC 9HA11. That 9HA11 is not involved is indicated by the fact that measurements involving this YAC did not differ significantly between patient and control samples.
Figure 3.30. Double FISH performed on patient nuclei.

a) and b) Combination of YACs 19AA9 (red) and 37HG4 (green) hybridised to a patient lymphoblastoid cell nucleus. Only one pair of signals is well separated.

c) Combination of YACs 19AA9 (red) and 37HG4 (green) hybridised to a normal lymphoblastoid cell nucleus. Both pairs of signals are well separated.
Table 3.5. Parallel FISH analysis of contiguous YACs encompassing the APC region on patient nuclei and normal control nuclei.

<table>
<thead>
<tr>
<th>YAC combination</th>
<th>Physical separation of YACs (kb)</th>
<th>Total number of signals</th>
<th>Unresolved signals (%)</th>
<th>Mean separation (microns)</th>
<th>Adjusted mean separation (microns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Nuclei</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9HA11 + 24ED6</td>
<td>180kb</td>
<td>56</td>
<td>13%</td>
<td>0.45</td>
<td>0.53</td>
</tr>
<tr>
<td>19AA9 + 37HG4</td>
<td>300kb</td>
<td>42</td>
<td>17%</td>
<td>0.58</td>
<td>0.66</td>
</tr>
<tr>
<td>9HA11 + 37HG4</td>
<td>530kb</td>
<td>50</td>
<td>12%</td>
<td>0.66</td>
<td>0.75</td>
</tr>
<tr>
<td>9HA11 + 39GG3</td>
<td>630kb</td>
<td>58</td>
<td>3%</td>
<td>0.69</td>
<td>0.71</td>
</tr>
<tr>
<td>Patient Nuclei</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9HA11 + 24ED6</td>
<td>180kb</td>
<td>40</td>
<td>25%</td>
<td>0.44</td>
<td>0.59</td>
</tr>
<tr>
<td>19AA9 + 37HG4</td>
<td>300kb</td>
<td>48</td>
<td>40%</td>
<td>0.44</td>
<td>0.73</td>
</tr>
<tr>
<td>9HA11 + 37HG4</td>
<td>530kb</td>
<td>44</td>
<td>9%</td>
<td>0.76</td>
<td>0.84</td>
</tr>
<tr>
<td>9HA11 + 39GG3</td>
<td>630kb</td>
<td>66</td>
<td>6%</td>
<td>0.78</td>
<td>0.84</td>
</tr>
</tbody>
</table>

1- Estimated physical separation of various combinations of YACs (Kinzler et al., 1991a).
2- Unresolved signals are scored as those in which red and green signals were no longer distinct due to extensive overlap.
3- Mean separation refers to the average distance between signals measured after image capture. Overlapping signals are scored as zero microns separation.
4- The adjusted mean separation is the average measured distance between signals excluding those that could not be resolved.
Explanation proposed to explain the fluorescence in situ hybridisation and pulsed field gel electrophoresis data obtained from experiments on family HUD. *MCC* and *APC* are moved closer to one another by the inversion of a fragment containing the MCC gene.
3.5. Analysis of desmoid tumourigenesis.

3.5.1. Mutation analysis of desmoid tumours.

Although rare in the general population desmoid tumours (infiltrative fibromatosis) are relatively common in patients with FAP, affecting up to 13% of individuals (Jones et al., 1986). Mutation analysis was undertaken to determine whether the APC gene has a role in desmoid tumourigenesis. Where possible constitutional and tumour DNA samples were investigated using SSCP, heteroduplex analysis and PTT. In addition to the regions of APC analysed previously (section 3.1.) exons 1-11 were screened by PTT, and exons 5, 10, 12, and 13 were investigated by SSCP (additional analyses performed by Dr. P. Radice, Instituto Nazionale Tumori, Italy).

A germline APC mutation was detected in 10 out of 12 FAP patients that developed desmoid tumours (table 3.6.), and in each case the predicted outcome was truncation of the APC protein. A sample of DNA derived from a desmoid tumour was available from seven of these FAP patients and four were found to contain a somatic mutation of the APC gene, in each case this mutation was additional to a germline mutation (table 3.7.). Constitutional and tumour DNA samples were also available from five individuals that had developed a sporadic desmoid tumour. No germline mutation was identified in these patients, however, somatic mutations were detected in two of their desmoid tumours. Indeed two distinct somatic mutations were detected in desmoid tumour 2324 (table 3.8.). Like the germline alterations all of the somatic mutations were predicted to cause premature truncation of the APC protein, and were characterised by a similar proportion of deletion, insertion, and nonsense mutation to that found in the germline of FAP patients.

The detection of both germline and somatic APC mutations in four of the FAP associated desmoids, and the detection of two somatic mutations in a sporadic desmoid suggests that, as in colorectal tumourigenesis, the APC gene behaves as a classical tumour suppressor during the development of some desmoid tumours.
Figure 3.32. Single strand conformation polymorphism and heteroduplex analysis of desmoid tumours.

Detection of mutations in \textit{APC} exon 15 in five desmoid tumours by a combination of SSCP and heteroduplex analysis.
Table 3.6. Germline mutations of the APC gene detected in FAP patients with desmoid tumours.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Exon</th>
<th>Mutated codon</th>
<th>Nucleotide change</th>
<th>Previously reported</th>
</tr>
</thead>
<tbody>
<tr>
<td>2322*</td>
<td>15I</td>
<td>1543</td>
<td>del (GA) frameshift</td>
<td>No</td>
</tr>
<tr>
<td>2428*</td>
<td>15E</td>
<td>935</td>
<td>TAC to TAA stop codon</td>
<td>Yes</td>
</tr>
<tr>
<td>2443*</td>
<td>5</td>
<td>213</td>
<td>CGA to TGA stop codon</td>
<td>Yes</td>
</tr>
<tr>
<td>2444*</td>
<td>15H</td>
<td>1464</td>
<td>del (AGAG) frameshift</td>
<td>Yes</td>
</tr>
<tr>
<td>75*</td>
<td>15E</td>
<td>1085</td>
<td>del (AACA) frameshift</td>
<td>No</td>
</tr>
<tr>
<td>188*</td>
<td>15H</td>
<td>1461</td>
<td>del (AA) frameshift</td>
<td>Yes</td>
</tr>
<tr>
<td>200*</td>
<td>NONE DETECTED</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>282</td>
<td>15I</td>
<td>1537</td>
<td>ins (AG) frameshift</td>
<td>No</td>
</tr>
<tr>
<td>409</td>
<td>15I</td>
<td>1556</td>
<td>ins (A) frameshift</td>
<td>No</td>
</tr>
<tr>
<td>456</td>
<td>14</td>
<td>636</td>
<td>ins (GC) frameshift</td>
<td>No</td>
</tr>
<tr>
<td>GIB</td>
<td>NONE DETECTED</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEL</td>
<td>15H</td>
<td>1465</td>
<td>del (AG) frameshift</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*FAP patients for which DNA was also available from a desmoid tumour.
Table 3.7. Somatic mutations of the APC gene in FAP desmoid tumours.

<table>
<thead>
<tr>
<th>Tumour</th>
<th>Exon</th>
<th>Mutated codon</th>
<th>Nucleotide change</th>
<th>Previously reported</th>
<th>Germline mutation of APC also detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>2428</td>
<td>15I</td>
<td>1558</td>
<td>ins (A)</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>2443</td>
<td>15I</td>
<td>1534</td>
<td>del (G)</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>2444</td>
<td>15G</td>
<td>1309</td>
<td>del (AAAGA)</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>75</td>
<td>15G/H</td>
<td>UNCHARACTERISED</td>
<td></td>
<td>Yes</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.8. Somatic mutations of the APC gene in sporadic desmoid tumours.

<table>
<thead>
<tr>
<th>Tumour</th>
<th>Exon</th>
<th>Mutated codon</th>
<th>Nucleotide change</th>
<th>Previously reported</th>
<th>Germline mutation of APC also detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>2324</td>
<td>15E</td>
<td>1141-1148</td>
<td>23bp deletion</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>2324</td>
<td>15H</td>
<td>1470</td>
<td>CAA to TAA</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>2323</td>
<td>15H</td>
<td>1451</td>
<td>CGA to TGA</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>2325</td>
<td>NONE DETECTED</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2431</td>
<td>NONE DETECTED</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2442</td>
<td>NONE DETECTED</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.5.2. Investigation of loss of heterozygosity and microsatellite instability in desmoid tumours.

Four sporadic desmoids and seven desmoids from patients with histologically confirmed FAP were tested for a replication error positive (RER\textsuperscript{+}) phenotype characteristic of tumours with deficient DNA mismatch repair. A total of thirteen microsatellite loci were investigated, but no instability was detected suggesting that mutation of genes that function in mismatch repair is not a typical mechanism of desmoid tumourigenesis (Table 3.9.). Comparison of microsatellite loci in matched constitutional DNA and tumour DNA samples failed to provide evidence for loss of heterozygosity at any of the thirteen sites assessed. This included polymorphic loci in the vicinity of the $APC, MCC, DCC, TP53$, and $hMSH2$ tumour suppressor genes.
Table 3.9. Loci analysed for replication errors and loss of heterozygosity in desmoid tumours.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Sporadic desmoids</th>
<th>FAP desmoids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2324 2325 2431 2442</td>
<td>2322 2428 2443 2444 200 75 188</td>
</tr>
<tr>
<td>D2S119</td>
<td>+ + + +</td>
<td>+ + - - + + + +</td>
</tr>
<tr>
<td>D2S391</td>
<td>+ - + +</td>
<td>- + + + - + + +</td>
</tr>
<tr>
<td>D2S123</td>
<td>+ + + +</td>
<td>+ + + + + + + +</td>
</tr>
<tr>
<td>D4S175</td>
<td>+ + + +</td>
<td>+ + + + + + + +</td>
</tr>
<tr>
<td>D5S346</td>
<td>+ + + +</td>
<td>+ + + + + + + +</td>
</tr>
<tr>
<td>D9S156</td>
<td>+ + + -</td>
<td>+ + + - + - - -</td>
</tr>
<tr>
<td>D14S50</td>
<td>- + - +</td>
<td>+ + + + + + + +</td>
</tr>
<tr>
<td>D15S129</td>
<td>+ + + -</td>
<td>+ + - + - + + +</td>
</tr>
<tr>
<td>17 (TP53)</td>
<td>+ + + -</td>
<td>+ + + + + + + -</td>
</tr>
<tr>
<td>D18S61</td>
<td>+ - + +</td>
<td>+ + + + + + - +</td>
</tr>
<tr>
<td>DCC (AT)</td>
<td>+ + + +</td>
<td>+ + - + + + + +</td>
</tr>
<tr>
<td>D19S49</td>
<td>+ + - +</td>
<td>+ + + + - + + +</td>
</tr>
<tr>
<td>D22S351</td>
<td>- + - +</td>
<td>+ + + + + + + +</td>
</tr>
<tr>
<td>APC 15J</td>
<td>+ - - -</td>
<td>- - - + - + + -</td>
</tr>
</tbody>
</table>

+ Informative- constitutional DNA is heterozygous.
- Uninformative- constitutional DNA is homozygous, and LOH studies can not be performed.
3.6. Investigation of chromosome 5q rearrangement / deletion.

Deletion of the APC locus on chromosome 5q21-22 was investigated in six patients. Four patients had deletions that had been detected by routine cytogenetics, and in three cases FISH analysis confirmed that the APC gene had been lost from the deleted chromosome (table 3.10.). These gross chromosomal alterations were investigated with the YAC 37HG4 which spans the APC locus.

Patient KAR was initially referred to the North West Thames Regional Genetics Service with a diagnosis of Treacher Collins syndrome, however, while she possessed some features of this syndrome, she did not exhibit them all. Karyotyping revealed a deletion of 5q: del (5)(q15q23.1), the loss of genetic material from this region presumably explaining the dysmorphic features displayed by this patient. A short time after initial referral a change in bowel habit coupled with rectal bleeding led to further investigation and a diagnosis of FAP. The patient KAR has a daughter, MTR, who has inherited the 5q deletion. Analysis of at least 10 metaphase chromosome spreads per individual by FISH confirmed the loss of the APC region in KAR and MTR. Consequently MTR is at risk of FAP and should therefore receive regular screening by colonoscopy.

Patient BMP, a child showing mild dysmorphic signs, was also karyotyped by the North West Thames Regional Genetics Service. Her karyotype was found to be mosaic for two cell lineages: one having a normal female constitution, the other identical but for an interstitial deletion of chromosome 5 stretching from band q14 to band q21. The two lineages were said to be equally represented in the blood lymphocytes analysed, however, their proportions in other tissues were unknown. Further investigation by FISH was employed primarily to aid the counselling of this family, as standard cytogenetics could not determine whether APC had been deleted. Because of the mosaicism identified in EMP 20 metaphase spreads were examined. No evidence of APC deletion was observed, suggesting that this patient is not at increased risk of colorectal cancer.

At birth the patient KHA displayed a number of malformations including polydactyly, cleft palate, micrognathia, low set ears, bilateral talipes, microosmia,
carp mouth, and downslanting palpebral fissures. Cytogenetic investigation by the Cytogenetics Unit of University College Hospital revealed a chromosomal abnormality, a deletion of chromosome 5q spanning bands q22 to q23.3. Analysis of the APC region using FISH indicated that the APC gene had been deleted, supporting the original cytogenetic findings.

The remaining two patients investigated for deletion of the APC gene were members of confirmed FAP kindreds and had no obvious chromosomal abnormalities. Patient WIN was investigated because molecular analysis had indicated a surprising degree of homozygosity at polymorphic loci flanking and within the APC gene. Intragenic polymorphism in exon 11 was detected by Rsal digestion, while the codon 1493 (151) polymorphism was detected by SSCP. Additional analyses by K. Tsioupra and L. Bowles focused on polymorphisms in the 3' untranslated region of APC and in the 5' non-coding region as well as flanking markers PI227 and ECB27. The patient was homozygous at all loci. Furthermore mutation analysis which had successfully detected mutation in 67% of FAP kindreds (see section 3.1.), was unable to identify any APC mutation in this patient. The second FAP patient, OHA, was investigated because he displayed mental retardation in addition to colorectal polyposis. One of the most consistent phenotypic features of small chromosomal deletions is mental retardation or disturbance of normal behaviour. Both patients were initially investigated with YAC 37HG4. When this failed to reveal a deletion three individual cosmids, from a contig covering the 3' half of the APC locus (figure 3.33.), were employed, providing specific information regarding this region of APC. A high resolution approach using cosmids was considered more likely to detect microdeletions than investigation with YACs (Mandl et al., 1996), however no evidence for deletion was seen in either patient. This analysis was hampered by a rarity of metaphase spreads in the preparation from patient OHA, nevertheless two fluorescent signals could be detected in interphase nuclei using any one of the three cosmid probes (figure 3.35). The investigation was also limited by the unavailability of cosmids covering the 5' third of the APC gene. Consequently it is possible that a deletion removing this part of the APC gene could have gone undetected.
Figure 3.33. Location of YAC-37HG4 and cosmids used for investigation of the APC locus.
Figure 3.34. Fluorescent *in situ* hybridisation analysis of the APC locus using YAC 37HG4.

a) Patient KHA
46, XY, del (5)(q22q23.3)

b) Patient KAR/MTR
46, XX, del (5)(q15q23.1)

c) Patient EMP
46, XX/46, XX, del (5)(q14q21)

A single signal was visible on metaphase chromosomes from patients KHA, KAR and MTR indicating deletion of one copy of *APC*. However two signals were observed on chromosome preparations from patient EMP indicating that 5q deletion does not extend as far as the *APC* locus in this case.
Figure 3.35. Fluorescence *in situ* hybridisation analysis of the *APC* locus using cosmids ym62, ym72 and ym75.

Interphase nuclei from patient OHA showing that each cosmid probe hybridises to both copies of chromosome 5. Consequently it can be deduced that the 3' region of *APC* has not been deleted in this patient.
### Table 3.10. Patients investigated for deletion of the APC locus.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Deletion*/Homozygosity**</th>
<th>Colorectal polyposis</th>
<th>Dysmorphic features/mental retardation</th>
<th>FISH result</th>
</tr>
</thead>
<tbody>
<tr>
<td>KAR</td>
<td>31</td>
<td>46, XX, del (5)(q15q23.1)</td>
<td>Yes</td>
<td>Yes</td>
<td>APC deleted</td>
</tr>
<tr>
<td>MTR</td>
<td>11</td>
<td>46, XX, del (5)(q15q23.1)</td>
<td>No</td>
<td>Yes</td>
<td>APC deleted</td>
</tr>
<tr>
<td>EMP</td>
<td>Infant</td>
<td>46, XX/46, XX, del (5)(q14q21)</td>
<td>No</td>
<td>Yes</td>
<td>APC present</td>
</tr>
<tr>
<td>KHA</td>
<td>2</td>
<td>46, XY, del (5)(q22q23.3)</td>
<td>No</td>
<td>Yes</td>
<td>APC deleted</td>
</tr>
<tr>
<td>WIN</td>
<td>30</td>
<td>Homozygous at 5q21-22 loci</td>
<td>Yes</td>
<td>No</td>
<td>APC present</td>
</tr>
<tr>
<td>OHA</td>
<td>44</td>
<td>Mental retardation</td>
<td>Yes</td>
<td>Yes</td>
<td>APC present</td>
</tr>
</tbody>
</table>

*Large chromosomal deletions determined by routine cytogenetics.

**Homozygous at 6 loci flanking or within the APC gene, revealed by molecular analysis.

Fluorescent *in situ* hybridisation was also employed to further characterise a large germline rearrangement of chromosome 5 in family ALA. Although not segregating FAP members of this family have developed a range of malignancies including cancers of the breast, colon, and uterus. Chromosomal analysis using G-banding had previously revealed the rearrangement to be an inversion: inv (5)(q14.2q31.1). Results of the routine cytogenetic analysis do not support a role for the APC gene in this families cancer predisposition, as APC is centrally located on the inverted fragment, distant from the actual breakpoints. However, PFGE analysis carried out by Simon Gayther had revealed evidence of rearrangement in the vicinity of the APC gene in this family. The precise nature of the putative rearrangement could not be elucidated by PFGE.

To clarify the role of the inversion in this family YACs (9HA11, 19AA9, 24ED6, 37HG4, 39GG3) encompassing the APC region were labelled and simultaneously hybridised to patient chromosomes (figure 3.36(a)). No split signals, which would have indicated a breakpoint in this region, were observed. However, the
mutant chromosome carrying the inversion could be distinguished from the normal by virtue of a shift in the position of the signal. The \textit{APC} region was observed to be relocated distal to it's original position. This suggests that the proximal breakpoint is closer to 5q22 than the distal breakpoint. Further probes shed more light on the inversion: YAC78 mapping to 5q14 was shown to be unaffected by the rearrangement (figure 3.36(b).), whereas the \lambda phage probe GM2\*3A, which maps to 5q32-33, was consistently shifted towards the centromere on one chromosome, occupying a position equivalent to 5q14-15 (figure 3.36(c).). The fact that GM2\*3A is relocated so close to the presumed site of the proximal breakpoint suggests that the distal breakpoint is just telomeric of 5q32-33. These results were confirmed in a dual colour FISH experiment in which YACs from 5q21-22 and GM2\*3A were simultaneously hybridised to patient metaphase spreads (figure 3.36(d).), and indicate that while the proximal breakpoint determined by G-banding is correct the distal breakpoint should be reassigned. Despite revision of the original breakpoints there is no new evidence supporting a role for the APC gene in this family's predisposition to cancer. The DNA fragment of unusual size identified by PFGE may simply reflect a rare sequence variant segregating in this family that abolishes one of the restriction sites used for this analysis, but has no effect on phenotype.
Fluorescent *in situ* hybridisation analysis of patient chromosomes from family ALA:

a) APC contig: YACs 9HA11, 19AA9, 24ED6, 37HG4, 39GG3 mapping to chromosome 5q21-22 and showing a small distal shift in position.

b) YAC 78 mapping to 5q14, unaffected by the inversion in family ALA.

c) Phage probe GM2*3A (5q32-33) shifted towards the centromere on the inverted chromosome 5 (indicated by asterisk).

d) Dual colour FISH of GM2*3A (green) and APC YAC contig (red) hybridised to an ALA metaphase spread. Chromosomes are difficult to visualise because the DAPI (blue) counterstain can not be detected by the confocal microscope used. The mutant (*) and normal (n) chromosomes are shown enlarged (x2) to the left of the picture.
Figure 3.37. Structural abnormalities of chromosome 5 investigated during this study: patients ALA, KHA, KAR and MTR.

Arrows indicate the site of the APC gene and the suggested breakpoints of chromosomal rearrangement in patients ALA, KHA, MAR and MTR.
3.7. Prenatal Diagnosis of FAP.

FAP can be diagnosed before birth by detecting APC mutation in fetal samples. However, the uptake of prenatal testing remains low because termination of an affected pregnancy is not always seen as justified. During the course of this study only one patient requested prenatal diagnosis for FAP. The patient, III 1 from family COO, had also undergone prenatal diagnosis for FAP during an earlier pregnancy. The first pregnancy had been tested using linked polymorphic markers because the causative APC mutation, a 4 bp deletion in exon 14 of the APC gene had not been identified at the time. On this occasion prenatal diagnosis was performed at eleven and a half weeks following transcervical chorionic villus sampling (CVS), and direct mutation analysis was employed: DNA extracted from the sample was amplified by PCR and subjected to heteroduplex analysis. No mutation was detected and consequently the pregnancy was diagnosed as unaffected. This patient has since given birth to an unaffected child.
3.8. Preimplantation Genetic Diagnosis of FAP.


In response to a request for PGD from an infertile member of family HUD (HUD III 5) a strategy for the diagnosis of FAP in single embryonic cells was devised. Genetic analysis of a single cell is associated with a number of difficulties which necessitate extensive preliminary testing prior to the clinical application of any diagnostic protocol. To maximise the probability of successful diagnosis it was decided to amplify a fragment of \( APC \) containing an informative intragenic \( APC \) polymorphism (\( APC^{\text{poly}} \)) as well as a fragment encompassing the actual mutation site at codon 764 (\( APC^{\text{mut}} \)). To provide sufficient DNA for these two independent PCR amplifications isolated single cells were first subjected to whole genome amplification using primer extension preamplification (PEP). Aliquots of the resulting PEP product provided DNA templates for subsequent nested PCR of the two \( APC \) fragments. A maximum of 12 aliquots could be taken from each PEP product, and preliminary experiments demonstrated that diverse loci such as the cystic fibrosis transmembrane regulator (CFTR), \( \beta \)-globin, and the ZFX/ZFY locus could all be amplified from different aliquots of the same PEP product.

A total of 66 patient lymphocytes were isolated and subjected to PEP. Whole genome amplification was successful in 64/66 (97%) cases. Similar efficiencies were also obtained for single blastomeres biopsied from human embryos donated for research. Unsuccessful amplification was probably due to a failure in transferring the single cell to the microcentrifuge tube containing the PCR mixture. Two aliquots were taken from each of the 64 successfully amplified PEP products and were used to amplify \( APC^{\text{mut}} \) and \( APC^{\text{poly}} \) fragments (see section 2A.9(d.) for details of these fragments). This was achieved with amplification efficiencies of 96% and 92% respectively. Mutation detection by SSCP and heteroduplex analysis identified the mutant allele in 86% of \( APC^{\text{mut}} \) amplifications, while the polymorphic allele associated with the mutation was detected in 78% of \( APC^{\text{poly}} \) amplifications. Failure to identify the mutant allele was the consequence of allele specific amplification failure (allele drop-out). Allele drop-out (ADO) was not restricted to the affected allele, in some instances the mutant allele was detected and the normal was not (see...
table 3.11). In 3.1% of single cells the mutant allele could not be detected in either APC$^{\text{mut}}$ or APC$^{\text{poly}}$ fragments due to allele drop-out at both loci on the affected chromosome.
Analysis of isolated lymphoblastoid cells from a line established from an affected member of family HUD (HUD III 5).

Lane 1- Allele drop out affecting the mutant allele- Only the normal SSCP pattern is present with no heteroduplexes.

Lane 2- Detection of mutation and normal allele- Heteroduplex formation and a characteristic pattern of single strands reveals that both alleles have been successfully amplified.

Lane 3- Allele drop out affecting the normal allele- Only the mutant SSCP band is present with no heteroduplexes.
Figure 3.39. Detection of $APC$ polymorphism in members of family HUD.

Single strand conformation polymorphism analysis of $APC^{poly}$ fragments encompassing the common codon 1678 polymorphism in the $A\Delta C$ gene.

Lane 1 - Patient HUD III 5- affected female requesting preimplantation genetic diagnosis. ** indicates the allele associated with IAP in this heterozygous individual (genotype- 1.2).

Lane 2 - Control sample (genotype- homozygous 1.1).

Lane 3 - Control sample (genotype- homozygous 2.2).

Lane 4 - Husband of HUD III 5 (genotype- homozygous 2.2).
Table 3.11. Summary of preliminary single cell work.

<table>
<thead>
<tr>
<th>Fragment of the APC gene amplified</th>
<th>Amplification efficiency</th>
<th>Mutant allele detection efficiency</th>
<th>Amplifications with allele drop-out</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Proportion* affecting mutant allele</td>
</tr>
<tr>
<td>APC\textsuperscript{poly}</td>
<td>92%</td>
<td>78%</td>
<td>22%</td>
</tr>
<tr>
<td>APC\textsuperscript{mut}</td>
<td>96%</td>
<td>86%</td>
<td>14%</td>
</tr>
</tbody>
</table>

*Proportion of total amplifications

Amplifications with allele drop out affecting at least one APC fragment (APC\textsuperscript{mut} and/or APC\textsuperscript{poly}): 48%

Amplifications with allele drop out affecting both APC\textsuperscript{mut} and APC\textsuperscript{poly} fragments from different chromosomes: 6.3%

Amplifications with allele drop out affecting both APC\textsuperscript{mut} and APC\textsuperscript{poly} fragments from the same chromosome: 6.3%

To confirm that ADO was genuine and not simply the result of failure of mutation analysis all samples showing ADO for APC\textsuperscript{mut} were digested with the restriction endonuclease ApoI. This enzyme cleaves mutant but not normal DNA fragments, allowing the two alleles to be resolved by agarose gel electrophoresis. In all cases where ADO had been indicated by SSCP/heteroduplex analysis only one allele was visible after electrophoresis. Increasing the temperature of the denaturing phase of PCR has been reported to reduce ADO, however an increase from 94°C to 96°C had little impact on ADO in this study.
The reliability of diagnosis at one locus alone was considered insufficient for clinical application: 78% for $\text{APC}^\text{poly}$ and 86% for $\text{APC}^\text{mut}$. However if both fragments are assessed together then it is predicted that an affected embryo would be diagnosed as normal in only 3.1% of cases. In support of this figure the misdiagnosis rate observed during preliminary work on patient lymphocytes was also 3.1%. To reduce the probability of misdiagnosis still further it was decided that analysis should only be performed on embryos from which two cells could be biopsied. Assuming that both cells are independent in terms of ADO then the probability of misdiagnosis is approximately 0.1%.

3.8.2. Preimplantation Genetic Diagnosis of FAP: Clinical Application.

Ten oocytes were collected from the patient but only four embryos were normally fertilised with two pronuclei after intracytoplasmic sperm injection (ICSI). On the day of embryo biopsy two embryos had reached the 8-cell stage while the remaining two had reached seven cells. Two blastomeres were biopsied from all four embryos. Following PEP the $\text{APC}^\text{mut}$ fragment was successfully amplified in 7/8 blastomeres and after SSCP and heteroduplex analysis only one embryo (embryo 2) was considered to be normal at this site, both cells showing only the normal allele by SSCP and heteroduplex analysis (table 3.12.). One blastomere from embryo 3 appeared to be anucleate under the dissecting microscope and not surprisingly this cell displayed no amplification at either locus. Amplification of the $\text{APC}^\text{poly}$ fragment failed with the other blastomere from the same embryo, and analysis of the $\text{APC}^\text{mut}$ revealed only an affected allele. As it is not possible for these parents to produce a mutant homozygote this result indicates that allele drop-out of the normal allele had occurred. The $\text{APC}^\text{poly}$ fragment was successfully amplified in 6/8 cells (table 3.12.), and provided confirmatory results regarding the affected status of embryos 1 and 4, and the unaffected status of embryo 2 (table 3.12.). Embryo 2 was transferred on day 4 but unfortunately no pregnancy resulted.

The accuracy of the genetic test was assessed by analysing all the remaining cells from the embryos which were not transferred. The results obtained were concordant with the previous findings, all three embryos not transferred were carrying the mutant allele.
Figure 3.40. Detection of *APC* mutation in four embryos from family HUD.

<table>
<thead>
<tr>
<th>Embryo number</th>
<th>Cell 1</th>
<th>Cell 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mutant allele → Normal allele

Heteroduplex → Homoduplex

SSCP and heteroduplex analysis of *APC* fragments for the preimplantation diagnosis of FAP in family HUD.

Embryo 1 Both cells analysed display an heterozygous (affected) pattern of single strands as well as heteroduplexes.

Embryo 2 Both cells analysed display a normal (unaffected) SSCP pattern.

Embryo 3 In one cell an affected allele was detected by SSCP but no normal allele was identified. This suggests that allele drop out of the normal allele has occurred. Amplification failed in the second cell tested.

Embryo 4 A normal allele was detected in one of the two cells assessed, while the other cell was found to be heterozygous (affected). Thus allele drop out affecting the mutant allele seems to have occurred in the first cell.
Figure 3.41. Detection of \( APC \) polymorphism in four embryos from family HUD.

<table>
<thead>
<tr>
<th>Embryo number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SSCP and heteroduplex analysis of \( APC_{\text{poly}} \) fragments for the preimplantation diagnosis of FAP in family HUD.

Embryo 1  One cell appears heterozygous and therefore affected, while the second seems to be homozygous normal, probably as a consequence of allele-drop out.

Embryo 2  Both cells analysed display a homozygous normal SSCP pattern.

Embryo 3  Amplification of the \( APC_{\text{poly}} \) fragment failed in both cells assessed.

Embryo 4  Both cells sampled give a homozygous SSCP pattern but for opposite alleles. The most likely explanation for this observation is that both cells were actually heterozygous and that allele-drop out has occurred affecting a different allele in each case.
Table 3.12. Genetic analysis of preimplantation embryos tested for FAP.

<table>
<thead>
<tr>
<th>Embryo Number</th>
<th>Cell 1 Diagnosis of:</th>
<th>Cell 2 Diagnosis of:</th>
<th>Final Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>APC&lt;sup&gt;poly&lt;/sup&gt;</td>
<td>APC&lt;sup&gt;mut&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>polymorphism</td>
<td>mutation</td>
<td></td>
</tr>
<tr>
<td>Embryo 1</td>
<td>A</td>
<td>A</td>
<td>N* A</td>
</tr>
<tr>
<td>Embryo 2</td>
<td>N</td>
<td>N</td>
<td>N N</td>
</tr>
<tr>
<td>Embryo 3</td>
<td>F</td>
<td>A+</td>
<td>F F</td>
</tr>
<tr>
<td>Embryo 4</td>
<td>N*</td>
<td>A</td>
<td>A A</td>
</tr>
</tbody>
</table>

Results gained from the four biopsied embryos. Two cells were assessed per embryo at an intragenic APC polymorphic site (15J) and at the APC mutation site. Successful PCR allowed embryos to be diagnosed as affected (A) or normal (N). Failure of PCR amplification (F) was observed in 3 tests.

* Allele drop out of the mutant allele.
+ Allele drop out of the normal allele.
Chapter 4
Discussion
Discussion

4.1. Mutation analysis

The advent of the polymerase chain reaction has led to the development of a variety of techniques allowing the detection of single base alterations in fragments of genomic DNA. These methods have become increasingly important for the research of genetic disease, and indeed in molecular biology as a whole. Furthermore, mutation detection is now well established as a clinical procedure, applied to the diagnosis of genetic disease (e.g. Takahashi et al., 1994). Mutation analysis has long been applied to the detection of DNA variants that underlie rare inherited disease, however it is only recently that these protocols have become more widely applied. The discovery of mutations in cancer genes and genes implicated in multifactorial disease are largely responsible for shifting mutation detection into this more general arena.

The detection of single base changes can be employed to identify polymorphic sites in the genome, as well as mutations causing disease, thereby providing a resource of genetic markers for linkage analysis and aiding the localisation of disease genes (Gusella et al., 1983; Gitschier et al., 1985). Once a candidate gene has been isolated identical or related methods can be used for mutation analysis. The discovery of genetic alterations in a candidate gene, found solely in affected patients, provides compelling evidence for a relationship between the disease and the mutated gene. This strategy has been used for the isolation of a growing number of genes, and with the current popularity of positional cloning approaches for gene identification, the trend is likely to continue.

Methods that identify changes in DNA sequence can be employed for presymptomatic diagnosis. Individuals destined to develop diseases such as porphyria (Lee et al., 1995) or genetically predisposed to cancer (Olschwang et al., 1995) can often be identified before the onset of disease. If desired diagnosis can usually be performed at a prenatal stage (Xu et al., 1993; Schwartz et al., 1992). Indeed a small
number of genetic disorders, for example cystic fibrosis and Marfan syndrome, have even been diagnosed in embryos prior to implantation (Handyside et al., 1992; Harton et al., 1996). Thus methods for the detection of single base alterations are used from a stage before a disease gene is cloned right up until mutations of that gene are assessed clinically for diagnostic purposes.

As more mutations are identified within a disease gene the effect of mutation on phenotype is often better understood. It may become clear that additional prognostic information (age of onset, severity of symptoms, etc.) can be gained from detailed knowledge of the mutation carried by an affected individual, such as in cystic fibrosis (Gilber et al., 1995) and familial adenomatous polyposis (Caspari et al., 1994). Such information may assist clinicians in defining more appropriate strategies for the treatment of individual patients (Guldberg et al., 1995). Furthermore, by characterising mutations that cause disease an insight may be gained into the mechanisms responsible for mutation of the gene under investigation. A number of carcinogens have been found to induce specific mutation, such as a guanine to thymine transversion at codon 249 of the TP53 gene frequently found in lung cancers from uranium miners exposed to high levels of radon gas (Taylor et al., 1994). The discovery of a region of the gene which contains a disproportionate number of mutations relative to its size (a hotspot) may provide information about the critical regions of the protein that it produces. If a hotspot is identified then future mutational screening strategies can be targeted at the area of clustering before other areas of the gene are investigated, saving time and reducing expense.

There are a variety of mutation detection systems currently available each having advantages and drawbacks when compared to others. Generally the choice of which technique to employ is determined by the proportion of mutations that need to be detected (the efficiency of the method) and the funds and time available (the cost of the method). Ultimately an appropriate compromise between these factors must be reached.
4.1.1. Mutation analysis in FAP patients.

This study was undertaken shortly after the cloning of the APC gene on chromosome 5q21-22, and the first priority was analysis of the mutational spectra within this gene. The cloning of APC was followed by an intense period of mutation analysis conducted on constitutional DNA from FAP patients as well as DNA from sporadic colorectal cancers and other neoplasia. A variety of mutation detection techniques were employed by a large number of groups these included RNase protection (Nishisho et al., 1991; Miyoshi et al., 1992b, Nagase et al., 1992a), single strand conformation polymorphism (SSCP) (Cottrel et al., 1992; Paul et al., 1993, Varesco et al., 1993), denaturing gradient gel electrophoresis (DGGE) (Fodde et al., 1992, Olschwang et al., 1993a), heteroduplex mismatch analysis (Hamzehloei et al., 1994), direct sequencing (Powell et al., 1992), chemical mismatch cleavage (Powell et al., 1993; Prosser et al., 1994), and in vitro transcription/translation (TNT or PTT) approaches (van der Luijt et al., 1993; Powell et al., 1993; Prosser et al., 1994).

The first APC mutations reported were mainly small deletions and insertions and as a result the mutation detection systems initially selected for this investigation were single strand conformation polymorphism (SSCP) and heteroduplex analysis. Later an in vitro synthesised protein assay (protein truncation test- PTT) was also introduced to detect nonsense and frameshift mutations. Together these methods provide a means of detecting most alterations of DNA sequence. Constitutional DNA from 52 FAP patients was analysed using these techniques, while FISH performed on chromosomal preparations was used to investigate large genomic rearrangements involving chromosome 5. In total 60% of the APC coding region (over 5,000 bp) including exons 6, 8, 11, 14, and the 5’ half of exon 15 was analysed. Mutation was detected in 67% of FAP patients.

In excess of 730 different APC germline mutations have now been described (Beroud and Soussi, 1996). Nagase and Nakamura (1993) surveyed 174 published germline mutations in unrelated FAP patients and found mutations scattered throughout the 5’ half of the gene. The cohort of 52 patients assessed in this study provided a similar picture. Analysis of the 5’ half of APC revealed mutations in all exons of the gene assessed, with the exception of exon 11. However, mutations were
not evenly distributed within this region showing a distinct clustering between codon 1060 and codon 1465. Forty eight percent of FAP patients investigated had mutation in this region, an identical proportion to that found in the literature (Nagase and Nakamura, 1993). Five sites were found to be mutated in more than one unrelated FAP kindred, in particular 5 bp deletions at codon 1061 and 1309 were found to be responsible for approximately 10% and 19% of all cases of FAP respectively. Again this resembles the published frequencies of these mutations.

All mutations detected during this investigation are predicted to cause truncation of the APC protein, as are a striking 97% of published mutations causing FAP (Nagase and Nakamura, 1993). This suggests that in the colon the tumour suppressing function of the APC protein is relatively insensitive to missense mutation, and inactivation is only achieved by eliminating the carboxyl-terminus of the protein. The majority of germline mutations (85%) were found to be the consequence of small deletions or insertions. These disturb the reading frame of transcription, inevitably causing the introduction of a premature stop codon at some distance downstream. Deletions of between 1 and 5 bp accounted for the majority of frameshift mutations (64% of detected APC mutations). Insertions, of 1-2 bp, were responsible for a smaller proportion (21% of APC mutations). The preponderance of deletions and insertions, especially in exon 15 of APC, is thought to be a consequence of nucleotide slippage (Trinh and Sinden 1991). Repeated or palindromic sequences may form complex secondary structures facilitating intra-strand misalignment of repeats during DNA replication. Sequences of this kind are relatively common in the 5' half of APC exon 15. The frequently reported deletion at codon 1309 for example occurs within a homopolymeric run of bases (Groden et al., 1993). The relative mutation rate within this sequence is approximately 85 times higher than the average for the rest of the gene (Cottrell et al., 1992). Although slippage is the most likely explanation there are several alternative possibilities explaining the increased mutation rate at certain sites. These areas of the gene may have a chromatin structure, or position in the nucleus which leaves them accessible to carcinogens, or they may be susceptible to unequal sister-chromatid exchange.

Mutations that truncate the APC protein are also caused by point mutation. Single base substitutions leading to the introduction of a premature stop codon were responsible for 15% of the detected APC mutations. This is somewhat less than the
33% indicated by review of the literature, and may reflect differences in the region of the APC gene assessed (Miyoshi et al., 1992a; Nagase et al., 1992b; Nagase and Nakamura, 1993). Single base substitutions are found concentrated in the first 14 exons of APC, where they account for about 75% of all reported mutations. This contrasts with the 6.5 kb exon 15 in which only 20% of mutations involve substitution of a single base. The identification of mutation hot-spots in the 5' half of exon 15 coupled with the large size of the APC coding region (8,500 bp), has caused many groups to focus their mutation detection efforts exclusively on this region of APC. This was also the case in the present study which only investigated four of the first fourteen exons of APC. As a result the proportion of mutations identified that are single base substitutions is expected to resemble the frequencies observed in exon 15 rather than those for the whole gene, thus a deficiency of nucleotide substitutions relative to frameshift mutations is not unexpected.

Point mutation is also responsible for nucleotide transitions at splice consensus sequences which account for 3% of published germline APC mutations (Nagase and Nakamura, 1993). A single splice site alteration was detected in this study which involved a guanine to adenine transition at the first position of the splice donor following exon 8. The mutation: AAG/gta to AAG/ata affects the highly conserved first position of the splice donor sequence (Breathnach et al., 1987). Such an alteration is predicted to cause aberrant splicing. This may manifest as exon skipping, inefficient or out of frame splicing, or alternatively the splice donor may be rendered completely without function as has been reported for mutations of this type in a splice donor of β-globin (Treisman et al., 1982). In most cases, including this one, it is likely that splice site mutations give rise to truncated proteins and therefore have a similar effect to nonsense or frameshift mutation.

Missense mutations have also been reported in the APC gene, although they account for a remarkably small proportion (3% of all mutations) (Nagase and Nakamura, 1993). Due to their scarcity it is not clear whether all substitutions represent genuine disease causing mutations, or whether some may be rare variants linked to the disease allele, but not directly responsible for FAP phenotype. Four rare missense mutations were identified during this study, however in each case it was possible to demonstrate that the alterations were not associated with FAP. This was achieved by further mutation analysis leading to the identification of a protein
truncating mutation elsewhere in the gene, or by detection of the variant in unaffected family members. In all but one case the missense mutation identified was unique to a single family, and was only detected once in a screen of 50 unrelated individuals.

In 74% of FAP patients with a point mutation the change involves alteration of cytosine and in 44% of these cases it occurs at a CpG site (Nagase and Nakamura, 1993). Most of the C to T transitions at CpG sites are probably due to spontaneous or enzymatic deamination of 5-methylcytosine (Coulondre et al., 1978). Transitions occurring at CpG dinucleotides in the TP53 tumour suppressor gene have been observed in a wide variety of cancers (Hollstein et al., 1991). Four out of five point mutations detected during this study were transitions from cytosine, however none of these affected CpG sites.

The few studies that have involved complete screening of the entire APC gene have demonstrated that most studies fail to detect approximately one third of the mutations causing FAP (Miyoshi et al., 1992a; Nagase et al., 1992b; Groden et al., 1993; Olschwang et al., 1993b). Even the most extensive studies reported to date have only detected mutations in 80-90% of FAP patients (Powell et al., 1993; Prosser et al., 1994). Powell et al (1993) were able to detect mutations in 82% of unrelated FAP patients (51 out of 62) by assessing APC expression and by using a protein truncation test. Prosser et al. (1994) surpassed this figure using an array of techniques including fluorescent in situ hybridisation (FISH) to detect chromosomal alterations, reverse transcription-polymerase chain reaction (RT-PCR) for the purpose of identifying splicing defects, and also the almost 100% efficient mutation detection method chemical mismatch cleavage analysis. Despite this in depth analysis APC mutation remained unidentified in 10% of patients studied.

Limitations of the mutation analysis techniques employed for screening the APC gene may explain the failure to detect mutation in some FAP families. Small deletions, invisible to routine cytogenetics, may remove entire exons and therefore be refractory to PCR based strategies of mutation analysis. Such alterations are known to affect some FAP patients, indeed two deletions of 100 and 260 kb, which remove exons 2 to 15 and exons 10 to 15 respectively, were instrumental in the localisation and cloning of APC (Joslyn et al., 1991; Groden et al., 1991). Deletions and other rearrangements of this type could possibly be detected by pulsed field gel
electrophoresis (PFGE). However, no such changes were identified in 40 FAP patients analysed by PFGE and Southern blotting with cDNA probes encompassing the APC locus (Cottrell et al., 1992). Other possibilities are the use of Western blotting and antibody analysis for the detection of truncated APC proteins (Smith et al., 1993), and the use of fluorescence in situ hybridisation (FISH) using cosmid probes specific for individual regions of APC (Mandl et al., 1996).

Another possible explanation for failure to detect mutation in 100% of FAP patients is that mutations lie within regions of the gene not assessed. Such areas include introns, 5' untranslated regions, promoter sequences, and exons only recently discovered or as yet undiscovered. Extensive studies of APC expression have demonstrated a number of alternative transcripts and several exons that were not identified when the gene was first cloned (Oshima et al., 1993; Thliveris et al., 1994; Samowitz et al., 1995; Sulekova et al., 1995; Xia et al., 1995; Sulekova and Ballhausen, 1995; Kraus et al., 1996).

Although intronic mutations may have a significant impact on gene expression the large size of the APC locus (spanning over 300 kb) prohibits the searching for mutations across the entire region (Joslyn et al., 1991; Groden et al., 1991). The additional exons identified at the 5' end of the gene (Oshima et al., 1993; Thliveris et al., 1994) and between exons 10 and 11 (Xia et al., 1995; Sulekova and Ballhausen, 1995) are more accessible to mutation analysis, but have not been widely studied. Similarly there is little data on analysis of the 5' non-coding region. Mori et al. (1993) assessed this region in 28 unrelated FAP patients for whom no APC mutation had been identified, but detected no mutations. It seems certain that a small number of FAP patients will have mutation in regions associated with the control of transcription, but detecting them will probably have to rely on quantitative RT-PCR analysis of patient RNA.

Another explanation for the third of FAP patients for whom mutation in APC cannot be found is that they have colorectal polyposis as a consequence of mutation in a gene other than APC. This seems improbable as no evidence of genetic heterogeneity was reported during efforts to localise the gene responsible for FAP (Bodmer et al., 1987; Leppert et al., 1987). If a second gene were involved it would have to show tight linkage to the APC gene (5q21-22). The only obvious candidate in this region is the mutated in colorectal cancer (MCC) gene. This gene lies just 30-150
kb distal of \textit{APC} (Joslyn \textit{et al.}, 1991; Groden \textit{et al.}, 1991), and has been shown to carry mutations in a subset of sporadic colorectal cancers (Kinzler \textit{et al.}, 1991a). However no germline mutations of \textit{MCC} have ever been reported in FAP patients (Nishisho \textit{et al.}, 1991; Groden \textit{et al.}, 1993), and some evidence has cast doubt on whether it genuinely functions as a tumour suppressor in colorectal cancer (Curtis \textit{et al.}, 1994).
Figure 4.1. Distribution of published *APC* mutations in FAP patients.

(Nagase and Nakamura, 1993)
Figure 4.2. Distribution of germline APC mutations detected during this study.
4.1.2. Fluorescence in situ hybridisation for the detection of APC deletion.

In addition to the analyses of DNA sequence already discussed suspected APC deletions, impossible to confirm using routine cytogenetics, and rearrangements of chromosome 5q were investigated in seven patients. The use of fluorescent in situ hybridisation (FISH) provides a sensitive means of detecting alterations of chromosome structure affecting a specific region of the genome. FISH allows accurate mapping of DNA probes onto high resolution chromosome preparations. Rearrangements localised to the same region as a probe by routine cytogenetics can then be reassessed relative to this specific marker. DNA fragments of as little as 250 bp have been investigated using FISH (Richard et al., 1994), however the detection of such small fragments is technically difficult, standard protocols focus on the use of cosmid and YAC clones. These larger probes give sufficiently intense signals that they may be viewed in interphase nuclei as well as on metaphase chromosomes. This is advantageous as it allows the copy number of any specific chromosomal region to be determined even in instances where the sample cannot be cultured to produce good quality metaphase chromosomes. Such interphase cytogenetics has been applied to direct preparations from tumours and preimplantation genetic diagnosis of chromosome abnormalities (Matsuura et al., 1996; Harper et al., 1995).

Three of the four patients with deletion involving 5q were found to have deletion of the APC gene, although only patient KAR was old enough (31 years) to display colonic symptoms of the disease. All the patients had a variety of features not directly attributable to loss of the APC gene. These presumably reflect the loss of additional genetic material from chromosome 5q. Although Rivera et al. (1990) concluded that there were few features common to patients with 5q deletion there do seem to be some similarities. A long mid-face, receding hairline, prominent forehead, abnormal ears, high arched palate, and mild mental retardation were seen in patients KAR and MTR and have also been reported by a number of other authors (Hockey et al., 1989; Cross et al., 1992; Hodgson 1993). Cleft palate displayed by patients MTR and KHA has also been reported several times (Rivera et al., 1990; Ohdo et al., 1982; Lindgren et al., 1992; Silengo et al., 1981).

It is interesting to note that patient KHA displays the broadest spectrum of severe abnormalities despite having the smallest deletion of chromosome 5. This
suggests that there may be genes mapping to the region deleted in this patient alone, 5q22-23.3, that are responsible for these differences. Another possible explanation for the disparity in phenotypes could be the effects of genomic imprinting. Parental origin of the mutant chromosome is known for two of the patients sharing deletion of 5q22-23.1. Patient KHA inherited a deleted chromosome 5 from his father whereas patient MTR inherited her abnormal chromosome from her mother. If certain genes within the deleted region are imprinted such that maternally inherited copies are silent then patient KHA would have no expression of these genes whereas patient MTR would achieve normal expression levels. Alternatively, KHA, the only male with a deletion of 5q in this study, could be experiencing more severe effects due to his sex. However, there is no indication in the literature that deletion of 5q is associated with more severe consequences for males than females. Patient EMP is mosaic, approximately 50% of her cells having a normal chromosomal constitution. Not surprisingly this patient has the mildest dysmorphic features of the patients investigated.

One of the malformations displayed by patient KHA is polydactyly. This is intriguing in view of the proposed role of APC in some forms of apoptosis and the function of the *Drosophila* homologues of APC, β-catenin, and GSK3β in segment polarity and other aspects of cell fate (Peifer *et al.*, 1994). Furthermore, mice carrying an *Apc* mutation have been seen to display digit abnormalities (R. Fodde CAPP study group meeting). However, polydactyly is not a frequent finding in patients with deletion of the APC locus and may therefore be the consequence of deletion of genes other than *APC*.

Interestingly three of KH’s six siblings also had a range of dysmorphic features at birth. Investigation of the rest of the family (carried out by Dr. R. Hastings) revealed the father to be the carrier of a balanced intrachromosomal insertion with the karyotype 46, XY, ins (10;5)(q25;q22q23.3), and all the affected children to be carriers of the deleted chromosome 5. In addition one child, who displayed no gross abnormalities, carried the derivative chromosome 10, and therefore possessed three copies of 5q22-23.3. Clearly the presence of the intrachromosomal insertion will place any of the father’s future offspring at a high risk of an unbalanced chromosomal constitution.
Cytogenetically invisible deletions were investigated in two FAP patients. Patient WIN had remarkable levels of homozygosity for markers flanking and within the APC locus, which was suggestive of a deletion. The other patient, OHA, displayed mental retardation which may also have been the consequence of deletion. The use of a YAC spanning the APC locus (YAC 37HG4) failed to detect any rearrangement. However, the large size of the YAC means that a FISH signal could still be generated even if significant regions of the APC gene were deleted. For this reason a high resolution approach using cosmids covering the 3' half of the APC locus was employed for a more detailed assessment. This method has been successfully applied to the detection of cytogenetically invisible deletions in other FAP patients (Mandl et al., 1996). Once again no deletion was detected, although it is impossible to exclude the possibility that deletions occurred in the 5' half of APC. It may be that both of these patients have FAP as a consequence of undetected mutation in the APC coding sequence. The high levels of homozygosity detected in patient WIN could be the result of a consanguineous marriage or possibly uniparental disomy for chromosome 5. Uniparental disomy (UPD), the phenomenon where both copies of a chromosome are derived from a single parent, is often the result of spontaneous correction of a trisomic or monosomic conceptus (Bartsch et al., 1994). There is usually some persistence of the original cell line however, and this was not detected during FISH analysis. Furthermore, some impact on the patients phenotype might be expected if a chromosome as large as chromosome 5, which is likely to contain some imprinted genes, were provided by a single parent alone. No unusual features were observed in this FAP patient.

An additional family with a chromosome 5 inversion (q14.2q31.1), family ALA, was also investigated. While patients from this family do not have FAP they do display predisposition to a variety of tumours including colon, uterus, and breast. Pulsed field gel electrophoresis conducted by Simon Gayther had detected an aberrant sized fragment of DNA in the vicinity of the APC gene. This would appear to be unrelated to the large paracentric inversion in this family, unless the original cytogenetic findings had been incorrect. To further characterise the rearrangement(s) single and dual colour FISH using various probes were employed. The results supported the definition of the proximal breakpoint but indicated that the distal
breakpoint should be reassigned from 5q31.1 to 5q32-33. Despite this reassignment there still remains no evidence for involvement of APC in this families cancer predisposition. The unusual fragment observed during PFGE may have been an experimental artefact or the consequence of a second rearrangement unrelated to the large inversion. It is possible that the breakpoints of the inversion coincide with another gene which influences the development of cancer. For example the hMSH3 gene which functions in DNA mismatch repair maps to 5q. Mutations of hMSH3 have been detected in endometrial cancer (Risinger et al., 1996), while mutations of the homologous hMSH2 gene are found in the germline of up to 50% of patients with hereditary nonpolyposis colorectal cancer (HNPCC), a syndrome associated with colorectal and uterine cancers. Other possible candidate genes could be identified after further analysis using pulsed field gel electrophoresis (PFGE) and FISH to refine the breakpoint localisation in family ALA.

4.2. Association between site of APC mutation and phenotype of FAP patients.

As the number of APC mutations characterised has increased, a correlation between site of mutation within the APC gene and certain aspects of the FAP phenotype has become apparent (Nagase et al., 1992; Olschwang et al., 1993; Caspari et al., 1994). Even before the cloning of APC linkage analysis had suggested that classical FAP and Gardner syndrome, a disease characterised by osteomas, desmoid tumours, epidermoid cysts, as well as colonic polyps, are the consequence of mutation at the same locus (Leppert et al., 1990). This finding was suggestive of a diversity of mutant APC alleles with different consequences for FAP phenotype. The fact that the number of polyps developed and age of cancer onset is reasonably consistent among members of the same family, but may differ between families, also hinted at a genetic basis for the variation in phenotype (Utsunomiya et al., 1989). A correlation between genotype and phenotype is not without precedent, associations between position and/or type of mutation and severity of disease has been reported for a number of conditions including Duchenne and Becker muscular dystrophies, and in pancreatic disease occurring in cystic fibrosis patients (Dean et al., 1990, Beggs et al., 1991).
Early reports of mutation analysis in the APC gene did not provide any data to support the existence of a genotype-phenotype correlation. Paul et al. (1993) argued that identical APC mutations can result in a variable phenotype both within and between APC pedigrees. However their sample size was small and the age matched data insufficient for a convincing study, particularly in regard to any correlation between number of polyps and specific mutations. Varesco et al. (1993) also reported no correlation between genotype and phenotype in 12 APC patients. However phenotypic information was not available for some of the patients and only 2 mutations were found outside the mutation cluster region (codons 1286 and 1513). Problems of ethnic variation, increasing numbers of polyps with age, and definition and quantification of phenotypes make data from different sources difficult to compare. Despite these difficulties the existence of a genotype-phenotype correlation in FAP is now accepted, supported by a considerable volume of evidence.

The first association to be identified was a correlation between site of mutation within the APC gene and both the number of adenomas developed and age of colorectal cancer onset. Familial adenomatous polyposis patients with an APC mutation lying between codon 1255 and codon 1467 were reported to display more severe symptoms (Nagase et al., 1992a). Thousands of colonic polyps (profuse type), rather than the more classical hundreds of polyps (sparse type) (Bussey, 1975), are found in the colorectum of such patients and progression to malignancy is accelerated accordingly. The findings of his study generally support the initial report of Nagase et al. (1992) as have the findings of other groups (Caspari et al., 1994), defining a region of APC where protein truncating mutations are associated with profuse numbers of polyps. It is clear that the severe region in terms of age of onset/number of polyps begins no later than codon 1309, ten patients from this study have mutation at this codon and the nine for which phenotypic data is available are all severely affected. Nagase et al. (1992a) succeeded in identifying a precise 5' border to the profuse region after finding patients with mutations at codon 1249 and 1250 that had sparse and profuse phenotypes respectively. However, it was not clear from their data whether there is also a sharp 3' boundary between regions of APC that cause severe or average FAP symptoms or whether this border is blurred giving rise to a variable or intermediate phenotype. The detection of mutations at codon 1464 in patient 540 IV 1 who developed more than 2,000 polyps and at codon 1465 in three patients (from
families BLO, 522, VEL) with average phenotypes appears to define a precise 3' border for this region. This data is compatible with that of Nagase et al. (1992a) who detected a mutation at codon 1330 causing profuse numbers of polyps and identified two families with mutation at codon 1465 that had polyp numbers defined as sparse. Defining the severe region as encompassing codons 1250-1464 allows inclusion of 13 out of 14 severely affected patients from this study, and exclusion of 17 out of 17 patients with an average phenotype. Not surprisingly this association between genotype and phenotype is highly statistically significant (significant at the 0.001% level, $\chi^2$ test). A single family, family HUD, did not fit the genotype phenotype correlation. Individuals from this family were classed as severely affected having developed over 2,000 adenomas and cancer during their twenties, and yet had mutation at codon 764 of APC, a region associated with an average phenotype. Two additional families, MAR and DOU, have an identical mutation at this site, but present with average symptoms. Members of family HUD were subjected to further analysis and were shown to carry a second germline mutation in the vicinity of APC which may influence their phenotype (discussed fully in section 4.3.).

Interestingly, an association between cases of FAP resulting from fresh mutation and APC mutation between codons 1309 and 1464 was also noted. This clustering within the severe region explains the observation that 68% of new mutation patients had a severe phenotype compared to only 15% of patients in which FAP could be shown to affect several generations (significant at the 0.01% level, $\chi^2$ test). A large proportion of the new mutations in this region of APC were the consequence of mutation at codon 1309. Eight out of ten mutations at this site affected new mutation patients, emphasising how mutable this codon appears to be. Familial adenomatous polyposis is a disease which is usually lethal at a post-reproductive age and has a high new mutation rate (20-30% [Bulow, 1987]). Early onset of cancer in many of the patients with fresh mutations, and death before the end of normal reproductive life, may be responsible for maintaining APC mutations at a relatively low level in the population.

The recent detection of mutations at APC codon 1597 and 1978 has demonstrated that mutations at the 3' end of APC give rise to a mild phenotype (Scott et al., 1995; Friedl et al., 1996). An attenuated form of FAP is also seen in kindreds with mutations 5' of codon 157, while mutations 3' of codon 168 give rise to a
classical FAP phenotype (Spirio et al., 1993). The milder form of the disease is characterised by relatively few polyps (generally less than 100) and a later onset of cancer (Spirio et al., 1993; Wallis et al., 1994). The expression of colonic symptoms within these families is often very variable, some patients developing over 100 polyps and other rare patients showing non-penetrance. Further mutation analysis may allow the identification of additional regions of _APC_, associated with specific phenotypic features, for example there are suggestions that certain mutations of exon 11 may be associated with a severe phenotype (Eccles et al., unpublished). The regions of _APC_ that give rise to a mild phenotype were not assessed during this study, and this probably explains why no mutations were detected in the two families considered to have a mild phenotype. Patients from families GIB and WYA have a history of late onset of cancer and may therefore have mutation at the 3' end or at the extreme 5' end of the _APC_ gene.

Congenital hypertrophy of the retinal pigment epithelium (CHRPE) has also been found to show an association with site of mutation (Spirio et al., 1993; Olschwang et al., 1993b; Wallis et al., 1994; Caspari et al., 1995). Patients with mutation prior to exon 9 or beyond codon 1444 of _APC_ do not develop CHRPEs. While those with mutation in the intervening portion of the gene are positive for these benign lesions. Thus, the majority of FAP patients (approximately 90%) express CHRPE allowing this feature to be used as a diagnostic marker of the disease in this subset of families (Burn et al., 1991; Iwama et al., 1990). In this study only one family out of sixteen for which CHRPE and mutation status are known does not fit the association outlined above. Family 523 is CHRPE positive despite having mutation in exon 6 of _APC_. This may be the result of chance occurrence of CHRPE, small numbers of which are sometimes detected in individuals unaffected by FAP. Alternatively modifying loci, linked or unlinked to _APC_, may have some influence on CHRPE expression in FAP kindreds. The CHRPE negative status of families GIB and WYA are in accord with that expected for mutation in the 5' or 3' end of the _APC_ gene as suggested above.

Only negative reports of correlation between site of mutation and the extracolonic manifestations of FAP displayed in Gardner syndrome were described for some time (Nishisho et al., 1991; Groden et al., 1991 and 1993; Varesco et al., 1993; Nagase et al., 1992a; Paul et al., 1993). However, recent reports suggest that at
least one of these features, growth of desmoid tumours, is influenced by the region of the APC gene mutated (Caspari et al., 1995; Scott et al., 1996). A non-metastatic, but frequently life threatening form of tumour, desmoids are rare in the general population, but are found in approximately 13% of FAP patients (Jones et al., 1986). All FAP patients have an increased probability of developing desmoid tumours, however, a smaller group with mutation between codons 1445 and 1578 appear to be at a much higher risk with virtually all mutation carriers displaying desmoids (Caspari et al., 1995). Such individuals also frequently show osteomas, epidermoid cysts, and polyps of the upper gastrointestinal tract, although these features do not seem to be entirely confined to patients with mutation in the desmoid prone region (Caspari et al., 1995). Desmoid tumours were developed by six of the original 52 FAP patients investigated (15%). An addition six FAP patients with desmoid tumours were investigated in collaboration with Dr. P. Radice (Instituto Nazionale Tumori, Italy). Constitutional APC mutation was detected in ten of these twelve patients, and in six cases this was situated between codon 1445 and 1578. The remaining mutations were scattered throughout the 5' half of the gene.

Interestingly three of the most 3' APC mutations identified to date, at codon 1924, codon 1962, and codon 1987 (Eccles et al., 1996; Scott et al., 1996) cause extremely mild polyposis, with either none or a few late onset polyps, and yet cause highly penetrant familial desmoid disease.

A benefit of determining correlation between genotype and phenotype is that it allows mutation screening to be focused. If the patient’s phenotype is known then the most likely region of the gene to confer that phenotype can be searched for mutations first. This is particularly useful in a large gene such as APC with mutations identified throughout the coding region. For example, affected members of family GIB display desmoids, are CHRPE negative, and have a mild polyposis phenotype and may therefore have a mutation towards the 3' end of APC. Furthermore, the association between certain phenotypes and specific regions of the APC gene may indicate functionally important domains of the protein that it produces.
Figure 4.3. Genotype-phenotype correlation in the APC gene. (Caspari et al., 1994)
The mechanisms underlying genotype-phenotype correlation in the APC gene are not altogether clear. Presumably mutation at the 3' end of the gene confers an attenuated phenotype because most of the important functional domains are left intact, producing a protein that is at least partially functional. The attenuated phenotype caused by mutation at the 5' end of the gene is likely to result from alternative splicing which skips combinations of the first four coding exons of APC (Samowitz et al., 1995). In this case enough normal protein lacking the mutated exon may be produced to reduce the severity of the symptoms. The hypothesis that splicing could allow some normal protein to be produced is supported by findings of a mutation in the alternatively spliced region of exon 9 that leads to an attenuated phenotype (van der Luijt et al., 1995), and of a similar effect caused by a splice donor site mutation that allows exon skipping to occur (Varesco et al., 1994). Further attenuated phenotypes resulting from mutation lying 3' of exon 4 (Smith-Ravin et al., 1994) may be explained by alternatively spliced forms of APC that are as yet unidentified. Another possibility is that translation is reinitiated 3' of some mutations allowing production of a semi-functional protein (Gordon, 1992). This mechanism has been shown to function in bacteria (Sarabhai and Brenner, 1967), sometimes occurring downstream of mutations that cause a barrier to translation (e.g. frameshift or nonsense mutations), the same sort of mutations that are common in the APC gene of FAP patients and colorectal tumours. However, low molecular weight proteins containing the carboxyl region of APC have not been reported, despite a number of studies employing COOH-terminus specific antibodies (Boman et al., 1995; Makino et al., 1997).

Mutations that give rise to large numbers of polyps may exert their effects by forming non-functional dimers with normal APC (dimerisation of APC is discussed in section 1.7.4.). In this way wild-type APC could be sequestered, leaving only few dimers containing entirely normal protein and able to provide tumour suppressive protection (in theory 25%). If adenoma formation in FAP patients is due to loss or mutation of the normal allele, rather than a dominant negative mechanism, it becomes necessary to explain why patients with mutations that cause severe polyposis apparently acquire a second hit more rapidly than patients with mutation elsewhere in the gene.
If a severe phenotype is produced by a dominant negative interaction then mutations which confer an average phenotype may be explained by the production of unstable proteins that degrade before they can participate in dominant negative dimer formation. APC proteins under 80 kDa have not been detected in lymphoid lines suggesting that this may be true in some cases (Smith et al., 1993). If an average phenotype results from absence of the gene product produced by one APC allele then this could explain why many patients with deletions of the entire APC gene also tend to exhibit a classical FAP phenotype (Lindgren et al., 1992; Cross et al., 1992). Otherwise deletions would be expected to produce milder disease symptoms because no dominant negative association of mutant and normal proteins is possible. If however, mutations that give rise to average phenotypes do create proteins that interact with and inactivate normal APC a different explanation is required. Under these circumstances the similar phenotype developed by carriers of constitutional APC deletions may be explained by the deletion of other genes from 5q that usually accompanies loss of APC. Loss of this additional genetic material, including neighbouring genes such as the mutated in colon cancer (MCC) gene, may produce a more severe phenotype than would otherwise be expected in the absence of dominant negative interactions. Mutation at the 5' end of APC producing rapidly degrading proteins may create what are effectively null alleles that would be predicted to have similar consequences as deletion of the APC locus. As mentioned previously mutations at the 5' end of the gene actually cause a milder phenotype than observed in many deletion patients suggesting that the second hypothesis may be correct. Deletion of APC is also associated with a more proximal distribution of adenomas within the colon, and possibly with a tendency to develop sessile adenomas (Hodgson et al., 1994; Lynch et al., 1992; van der Luijt et al., 1995).

An average FAP phenotype could also result from proteins which lack regions that normally increase the stability of dimerisation and consequently the stability of dominant negative interactions also. Another possibility is that proteins conferring an average phenotype do not interfere with as many aspects of normal APC function as the larger proteins responsible for a severe phenotype. For example, the catenin binding domains, and further towards the carboxyl-terminus the GSK3β phosphorylation sites, are often deleted in the APC proteins that give rise to average phenotypes. However, mutants causing severe FAP retain the catenin binding sites
and also a varying number of GSK3β phosphorylation sites. The region of \textit{APC} which predisposes to desmoid tumours when mutated is situated in the middle of the sites phosphorylated by GSK3β, a region also responsible for down-regulation of β-catenin (Munemitsu \textit{et al.}, 1995). In FAP the normal function of these regions may be disturbed, or the mutant protein may occupy the substrates required by the normal \textit{APC} protein indirectly inhibiting it’s function.

Congenital hypertrophy of the retinal pigment epithelium (CHRPE) is another feature of FAP absent from patients with mutation at either end of the \textit{APC} gene, as described previously (Olschwang \textit{et al.}, 1993; Wallis \textit{et al.}, 1994). Mutation within exon 9, the 5’ cut off point for CHRPE expression, give rise to an intermediate CHRPE phenotype (Olschwang \textit{et al.}, 1993). Similar mechanisms to those already discussed may allow cells of the retinal pigment epithelium (RPE) to maintain constraints on growth despite carrying certain types of \textit{APC} mutation. However, regions of \textit{APC} associated with expression of CHRPE do not precisely match regions predisposing to large numbers of polyps, and the entire region causing desmoid formation is correlated with CHRPE negative status of patients. It may be that different \textit{APC} transcripts are produced in the RPE, or that currently unidentified functional domains, preserved in some mutant proteins but lost from others, play an important role in this tissue.

Much of the interfamilial variation in phenotype can be explained by the inheritance of different \textit{APC} alleles, however, it has not been established whether intrafamilial variation is the result of environmental differences or unlinked modifier loci (Paul \textit{et al.}, 1993; Giardiello \textit{et al.}, 1994). Extracolonic manifestations of FAP (other than CHRPE and desmoids) do not show an absolute correlation with mutation site (Nishisho \textit{et al.}, 1991; Groden \textit{et al.}, 1991 and 1993; Varesco \textit{et al.}, 1993; Nagase \textit{et al.}, 1992; Paul \textit{et al.}, 1993). It has been suggested that such features may be influenced by hormonal fluctuations. The first appearance of polyps in FAP patients usually occurs during puberty (Bulow \textit{et al.}, 1996). Another possibility is that genes other than \textit{APC} are responsible for intrafamilial variation.

Treatment of mice with the mutagen ethylnitrosourea resulted in the generation of the \textit{Min} (multiple intestinal neoplasia) mutation, and a strain of mice predisposed to intestinal tumours (Moser \textit{et al.}, 1990). The affected gene was shown
to be the murine homologue of APC (Apc), and the alteration a nonsense mutation at codon 850 (Su et al., 1992). Mice heterozygous for the Min allele of Apc (Apc\textsuperscript{Min}) develop numerous intestinal tumours at an early age and usually die before reaching 150 days of age (Moser et al., 1990). Breeding experiments conducted with mice carrying the Apc\textsuperscript{Min} allele revealed that the genetic background has a dramatic effect on the number of intestinal polyps developed. Quantitative trait loci studies subsequently led to the identification of a modifier gene, unlinked to Apc, which alters the expressivity but not the penetrance of the Apc\textsuperscript{Min} mutation (Moser et al., 1992; Dietrich et al., 1993). Evidence suggests that this locus, modifier of Min 1 (Mom1), is in fact the secretory phospholipase A2 gene (Pla2s) (MacPhee et al., 1995). In the mouse Pla2s maps to the same chromosomal region as Mom1 (distal chromosome 4) and shows 100% concordance between allele type and tumour susceptibility (MacPhee et al., 1995). It has been hypothesised that Pla2s functions in lipid homeostasis and inflammatory response (reviewed in Pruzanski and Vadas 1991) both of which could conceivably influence the development of intestinal neoplasia.

The human homologue of Pla2s and two other related genes map to 1p35-36 (Johnson et al., 1990), a chromosomal region that frequently shows somatic loss in colorectal cancer and other human neoplasia (Mathew et al., 1987; Leister et al., 1990). Investigation of these genes has so far suggested that they are not associated with the phenotypic variation seen between FAP patients (Dobbie et al., 1996; Spirio et al., 1996). However, a recent investigation of 14 microsatellite markers in a large FAP family, in which affected individuals carry the same mutation but have differing phenotypes, suggests that a modifying locus situated on chromosome 1p35-36 does exist (Dobbie et al., 1997).

A second modifier locus, not yet assessed in humans, suppresses tumour development in the mouse by causing DNA hypomethylation (Laird et al., 1995). It has also been proposed that DNA methyltransferase has a direct role in mutagenesis facilitating the common C to T transitions observed in colorectal cancers (Laird et al., 1995). The inheritance of null alleles of DNA methyltransferase has been shown to reduce the number of intestinal tumours developed by Apc\textsuperscript{Min} mice (Laird et al., 1995). The methylation of individual loci is frequently altered in tumours, some showing increased levels of methylation and others showing reduced levels (e.g. Ohtani-Fujita et al., 1993; Issa et al., 1994). Despite normal or high levels of DNA
methyltransferase tumour cell loci are generally hypomethylated relative to normal cells (Feinberg et al., 1988; El-Deiry et al., 1991). Hypomethylation of DNA is usually correlated with increased genetic activity (Yeivin and Razin, 1993). Furthermore, recent findings suggest that demethylation of the genome results in errors of chromosome segregation during mitosis (Lengauer et al., 1997). An increase in chromosomal translocations and aneuploidy was shown to affect methylation deficient colorectal cancer cell lines (Lengauer et al., 1997). It has been suggested that loss of methylation competence results in genomic instability, accelerating progression towards a malignant state. Thus there are two mechanisms of genomic instability that can influence the formation of colorectal tumours: mismatch repair deficiency, resulting in point mutations, microdeletions, and microinsertions; and methylation deficiency causing losses and gains of entire chromosomes.

4.3. Familial adenomatous polyposis families with atypical phenotypes.

Patients from family HUD have a germline rearrangement in the vicinity of the APC and MCC genes. This is in addition to a protein truncating mutation at codon 764 of APC identified by SSCP and heteroduplex analysis. The rearrangement was detected in our laboratory by S. Gayther using PFGE the results of which were consistent with an inversion of approximately 150 kb bringing the MCC and APC genes closer together (Gayther et al., 1993). Mutation at codon 764 of the APC gene is predicted to cause an average polyposis phenotype, indeed two other families (MAR and DOU) were found to carry this mutation and display typical numbers of polyps (<500) and cancer onset around 50 years of age. Individuals from family HUD however show early onset of colorectal cancer (around 21 years), numerous polyps (>2,000) and develop additional extracolonic tumours (2 cases of pancreatic cancer, 1 case of peri-ampullary cancer). It was hypothesised that the rearrangement of the APC-MCC region had caused the expected FAP phenotype to be modified in this family.

To shed further light on the rearrangement fluorescent in situ hybridisation (FISH) was employed. For the purposes of this experiment the YACs spanning the
APC and MCC loci, that had been employed for analysis of APC deletions, were used as probes in a two colour FISH experiment (Kinzler et al., 1991b). The simultaneous hybridisation of pairs of YACs, detected in different colours, allowed rearrangement to be visualised as a difference in the separation of signals on normal nuclei compared to those on patient nuclei. After the localisation of the YACs was confirmed and chimerism was excluded a pilot study was undertaken to determine the maximum resolution of interphase FISH using these probes. Clearly the resolution of signals on metaphase chromosomes (not less than 1,000 bp) would be insufficient for independent visualisation of each YAC (Lawrence et al., 1990). Combinations of different YACs from this APC-MCC contig were simultaneously hybridised to normal lymphocyte nuclei. It was thought that the rearrangement in this family might even fall within one of the YACs which could result in a split signal when observed following FISH.

The pilot experiment demonstrated that the mean separation of signals rises with increased separation of YACs (estimated by physical mapping), and that this approximates to a linear relationship. A roughly linear relation of genomic and interphase separations had previously been reported for probes up to 1,000 kb apart (Lawrence et al., 1992). However the influence of increased physical separation has a progressively less pronounced affect on mean signal separation as distances increase, particularly for probe separations of over 600 kb (Trask et al., 1991; Lawrence et al., 1992). The gradient of genomic distance against signal separation was also seen to decrease in this study but more rapidly than expected, a fact which may have several explanations. The observation may simply be an artefact, with only five data points a single erroneous result could significantly distort the data. Alternatively a more extensive graph covering a greater range of separations might show a series of linear regions separated by transitions corresponding to increasing orders of chromatin packaging. Different regions of the genome may be more or less condensed as a result of factors such as methylation and transcriptional activity, the extent of any such difference is not known and may vary between different tissues.

The maximum observed separations for each pair of probes was closely associated with the estimated YAC separation. However it was considered unwise to place too much emphasis on these figures as they relied on a single observation rather than an average of many independent observations.
The number of overlapping signals is also related to the actual separation of the YACs. Not surprisingly probes which lie close together are found to overlap much more frequently than probes lying further apart. This would cause no problem except for the fact that the relationship is not linear, a fact also noticed by Trask et al (1989). All separations of more than 300 Kb give approximately the same number of overlaps. The large number of probes lying close together or overlapping is the result of the gradual breakdown of the resolution of the technique. However one cannot simply exclude the overlapping signals from calculations of the mean separation as this gives unrealistically high values for low separations while affecting values for more distant probes little. The results of the pilot experiment indicated that the relationship of distance between signals and actual genomic distance should be linear enough to allow detection of any rearrangement altering probe separation by more than 50 Kb.

Comparison of maximum separation of signals on patient material provided no useful data. The reason for this is that the germline rearrangement only lies on one chromosome, the other chromosome is normal and gives separations of the same length as seen in the control. The presence of the normal chromosome in patient nuclei meant that any measurements taken do not reflect the separation of signals on the mutant chromosome alone. They represent an average of values for the normal and rearranged chromosomes.

As expected the measurements obtained for the normal control in this experiment do not precisely mirror those of the pilot experiment. This is presumably because of differences in the culturing or harvesting of the cells or in the FISH technique resulting in differences in the condensation or arrangement of the chromosomes within the interphase nuclei.

Each of the YAC combinations was subjected to statistical analysis to determine whether there was any difference between the separation of signals in the control and in the patient. A standard t-test revealed that there was no significant difference between any of the YAC combinations save one. The combination of 37HG4 and 19AA9 gave significantly closer together signals in patient nuclei (P<0.01). It had been noticed during the collection of measurements that the signals for this pair of YACs were never well separated on both chromosomes, most often
one pair of signals was overlapping or only just resolvable. In the control nuclei the signals were often well separated on both chromosomes. This result confirmed the hypothetical rearrangement and demonstrated that inversion of a fragment containing the MCC gene was responsible.

Although the rearrangement is not the cause of FAP in this family it may be influencing the severity of APC symptoms. Family HUD is the only severely affected family identified during this study that carries a mutation 5' of codon 1309. It may be that the inversion affects the expression of APC or neighbouring genes such as MCC. One way in which the rearrangement might be able to affect APC expression is by interfering with the splicing of the gene. The APC gene produces several different transcripts most of which differ only in untranslated regions, however one transcript involves intergenic splicing of APC with a neighbouring gene SRP 19 (the 19kDa signal recognition protein) (Horii et al., 1993). The transcript produced contains the first 13 exons of APC linked to a short open reading frame in SRP 19. It is quite likely that the SRP 19 gene has been interrupted or relocated by the inversion in this family, as it lies in between the APC and MCC genes. The clinical significance of the APC-SRP transcript, which is found in abundance equal to that of APC-APC transcripts (Horii et al., 1993), is unknown. However one could speculate that loss of APC-SRP transcript in addition to a mutation disrupting the production of APC-APC mRNA could result in a more severe form of FAP. Although it has been demonstrated that the inversion lies on the same chromosome as the codon 764 mutation (Gayther et al., 1993) APC-SRP transcripts would not be truncated by this mutation as exon 15 is not contained within this transcript. Another possibility is that after inversion APC may be able to interact with MCC, perhaps via splicing as it normally would with SRP19. The inversion shifts MCC into the same transcriptional orientation as APC so this could be a possibility. The MCC gene has been reported to contain mutation in a small number of sporadic colorectal tumours (Nishisho et al., 1991). Moreover MCC contains heptad repeat motifs homologous to those found in APC, which are thought to facilitate protein-protein interactions and may allow the protein products to co-operate in the same biochemical pathway (Groden et al., 1991; Kinzler et al., 1991b; Bourne et al., 1991a). However, several investigations of the protein binding properties of APC have not provided evidence that such an interaction
occurs (Rubinfeld et al., 1993; Su et al., 1993; Su et al., 1995; Rubinfeld et al., 1996; Matsumine et al., 1996).

There are many experiments that could lead on from this point, the breakpoints of the inversion could be better defined or cloned after more refined physical mapping approaches. Analysis of APC transcripts could be undertaken by direct study of the mRNA or by reverse transcriptase PCR (RT-PCR) to reveal whether SRP or MCC are involved in this phenotype. Alternatively the APC protein could be investigated by Western blot analysis using monoclonal antibodies specific for APC (Smith et al., 1993). Further experiments could also utilise newer high resolution FISH protocols to examine the rearrangement in more detail. Such methods include FISH on DNA halo preparations (Wiegant et al., 1992), said to have a resolution of 10-200 kb, and FISH on the so called DIRVISH DNA preparations with a resolution of at least 5-700 kb (Parra and Windle, 1993). For our purposes the DIRVISH method is likely to be more appropriate as the extended DNA strands are in a linear form allowing measurements to be made without difficulty, halo preparations produce extended DNA loops which are less easy to measure.

Patients from family 82, like those of family HUD, have unexpected features not attributable to their detected APC mutation. Affected individuals from this family not only suffer from colon cancer but also from extra-colonic tumours not usually associated with FAP. These include ovarian, endometrial, and pancreatic cancers. A mutation at codon 232 of the APC gene which introduces a stop codon has been shown to segregate within this family. Mutation at this site usually gives rise to an average FAP phenotype, as observed in family 523 that has an identical APC mutation.

Some of the extracolonic tumours developed by members of family 82 are frequently observed in patients with hereditary nonpolyposis colorectal cancer (HNPPCC). Following the cloning of the hMSH2 gene, responsible for almost 50% of HNPPCC cases (Leach et al., 1994; Fischel et al., 1993; Kolodner et al., 1994), linkage analysis was employed to investigate whether this gene has a role in the cancer predisposition of family 82. This analysis was not entirely conclusive indicating that two haplotypes, H3 and H4, were each carried by three of the four patients with
extracolonic malignancy. These haplotypes were also evident in patients unaffected by extracolonic malignancy (three with H3 and two with H4).

The identification of individuals unaffected by extracolonic neoplasia that possess haplotype H3 or H4 does not exclude the possibility that a mutant hMSH2 gene is influencing the phenotype in this family. Mutations of hMSH2 that cause HNPCC are only 0.73% penetrant (Scapoli et al., 1994), furthermore two of the three unaffected family members with haplotype H3 are under the age of at which HNPCC cancers usually become manifest. If haplotype H3 were associated with cancer predisposition the occurrence of extracolonic malignancy in patient 82 IV 6, an individual not carrying this haplotype, can readily be explained by the high incidence of sporadic cancer in the population as a whole. What is more the cancer in question, non-Hodgkins lymphoma, is not part of the typical HNPCC spectrum of malignancies. Conversely if haplotype H4 were associated with extracolonic cancer rather than H3 then the thyroid cancer developed by 82 IV 4, could be explained as a sporadic case or attributed to this patient's APC mutation. Thyroid cancers occur at an increased rate in FAP patients (Bulow et al., 1988). Although the lymphoma developed by 82 IV 6 is not typical of HNPCC patients high frequencies of lymphomas have been reported in Msh2'' mice (Reitmair et al., 1995). This suggests that the function of this gene is not entirely redundant in this tissue, at least not in the mouse.

Hereditary nonpolyposis colorectal cancer (HNPCC) displays genetic heterogeneity with mutation of several different genes capable of producing the same phenotype. Principal amongst these are hMSH2 (causing about 50% of HNPCC cases), hMLH1 (30%), PMSI (5%) and PMS2 (5%) (Fischel et al., 1993; Leach et al., 1993; Papdopolos et al., 1994; Bronner et al., 1994; Nikolaides et al., 1994; Kolodner et al., 1994; Kolodner et al., 1995). In approximately 10% of cases the genetic cause is yet to be determined. Thus it is possible that a gene other than hMSH2, but still associated with HNPCC, could be affecting the phenotype in family 82.

A characteristic of HNPCC regardless of which gene is mutated is microsatellite instability, the so called replication error (RER^) phenotype (Aaltonen et al., 1993). This is the accumulation of mutations at simple repeated motifs of 1-4 bp (microsatellites) throughout the genome. Such loci are inherently more mutable than other genomic sequences but still have relatively low mutation rates in normal cells (5 x 10^-4 to 5 x 10^-5 mutations per cell division) (Hearne et al., 1992). The wide
array of errors seen in RER\(^+\) tumours result from failure to correct DNA mismatches occurring during replication. Detection of microsatellite instability in a tumour provides an indication that one of the genes involved in mismatch repair and HNPCC has been mutated. Matched constitutional and tumour DNA samples were available from two patients from family 82 that developed extracolonic malignancy. These tumours, a pancreatic tumour with lung metastases and an endometrial carcinoma, were found to display no evidence of microsatellite instability at thirteen loci investigated. This data suggests that the pancreatic and endometrial tumours can be considered mismatch repair proficient (RER\(^+\)). Most tumours with an RER\(^+\) phenotype would be expected to display instability at several loci tested. It could be argued that the PCR-SSCP technique used to detect microsatellite alterations is not 100% efficient, however the method succeeded in identifying a large amount of variation between different individuals and also in two colorectal tumours previously identified as RER\(^+\). Another possible way in which replication errors could have gone undetected is if the tumour samples were contaminated with excessive quantities of normal cells. This seems unlikely in the case of the pancreatic cancer as the region sampled came from deep within the tumour. However, the proportion of normal cells in the endometrial tumour is not known.

As well as providing data on microsatellite instability analysis of hypervariable loci also allows loss of heterozygosity (LOH) to be assessed. LOH is observed to effect specific regions of the genome in a wide variety of tumours. The sites affected correspond to regions that contain tumour suppressor genes inactivated in the tumour. No LOH was detected on ten different chromosomes despite the use of polymorphisms in the vicinity of a number of well characterised tumour suppressor genes including: APC, p53, DCC, and hMSH2. It may be that none of these tumour suppressors were inactivated during the formation of the pancreatic and endometrial cancers. Alternatively inactivation may have been accomplished by a mechanism other than allelic deletion. A third possibility, already discussed in relation to mismatch repair deficiency, is that normal cells accidentally included in the tumour sample obscured traces of allele loss.

These investigations provided no strong evidence in support of a role for hMSH2, or any other gene associated with HNPCC, in the phenotype of family 82. It may be that the extracolonic cancers observed in this kindred are the result of a chance
clustering of sporadic cancer. Alternatively a gene other than \textit{hMSH2} may be responsible for conferring a predisposition to neoplasia. As previously mentioned the thyroid cancer developed by 82 IV 4 may be a consequence of this patient's \textit{APC} mutation. Other genes that may be involved include the BRCA2 and p16 tumour supressor genes. Carriers of BRCA2 mutations are at an increased risk of ovarian cancer, and lymphomas and melanomas have also been reported in BRCA2 kindreds (Grimmond \textit{et al.}, 1996). Furthermore somatic mutations of the BRCA2 gene have been detected in sporadic pancreatic tumours (Flanders and Foulkes, 1996). All these forms of neoplasia are found in family 82, although the lack of any breast cancer, the principal consequence of BRCA2 mutation, argues against a direct role for this gene. Mutations of the p16 gene have been implicated in familial melanoma (Zuo \textit{et al.}, 1996) and to a lesser extent in thyroid cancer, non-Hodgkins lymphoma and ovarian cancer (Uchida \textit{et al.}, 1995; Shi \textit{et al.}, 1996; Kanuma \textit{et al.}, 1997; Pinyol \textit{et al.}, 1997). Further investigation will be required to ascertain whether either of these genes influence cancer predisposition in family 82.

4.4. The \textit{APC} gene in sporadic and extra-colonic neoplasia.

Somatic mutations of the \textit{APC} gene detected in sporadic colorectal tumours are found throughout the gene. However a pronounced clustering of mutation has been observed in a defined region of the gene, the mutation cluster region (MCR), which lies between codons 1286 and 1513 (Miyoshi \textit{et al.}, 1992). The MCR accounts for only 8% of the \textit{APC} coding sequence and yet approximately two thirds of somatic mutations are found in this region. Somatic mutation is characterised by a similar proportion of nonsense and frameshift mutation to that reported for the germline. However, it is interesting to note that American populations show a significant increase in somatic point mutation at CpG sites relative to Japanese populations (Nagase and Nakamura, 1993). This may represent a difference in exposure to carcinogens, possibly due to differences in diet.

The complete inactivation of the \textit{APC} protein, which is likely to result from many of the truncating mutations, supports the hypothesis that \textit{APC} acts as a tumour
suppressor. A preponderance of truncating mutations has been reported for a number of other tumour suppressors such as BRCA1 (Stoppa-Lyonnet et al., 1997) and RBL (Lohmann et al., 1996). The deletion of the normal APC allele in the adenomas of FAP patients has been detected by loss of heterozygosity studies (LOH), suggesting that both copies of APC must be inactivated for a colorectal adenoma to develop (Ichii et al., 1992). Taking into account the limitations of mutation detection techniques Nagase and Nakamura (1993) concluded that almost all the 138 colorectal adenomas and carcinomas that they surveyed were likely to carry mutations of both APC alleles. This indicates that the 'two-hit' mutational model of a tumour suppressor gene applies to APC during colorectal tumourigenesis (Knudson, 1971). Furthermore a similar frequency of APC mutation in adenomas compared to carcinomas suggests that inactivation of APC occurs during the earliest stages of tumour progression (Ichii et al., 1992).

Somatic mutation of the APC gene has also been detected in sporadic neoplasia arising from tissues other than the colon. The other tissues of the gastrointestinal tract and tissues prone to tumour development in FAP patients, such as desmoids, were considered most likely to be affected by APC mutation.

Desmoid tumours are rare in the general population, representing less than 0.1% of all human tumours (Reitamo et al., 1986). However the incidence in FAP patients is much increased and desmoids are found in 8-13% of patients, where they represent a substantial cause of mortality (Gurbuz et al., 1994; Caspari et al., 1994). Most desmoids occur intra-abdominally or in the abdominal wall, and frequently appear following surgery (Gurbuz et al., 1994). The inheritance of a mutant APC allele can also cause highly penetrant familial desmoid disease if the mutation is at the extreme 3' end of the gene (codon 1924-codon 1987) (Eccles et al., 1996; Scott et al., 1996). During the course of this study analysis of 7 desmoid tumours from FAP patients and 5 sporadic desmoids was undertaken to assess the role of the APC gene in the development of this variety of tumour.

Molecular studies have recently demonstrated the clonal nature of desmoid tumours (Li et al., 1996; Alman et al., 1997). Furthermore in FAP patients it has been shown that the wild-type APC allele is frequently silenced in these tumours, suggesting that APC undergoes similar mutation in desmoid and colorectal
tumourigenesis (Sen Gupta et al., 1993; Miyaki et al., 1993; Palmirotta et al., 1995). The APC gene may also have a similar role in the formation of sporadic desmoids. This is suggested by reports detailing the loss of 5q in a sub-group of sporadic desmoid tumours (Bridge et al., 1992; Bridge et al., 1996).

Somatic mutation of the APC gene was detected in 4 out of 7 desmoids (57%) from FAP patients and in each case this alteration was additional to a previously identified constitutional mutation. All mutations were predicted to result in truncation of the APC protein, and thus these results are consistent with other reports that the APC gene is entirely inactivated in most FAP related desmoid tumours (Miyaki et al., 1993; Palmirotta et al., 1995).

No germline mutation was detected in any of the five patients with sporadic desmoids. Analysis did however reveal somatic mutation of APC in two of the tumours. Indeed, tumour 2324 was found to contain two distinct APC mutations. Once again the mutations were predicted to truncate the APC protein. A fragment of APC encompassing both of the mutation sites in tumour 2324 was amplified by PCR and cloned into plasmid vectors. Subsequent sequence analysis revealed that the two mutations segregate into different recombinant clones, demonstrating that both APC alleles are mutated in this tumour (analysis performed by Giarola et al., submitted). These results seem to indicate that APC has a critical tumour suppressive function in the tumourigenesis of sporadic desmoids as well as those associated with FAP. However, the five sporadic tumours investigated here formed part of a larger study of 16 sporadic desmoids which reaches different conclusions (Giarola et al., submitted). The analysis of an additional 11 sporadic desmoids, employing identical mutation analysis techniques to those used in this study, failed to identify any further mutations of the APC gene. Thus, it seems that mutation of APC is only important in a minority of sporadic desmoids, 12.5% in the study of Giarola et al. Presumably other genetic mechanisms have more significance or occur more frequently in sporadic desmoid tumourigenesis. An alternative possibility is that sporadic desmoids do contain APC mutation, but the screening strategy used in this study and that of Giarola does not readily detect them. Missense mutations within regions only assessed by PTT would not be detected, although the vast majority of somatic APC mutations in colorectal cancers are protein truncating (Nagase and Nakamura, 1993). Another possibility is that somatic mutations are clustered in regions of the gene not assessed during this
study, such as regulatory regions. The \textit{APC} mutations found in FAP desmoid tumours display a clustering similar to that observed in MCR of colorectal tumours. The region encompasses codons 1399 to 1584 overlapping approximately half the area covered by the MCR and continuing for an additional 213 bp 3' of this region. Four of the seven somatic mutations detected were located within this area, suggesting that this is also a significant region for somatic mutation. Interestingly four out of four desmoids for which two separate mutations had been characterised had at least one mutation within this region. A fifth desmoid, 75, had one mutation at codon 1085 and a second uncharacterised alteration between codons 1360 and 1377 detected by heteroduplex analysis. If this mutation is a deletion or an insertion, as seems likely, then the protein may not actually be truncated until after codon 1399. A necessity of mutation at or near to codon 1444 in \textit{APC} mediated desmoid formation has been reported previously (Palmirotta \textit{et al.}, 1995).

Although evidence suggests that inactivation of the wild-type \textit{APC} allele is selected for during desmoid tumourigenesis in FAP patients an alternative genetic mechanism may act in the formation of most sporadic desmoids. It is possible that genes that interact with APC, such as \(\beta\)-catenin, may be the targeted for mutation. \(\beta\)-catenin has been shown to be mutated in two colorectal cancer cell lines in which no \textit{APC} mutation was detected (Morin \textit{et al.}, 1997).

Giarola \textit{et al.} also noted that the two sporadic desmoids in which \textit{APC} mutation had been detected were the only non-FAP tumours (out of 16) affecting the abdominal wall. Furthermore, review of the literature revealed that 10 out of 15 FAP related desmoids, in which somatic \textit{APC} mutation had been detected, also occurred in the abdominal wall (Giarola \textit{et al.}, submitted; and references therein). This suggests that the somatic mutation of \textit{APC} may have greatest significance if it occurs in this specific anatomical region. Although it could also be argued that the abdominal desmoids common in FAP patients are initiated by abdominal surgery such as colectomy.

To further investigate desmoid tumourigenesis four sporadic and seven FAP-related tumours were investigated for loss of heterozygosity (LOH), and also for replication errors characteristic of mismatch repair deficiency. As previously discussed (section 4.3.) the replication error positive (RER\(^{+}\)) phenotype is important
in the tumourigenesis of approximately 15% of colorectal cancers as well as in a variety of other neoplasia (Liu et al., 1995).

Analysis of thirteen microsatellite loci revealed no evidence of mismatch repair deficiency in any of the desmoids investigated. Furthermore no loss of heterozygosity was detected at any of these loci. As with analysis conducted on the tumours of patients from family 82, failure to detect any evidence of replication errors or loss of heterozygosity could be the consequence of contaminating normal cells. The infiltrative nature of desmoids may increase the likelihood of this sort of problem occurring, however the detection of APC mutation does not seem to have been impaired by any contaminants. The lack of any LOH in the vicinity of APC was expected in four of the seven FAP related desmoids and in one of the sporadic desmoids because mutations inactivating both APC alleles had already been detected. Apart from the loss of 5q the only consistent chromosomal abnormalities reported in desmoid tumours involve trisomy of chromosomes 8 and 20 (Mertens et al., 1995; Fletcher et al., 1995; Qi et al., 1996). Increases in chromosome copy number could not be detected by the methods employed during this study. Analysis of microsatellite loci using quantitative fluorescent PCR would provide this additional data as well as information on allele loss and replication errors. Consequently this seems to be the most effective method for any future analysis of these tumourigenic mechanisms.

Patients suffering from FAP are not only at an increased risk of desmoids and colorectal tumours, but of a variety of other forms of malignancy also. These include periampullary and thyroid tumours and suggest that APC may also have a tumour suppressive function in these tissues. Accordingly a high level of somatic APC mutation has been reported in periampullary adenomas developed by FAP patients (Bapat et al., 1993; Miyaki et al., 1993). As in sporadic colorectal cancer the mutations are predicted to cause truncation of the APC protein, and are clustered around the MCR. Thyroid tumours, which occur at a frequency of 2% above the population average in females affected by FAP (Bulow et al., 1988) have also been investigated, however no mutation was detected in two studies of sporadic thyroid cancer (Curtis et al., 1994; Colletta et al., 1994), and in a third mutation was only detected in 2 out of 87 cases (2.3%) (Zeki et al., 1994). Thus it seems that as in the
formation of sporadic desmoid tumours *APC* does not play a critical role in the development of sporadic thyroid tumours, at least not in the majority of cases.

Tumours of the gastrointestinal tract and digestive organs have been analysed for *APC* mutation with mixed results. No mutation was found in 49 oesophageal cancers (Ogasawara et al., 1994) despite the detection of 5q LOH (Boynton *et al* 1992). However, mutations were identified at appreciable frequencies in oral squamous-cell carcinomas and gastric cancers. Mutation of *APC* in 12.5% of squamous-cell carcinomas, coupled with loss of heterozygosity detected in 72.7% of informative samples, suggests that *APC* does behave as a tumour suppressor in this tissue (Uzawa *et al*., 1994). The picture is less clear in gastric cancer, in which genetic alterations underlying carcinogenesis are poorly understood. Several factors suggest a role for *APC* in this form of malignancy: some gastric cancers are considered to originate from the intestinal metaplasia; FAP patients are at least ten times more likely to develop gastric cancer than the population average; and chromosome 5q shows frequent loss of heterozygosity in a subset of gastric cancers (Sano *et al*., 1991). Histopathological criteria define two categories of gastric carcinoma; undifferentiated (diffuse) type and differentiated (intestinal) type. There is some discrepancy in reports of the prevalence of *APC* mutation in well differentiated gastric cancers, which probably results from difficulties in classification of tumour type. Between 0% and 41% are said to contain a mutation (Nakatsuru *et al*., 1992; Horii *et al*., 1992; Ogasawara *et al*., 1994; Sud *et al*., 1996). Mutation of *APC* is also found in 0-5% of poorly differentiated gastric tumours, and has been reported in a precancerous flat adenoma (Nakatsuru *et al*., 1992; Nakatsuru *et al*., 1993; Horii *et al*., 1992; Ogasawara *et al*., 1994). This suggests that mutation of *APC* is an early event in the formation of a minority of gastric tumours, predominantly of the differentiated (intestinal) type, in Japanese patients. A study of gastric cancers in British patients has indicated a very low (4%) frequency of *APC* mutation (Sud *et al*., 1996). Discrepancies also exist in reports of the frequency of *APC* mutation in pancreatic cancer. Importantly all studies detected *APC* mutation, however the frequency reported varies from 2.5% to 30% (Horii *et al*., 1992; Yashima *et al*., 1993).

Ovarian and breast cancers have also been assessed for *APC* mutation. No genetic alterations were detected in ovarian tumours (Allan *et al*., 1994), but
mutations were detected in 2/35 (6%) breast tumours (Kashiwaba et al., 1994). Allele loss on chromosome 5q21 has been noted in 28% of breast cancers, suggesting that there is a tumour suppressor gene in this region (Thompson et al., 1993).

An interesting feature of APC mutations detected in gastric, squamous-cell, pancreatic, and thyroid cancer is that the proportion of missense mutations is not the same as that found in colorectal tumours. In gastric cancer missense mutations account for 25-50% of APC mutation (Nakatsuru et al., 1992; Horii et al., 1992), and in squamous-cell carcinomas 60% (Uzawa et al., 1994). In the small number of pancreatic, thyroid, and breast cancers with APC mutation missense mutations are the predominant type of alteration (Yashima et al., 1993; Zeki et al., 1994; Kashiwaba et al., 1994). This is in stark contrast to APC mutations in colorectal cancers only 5% of which are missense (Nagase and Nakamura, 1993). Furthermore, not all APC mutations in extracolonic neoplasia group in the MCR. Mutations tend to lie 3' of the MCR in squamous cell carcinomas, and 5' of the MCR in breast cancers. The majority of mutations in gastric cancer are found in the MCR, but the overrepresentation of mutations in this area is not as striking as that observed in colorectal cancer. The difference in mutation type and localisation could be the consequence of specific carcinogens that some tissues are exposed to but not others.

Carcinogens often cause characteristic types of mutation such as the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) which induces deletion of a single guanine at GGGA sequences in the rat APC gene (Kakiuchi et al., 1995). Alternatively tissue specific differences in the structure or function of the APC protein might be responsible for selection of cells carrying mutation in different regions of the gene. Variation in structure could result from alternative splicing in different tissues (Horii et al., 1992).

4.5 Performance of mutation detection techniques.

Because several different mutation detection protocols were employed during the course of this study it has been possible to compare the relative efficiency of each technique. A total of 33 distinct APC mutations were detected in desmoid tumours and constitutional DNA from FAP patients following analysis of 60% of the coding sequence. Overall the performance of single strand conformation polymorphism
(SSCP) and heteroduplex analysis were similar: 42% of variants were detected by SSCP alone; 40% were detected by heteroduplex analysis alone; and 18% could be detected by both methods. However, more detailed analysis reveals that the types of mutation efficiently detected by these techniques differ considerably. For the purpose of identifying a variety of mutations including deletions, insertions, and substitutions heteroduplex analysis and SSCP appear to be complementary. Heteroduplex analysis detects small deletions and insertions with high efficiency, but is relatively insensitive to single base pair substitutions, while for SSCP the converse is true (see table 4.1.). From this it can be concluded that the use of SSCP and heteroduplex analysis in tandem increases the likelihood of detecting a mutation in the APC gene. The detection rate per patient using these techniques alone was 58%. Considering that the region assessed in this study accounts for only 60% of the coding sequence, estimated to contain about 75% of APC mutations, the mutation detection strategy employed appears to be working satisfactorily. Furthermore this efficiency compares favourably with other studies using heteroduplex analysis and/or SSCP. Varesco et al. (1993) detected mutation in 29% of FAP patients following SSCP, while Dobbie et al. (1996) identified mutation in 61% of cases after SSCP and PTT analysis, both groups investigated the entire APC coding sequence.
Table 4.1. Relative efficiencies of SSCP and heteroduplex analysis for the
detection of frameshift mutations and single base pair substitutions in desmoids
and FAP patients.

<table>
<thead>
<tr>
<th>Mutation detection technique</th>
<th>Type of mutation</th>
<th>Deletion/Insertion</th>
<th>Substitution</th>
</tr>
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<tbody>
<tr>
<td>Heteroduplex Analysis</td>
<td></td>
<td>13</td>
<td>2</td>
</tr>
<tr>
<td>SSCP</td>
<td></td>
<td>6</td>
<td>11*</td>
</tr>
</tbody>
</table>

*Substitutions include polymorphisms and rare variants not associated with FAP, as well as disease causing mutations.

Various reports have quoted SSCP efficiencies varying from 35% to almost 100% (Sarkar et al., 1992; Orita et al., 1989b), although most studies suggest a detection level of between 75% and 90% can be expected for fragments of near optimal size (Orita et al., 1990; Condie et al., 1993; Sheffield et al., 1993; Hayashi and Yandell, 1993). Although SSCP is generally robust a variety of elements can influence the likelihood of detecting a sequence variant. The principal factors affecting the conformation adopted by single strands of DNA, and consequently mutation detection efficiency, are electrophoretic conditions and length and base sequence of the DNA fragment under analysis. In this study amplified fragments from exon 15 of APC were often about 400 bp in length, approaching the upper size limit for satisfactory mutation detection and well over the 150 bp suggested to be optimal (Sheffield et al., 1993). However, the protocol used has successfully detected single base substitutions even in the largest of fragments assessed (465 bp), and complementary mutation detection techniques such as PTT and heteroduplex analysis have not indicated a reduced efficiency of SSCP for these large fragments.

Electrophoretic conditions that effect SSCP efficiency include: temperature of electrophoresis; concentration and pore size of the polyacrylamide gel; and ionic concentration of the running buffer (Spinardi et al., 1991; Hayashi and Yandell, 1993;
Glavac and Dean, 1993). The effect of different experimental conditions on the efficiency of SSCP is reflected in the range of mutation detection rates reported in the literature. In many cases several different sets of conditions used in tandem has been found to increase the detection efficiency (Michaud et al., 1992; Hayashi and Yandell, 1993; Glavac and Dean, 1993). During this study the use of the Phastsystem™ (Pharmacia), a semi-automated electrophoresis system allowing accurate control of the gel temperature during electrophoresis, provided reliable, reproducible analysis at three different temperatures (4°C, 10°C, 15°C). Even small changes in temperature (+/- 5°C) had a significant impact on the migration of single stranded DNA, presumably as a consequence of altered single strand conformations. Several mutations were identified that could only be detected at one of the three temperatures, and would have been missed if only a single set of conditions had been used.

Radioactive forms of SSCP appear to be less sensitive than their non-isotopic equivalents (Ainsworth et al., 1991; Dockhorn-Dworniczak et al., 1991) mainly because autoradiography provides poor resolution of closely situated bands. The use of the Phastsystem™ for automated silver staining is said to improve efficiency of non-isotopic detection still further (Dockhorn-Dworniczak et al., 1991).

It may have been possible to further improve SSCP efficiency by adopting a modified protocol. Analysis using purified PCR products may have helped by removing excess primers that can interact with the single strands of DNA (Cai and Touitou, 1993). Alternatively electrophoresis could have been performed using different gel matrices, or at a greater number of different temperatures. A number of high sensitivity methods based on the principle of SSCP have been reported including RNA-SSCP (Sakrar et al., 1992), dideoxy fingerprinting (ddF) (Sarkar et al., 1992b), amplification refractory mutation system-SSCP (ARMS-SSCP) (Lo et al., 1992), and the use of fluorescent sequencing technology (Iwahana et al., 1995). However, these techniques are more expensive and time consuming. Perhaps the greatest virtue of SSCP is it’s simplicity, and a small improvement in mutation detection did not seem adequate recompense for the increase in complication and cost.

Mutation detection techniques that rely on heteroduplex formation, such as the direct heteroduplex analysis reported here and indirect methods such as RNase
protection (Nishisho et al., 1991; Miyoshi et al., 1992b, Nagase et al., 1992a), appear to perform particularly well when applied to the APC gene. Presumably this is the consequence of the high proportion of small insertions and deletions that characterise APC mutation (Nagase and Nakamura 1993). This type of alteration creates heteroduplexes with more significant areas of mismatch than those produced by substitutions, and are consequently easier to detect. The efficiency of heteroduplex analysis can be improved by adding additional fragments of almost but not quite identical sequence (Wood et al., 1993), or by performing electrophoresis using weakly denaturing conditions (Glavac and Dean, 1995). However, these improvements interfere with SSCP and could not therefore be incorporated into the simultaneous SSCP and heteroduplex analysis protocol used during this study.

The protein truncation test proved to be highly effective as a rapid screen of the APC region most frequently subject to mutation. Not only was it possible to analyse a large (2 kb) fragment in a single experiment, but the size of truncated protein products gave an indication of where within the fragment mutation had occurred, allowing sequencing efforts to be targeted efficiently. Analysis of an APC fragment stretching from codon 1034 to 1706 successfully detected all but two protein truncating mutations that had been identified by SSCP/heteroduplex analysis. The exceptions were mutations at codons 1061 and 1085. Failure of detection may be the consequence of instability of the mRNA or polypeptide produced leading to rapid degradation. Alternatively there may be unforeseen technical limitations to the detection of the low molecular weight proteins that these mutations are predicted to produce. In addition to previously identified mutations PTT also detected 7 alterations that had eluded earlier SSCP and heteroduplex analysis. The detection of 21 of the 23 truncating mutations situated between codon 1034 and 1706 reveals PTT to be the most efficient technique for the detection of mutation in this region of APC.

In theory PTT should detect any frameshift mutations leading to protein truncation as well as all nonsense mutations. As 97% of recorded APC mutations fall into one of these categories it is particularly appropriate to use such a method in this case. PTT is not the only method for detecting truncated proteins to have been applied to APC. Varesco et al. (Varesco et al., 1993) used a functional assay in which in frame segments of APC were cloned into a marker gene (beta-galactosidase) and its
functional activity assessed via a colourimetric method (Varesco et al., 1993). A number of other authors have employed APC-specific antibodies to detect proteins of reduced size (e.g. Smith et al., 1993; Boman et al., 1995).

Restriction endonuclease digestion was employed to detect four separate mutations that together account for almost 10% of all reported APC mutations (Ando et al., 1993). Digestion of fragments of APC exon 6 with the enzyme AccI revealed two families (82 and 523) that carry a nonsense mutation at codon 232 of APC which SSCP and heteroduplex analysis had failed to detect. No mutations were detected using any of the other enzymes.


4.6.1. Presymptomatic Screening of FAP Patients.

Direct detection of mutation or the construction of an informative haplotype for linkage in patients suffering from FAP is desirable because once found the inheritance of the disease mutation/haplotype can be traced through the affected family. The information gained reveals which individuals have inherited the APC mutation and are therefore at risk of developing colon cancer. Family members diagnosed can then receive improved counselling, and more appropriate screening regimens can be devised. This can help to reduce the frequency of endoscopic examinations, which are usually carried out annually from puberty to the age of 50. Regular examination by flexible sigmoidoscopy allows early detection of adenomas and surgical intervention before the onset of malignant disease (Vasen et al., 1990). Individuals not carrying the mutation are freed from the concern that they may have inherited the disease, they can plan families without fear of transmitting FAP to their children, and no longer need to undergo frequent examination. Examination at 18, 25, and 35 years has been suggested to cover the possibility of a false negative result (Petersen et al., 1993).

Several intragenic and closely linked polymorphic DNA markers are available for presymptomatic diagnosis of FAP by linkage methods (Nakamura et al., 1988; Dunlop et al., 1991; Burn et al., 1991; MacDonald et al., 1992). The apparent lack of any genetic heterogeneity has simplified presymptomatic diagnosis for APC families,
while the growing number of markers in the APC region have made testing more reliable and more informative (Petersen et al., 1991; Spirio et al., 1993). For this reason the detection of intragenic polymorphisms during the course of mutation analysis was valuable. Despite the number of polymorphisms now identified in the vicinity of APC linkage approaches still sometimes encounter problems due to unavailable critical family members, uninformative pedigree structures, and uninformative markers are still observed in some cases.

An indication of whether or not an individual is likely to be affected by FAP can also be gained by looking for congenital hypertrophy of the retinal pigment epithelium (CHRPE). FAP patients usually possess multiple bilateral CHRPEs which are present from an early age (Chapman et al., 1989, Romania et al., 1989). This method of screening is less of an ordeal than colonoscopy but is complicated by the presence of low numbers of CHRPEs in some unaffected individuals, and by the variable number of CHRPEs in FAP patients (none are evident in 10-30% of patients) (Traboulsi et al., 1988).

Difficulties encountered in linkage analysis or diagnosis using CHRPE status pose no problem for mutation screening which provides a definitive DNA test. Unfortunately it is not yet possible to identify 100% of mutations causing FAP, the most successful studies to date have only detected mutation in 87-90% of patients after exhaustive analysis (Powell et al., 1993; Prosser et al., 1994). Consequently alternative forms of diagnosis will likely remain important for diagnosis of FAP for some time to come.

4.6.2. Prenatal diagnosis of FAP.

The molecular genetic basis of a growing number of inherited cancer syndromes is now understood but this in turn creates problems for clinical management since there are very few measures available to prevent the onset of cancer in such individuals (Bryant, 1996). In general, prenatal diagnosis is offered to families at risk of having children with genetic disease and the pregnancy may be terminated if affected. However, termination of pregnancy after conventional prenatal diagnosis (amniocentesis or chorionic villus sampling, CVS) for couples carrying a cancer predisposing mutation is controversial and may be unacceptable to some
couples. Affected children are otherwise healthy and may remain so for many years. A survey of attitudes to prenatal testing in patients with FAP has revealed that only 24% of affected individuals would be prepared to terminate an affected pregnancy (Whitelaw et al., 1996). However the same study also found that 10% of patients, who had opted not to have children, believed that prenatal testing would enable them to contemplate having a family.

During the course of this study only one patient (COO III 1) requested prenatal diagnosis. This patient had already undergone prenatal diagnosis of FAP during an earlier pregnancy. On that occasion linked polymorphic markers had been used to demonstrate that the fetus was unaffected. However, as the specific *APC* mutation, a 4 bp deletion at codon 625, had since been identified a direct mutation detection strategy was considered more appropriate for the second pregnancy. Transcervical chorionic villus sampling (CVS) was followed by DNA extraction from fetal cells and PCR amplification of a fragment encompassing codon 625. Mutation detection employed heteroduplex analysis and revealed that the fetus was not carrying the mutation responsible for disease in family COO. A survey of patient attitudes has revealed that although uptake of prenatal diagnosis remains low it seems that most patients would be keen to reduce the risk of an affected pregnancy if the option were available (Whitelaw et al., 1996).

4.6.3. Preimplantation genetic diagnosis (PGD) of FAP.

An alternative to prenatal diagnosis that some FAP families are keen to pursue is preimplantation genetic diagnosis (PGD). This involves the use of *in vitro* fertilisation (IVF) techniques in order to generate multiple embryos from the couple at risk of passing on a genetic disorder. The embryos are usually grown in culture until day three post fertilisation at which time 1-2 cells are biopsied. These cells are then subjected to genetic analysis using either FISH or PCR and mutation analysis. If a biopsied cell is found to be normal then it can be inferred that the remainder of the embryo is also normal. Unaffected embryos (no more than two) are transferred back to the mother’s uterus, approximately 25% of cycles leading to a pregnancy (Harper, 1996). Any pregnancy therefore will be unaffected and the possibility of termination following diagnosis at later stages of pregnancy avoided.
A number of inherited diseases have been successfully approached by PGD, including cystic fibrosis, Lesh-Nyhan syndrome, and Tay-Sachs disease (Harper and Handyside, 1994; Harton et al., 1996). By September 1995 34 babies had been born worldwide following preimplantation genetic diagnosis (Harper, 1996).

An infertile female patient from family HUD (HUD III 5) requested PGD for FAP. At the time of diagnosis she was 34 years old, and had never had a pregnancy. As discussed previously this family has an \( APC \) mutation at codon 764 and displays a severe FAP phenotype. Two of the patient’s affected sisters died in their early twenties from extracolonic malignancy despite having had earlier colectomies. The patient also had a total colectomy following the development of cancer at age 21, and it appears to be this operation that caused her infertility, resulting in blocked Fallopian tubes. As a consequence patient HUD III 5 requires IVF treatment to conceive. Before any diagnosis could be attempted a strategy for the detection of the codon 764 mutation in single embryonic cells had to be devised.

Any method reliant upon single cell PCR must overcome a number of difficulties if it is to be successfully applied. The large number of cycles required for sufficient amplification of a single genome exacerbates problems encountered during PCR of relatively large quantities of DNA. The risk of contamination with spurious DNA sequences is a particularly pronounced problem which must be avoided by the implementation of stringent experimental practices. Extraneous DNA from sperm or maternal granulosa cells is a potential source of contamination encountered when sampling embryonic cells following IVF procedures. Excess sperm are avoided by the use of intracytoplasmic sperm injection (ICSI) to introduce a single sperm into the cytoplasm of the oocyte. Nested PCR is usually employed to enhance the specificity of PCR and also to reduce the risk of 'carry over' contamination from earlier amplifications. As well as these difficulties preimplantation diagnosis must also overcome problems unique to single cell PCR. Perhaps the most significant of these is that of allele dropout, a phenomena whereby only one of the two alleles present in a heterozygous sample is successfully amplified (Ao et al., 1995). Such an event may lead to misdiagnosis of heterozygous embryos, and is the most significant obstacle to the diagnosis of dominant disorders from small quantities of DNA. ADO has been seen to affect over 20% of single cell amplifications, although rates are generally
the APC mutation site (codon 764); and an intragenic polymorphism (codon 1678). Preliminary experiments conducted on isolated lymphocytes from HUD III 5 indicated that although rates of ADO for each individual amplicon are too high to perform a diagnosis (14% ADO at the mutation site, 22% at the polymorphic site) the probability of ADO affecting the mutant allele at both of these sites was only 3.1%. If two cells were sampled per embryo then the misdiagnosis rate drops to 0.1%. Sampling of two cells rather
somewhat lower than this (Ray et al., 1996). The exact cause of allele dropout has remained elusive. Initial hypotheses have suggested that DNA degradation, imperfect PCR conditions, or incomplete cell lysis could be responsible, and experimental evidence in support of the latter two ideas has been presented (Ray and Handyside 1996; El-Hashemite et al., 1997).

The efficiency of PGD is limited, in the main, by the low pregnancy rates achieved using assisted conception. Consequently it is unlikely that pregnancy rates following PGD will exceed 20-30% in the near future. Although misdiagnosis has occurred there has been a general increase in accuracy rates for PGD. Improved diagnostic strategies have accompanied an increased understanding of the problems associated with single cell analysis. Recent estimates of the risk of misdiagnosis of cystic fibrosis based on results from several hundred blastomeres varied from 0.1%-2% depending on whether one or two cells were analysed (Ray and Handyside, unpublished). Whatever the difficulties faced by single cell diagnosis, the growing demand for PGD continues to drive research into strategies for the diagnosis of further inherited diseases.

A strategy to overcome the problem of ADO was developed during the course of this study and involved whole genome amplification (PEP) followed by independent analysis of two informative loci than just one does not seem to reduce embryo viability, and has been employed for the diagnosis of chromosomal abnormalities in human preimplantation embryos (Conn et al., 1997). The design of similar strategies to circumvent the problem of ADO should be possible for most dominant disorders.

If ADO occurs at the nested PCR stage of the diagnosis it could probably be overcome by taking several aliquots of each PEP product and amplifying each of them separately. The probability of ADO affecting all aliquots would be very low. Alternatively if PEP does not amplify the APC gene efficiently, producing only a few new copies of the APC fragments, then the aliquot taken to set up the nested PCR (1/12 volume) may not contain DNA fragments representing both alleles. The first possibility was tested by amplifying several aliquots from each PEP product, while the second was investigated by using larger (1/8 volume) aliquots of PEP product to set up each nested PCR. Neither of these approaches reduced ADO and consequently it
seems likely that the majority of allele specific amplification failure had already occurred during the PEP procedure. The two fragments being amplified, APC\textsuperscript{mut} and APC\textsuperscript{poly} were entirely independent in terms of ADO. Allele drop-out at one locus did not correlate with an increased probability that the other locus would experience ADO. Interestingly both fragments of the APC gene had a higher incidence of mutant than of normal chromosome ADO. The reason for this phenomenon is yet to be fully elucidated. Differences in DNA sequence between normal and mutant alleles could cause differences in DNA secondary structure and in amplification efficiencies, however this seems unlikely as mutant and normal sequences are more than 99% identical. Total failure of the PEP sample to amplify coupled with excessive normal contamination could also produce this result, however numerous control blanks indicated only a low level of sporadic contamination (3/30 negative control blanks contaminated). Furthermore analysis of contaminants revealed that mutant sequences were responsible for a similar proportion of total contamination as normal sequences. This also suggests that previous PCR amplifications provide the principal source of contamination for single cell PCR.

Ten oocytes were collected from HUD III 5 and four were normally fertilised after ICSI. Only one embryo was diagnosed as normal and this was transferred back to the mother, HUD III 5, but no pregnancy resulted. The pregnancy rate following single embryo transfer is reported to be only 10%, disproportionately lower than if two embryos are available (Giorgetti \textit{et al.}, 1995). The failure to obtain more embryos for the procedure is due to the low normal fertilisation rate. In a recent study, the numbers of oocytes collected and normally fertilised (56%) in these mostly fertile couples undergoing PGD were similar to the numbers after IVF with infertile couples (Ao \textit{et al.}, 1996).

The accuracy of the genetic test was assessed by analysing the remaining embryos which were not transferred. The results were concordant with the previous findings, all three embryos not transferred were carrying the mutant gene.

At present a few drastic surgical measures, that may have a significant impact on the quality of life, are available to prevent cancers from occurring in predisposed individuals. Oophorectomy together with prophylactic bilateral mastectomy may be
employed for carriers of a BRCA 1 gene mutation and colectomy may increase life expectancy for carriers of a mutated APC gene, but their relative risk of dying early is still increased threefold over that of the general population (Whitelaw et al., 1996; Struwing et al., 1995).

Using strategies such as that used for patient HUD III 5 it is possible to detect mutations in cancer predisposing genes at the single cell level with confidence. However, the ethical issues concerning embryo selection for diseases which are late onset and are not immediately life threatening remain controversial. The severity and age of onset of many cancer predisposition syndromes can vary dramatically from family to family making it difficult to formulate universally applicable guidelines. The family history and severity of symptoms experienced will undoubtedly influence the individual patient’s attitude to prenatal/preimplantation testing. Since surgical intervention does not completely eliminate the risk of cancer, and because there is patient demand for measures that prevent the inheritance of cancer predisposing mutations the development of preimplantation genetic diagnosis does not seem inappropriate (Whitelaw et al., 1996).

4.7. Conclusions.

The genetic analysis of cancer susceptibility syndromes has had unprecedented success in increasing our understanding of carcinogenesis, shedding light on the mechanisms underlying the formation of sporadic tumours as well as those resulting from inherited predisposition. Perhaps most importantly of all an insight into the pathways regulating cell division, growth and death in normal tissues has also been gained. The RB1 gene, for example, that is responsible for the inherited cancer syndrome retinoblastoma and has a role in the formation of sporadic retinal tumours, is now known to be important in the regulation of the cell cycle (Weinberg et al., 1995). The adenomatous polyposis coli (APC) gene, mutation of which influences the development of inherited and sporadic forms of colorectal cancer, has been found to bind a number of previously characterised proteins which suggest diverse roles for this large protein. It seems likely that APC expression influences cell fate, probably via the vertebrate homologue of the wingless signalling pathway critical in Drosophila
development. The APC gene has also been implicated in cell adhesion, a function that has also been suggested for other tumour suppressor genes mutated during colorectal tumourigenesis such as the deleted in colon cancer (DCC) and β-catenin genes. It may be that cell-cell interactions mediated by these genes are particularly important in the maintenance of epithelial sheets.

One intriguing feature common to genes associated with inherited cancer predisposition is that inherited oncogenic mutations do not produce tumours in all tissues in which the gene is expressed. This is true of APC which is widely expressed and yet only causes predisposition to cancer in a small number of tissues, principally in the colonic epithelium. Such genes may have tissue specific functions or be redundant in certain tissues, other genes fulfilling related or identical roles.

Although many of the genes critical in the development of a wide range of malignancies have now been isolated there still remain low penetrance tumour susceptibility genes that have not yet been identified. In addition to these there are undoubtedly some genes that function in tumour formation that will not be found in the form of a heritable predisposition to cancer due to embryonic lethality.

As well as aiding our understanding of basic biological questions pertaining to tumour formation genetics has also provided a powerful tool for the clinical assessment and management of individuals predisposed to cancer. Detection of a germline mutation within one member of an affected family allows other carriers to be identified with ease. Once accomplished appropriate strategies for the surveillance of the predisposed tissue can be devised improving the likelihood that tumours will be detected early and that surgical intervention will be successful.

Certain mutations have also been found to influence the likely prognosis of sporadic and inherited forms of neoplasia. Such as loss of chromosome 17p (\textit{TP53}) in colon cancer (Rodrigues \textit{et al.}, 1990; Kern \textit{et al.}, 1989). In the future detection of such mutations may allow precise therapies to be tailored to individual tumours. The use of nonsteroidal anti-inflammatory drugs (NSAIDs) have been shown to reduce the incidence of colorectal cancer and cause regression of polyps in familial adenomatous polyposis (FAP) patients (Jacoby \textit{et al.}, 1996). Such treatments could soon be supplemented by new therapies specifically targeting the genes now known to be involved in colorectal tumour formation. Indeed it now appears that this is precisely
how NSAIDs function, suppressing tumour formation by inhibiting the prostaglandin H synthase-2 (COX-2) gene which is frequently over-expressed in colorectal cancer (Oshima et al., 1996). However, until more effective treatments are available colectomy and ileorectal anastomosis will likely remain the most effective preventative treatment for FAP patients.

Detection of inherited mutations in cancer susceptibility genes not only allows presymptomatic diagnosis, but also the possibility of prenatal and even preimplantation diagnosis of inherited tumour predisposition. Although few patients have so far requested mutation detection at prenatal stages it seems likely that a significant proportion of families will be interested in these forms of diagnosis, particularly if techniques such as preimplantation genetic diagnosis are publicised.

The development of methods such as the protein truncation test (PTT) and immunological assays for the rapid detection of mutations in tumour suppressor genes should help to make screening of asymptomatic family members more practical, and are particularly important in large genes such as APC that have a heterogeneous spread of mutations throughout the coding region.

The discovery of mutations in several different tumour suppressor genes and oncogenes in individual tumours has confirmed earlier suspicions that carcinogenesis is a multi-step process, effectively an evolutionary process at the somatic cell level involving mechanisms of mutation and selection. Interestingly the simultaneous disruption of genes functioning in cell-cycle and apoptotic pathways may produce the most pronounced affects on the tumourigenic phenotype. Genes that have been shown to combine in this way include RBI and TP53 (Williams et al., 1994) and MYC and TP53 (Blyth et al., 1995).

The majority of tumour suppressor genes were originally thought to act as 'gatekeepers' having a direct influence over cellular proliferation. However with multiple genetic events necessary for carcinogenesis it is clear that factors affecting the mutation rate and efficiency of DNA repair will also influence cancer formation. Exposure to radiation or carcinogens that damage DNA has long been known to increase cancer risk, however now it is also clear that genes involved in the maintenance of genomic integrity ('caretakers') are responsible for many cases of
inherited predisposition to cancer, and for a significant number of sporadic cases also. Examples of such genes include those that confer sensitivity to radiation such as the ataxia telangiectasia gene (ATM), the nucleotide-excision-repair genes that are responsible for xeroderma pigmentosum, genes that are involved in the metabolism of carcinogens and most recently discovered the genes underlying hereditary nonpolyposis colorectal cancer (HNPCC) which are necessary for efficient repair of DNA mismatches. It is also thought likely that the BRCA1 and BRCA2 genes responsible for breast cancer susceptibility act as caretakers, although how they achieve this is not entirely clear at present. The identification of tumours that have mutations of caretaker genes such as those associated with DNA repair may have clinical significance. Chemotherapeutic agents which induce the form of DNA damage that the tumour cells can no longer repair should respond favourably.

In addition to genetic instability resulting from deficiency of DNA mismatch repair the recent identification of differences in the methylation competence of different colorectal cancer cell lines has shed light on another mechanism of genetic instability and tumourigenesis which may prove to be even more significant affecting chromosomal segregation.

Animal models of inherited cancer susceptibility provide versatile tools for investigating the biochemical and biological consequences of cancer susceptibility gene mutation. Breeding experiments provide an opportunity to examine the existence of modifying loci, as in mice carrying mutation of the murine APC gene which develop a varying number of intestinal tumours depending on which mouse strain (genetic background) is used. A number of mouse models for inherited cancer syndromes have now been generated (reviewed in Jacks et al., 1996). Although not all the mice closely mirror the features of the human disease as do those with APC mutation.

Extensive research in the field of cancer genetics conducted over recent years has yielded a vast amount of data, with implications for cell and molecular biology as well as for clinical practise. Research is now focusing on the full characterisation of the tumour susceptibility genes already identified, on the weakly penetrant genes not yet characterised, and on the rare pedigrees in which the inheritance of forms of cancer not associated with any of the common predisposition syndromes segregate as a single
Mendelian trait. This research will undoubtedly illuminate still further the mechanisms underlying cell growth control and normal tissue homeostasis opening up yet more possibilities for therapeutic intervention.
Chapter 5

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Publications arising from this thesis.


