MOLECULAR ANALYSIS OF THE
ADENOMATOUS POLYPOSIS COLI GENE REGION

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The *adenomatous polyposis coli (APC)* gene has been mapped to the the long arm of human chromosome 5. Mutations of the *APC* gene are responsible for familial adenomatous polyposis (FAP); a dominantly inherited predisposition to colorectal cancer.

In order to isolate DNA probes in the *APC* gene region human:hamster somatic cell hybrids were made in which human chromosome 5 is retained. These were derived from three individuals: one patient with FAP and an interstitial deletion of chromosome 5, one patient with an interstitial deletion of chromosome 5 of unknown FAP status and a patient with FAP and mental retardation with no visible karyotypic abnormality. The two human chromosome 5s were segregated in these hybrids and they have been used to construct a mapping panel.

A panel of 150 radiation hybrids was made from somatic cell hybrid PN/TS-1, in which the only human genetic material is chromosome 5. These hybrids were used to order probes from the *APC* gene region. A lambda library was constructed from a radiation hybrid retaining genetic material in the *APC* gene region and the human clones mapped on the chromosome 5 deletion hybrid panel.

The retinoblastoma model suggests that inherited cancer genes may also be involved in the development of sporadic tumours. This hypothesis is supported by the loss of genetic material on chromosome 5 in sporadic, non-familial, colorectal cancer. As the majority of colorectal carcinomas are thought to arise from pre-existing adenomatous polyps studies of loss of heterozygosity for markers on chromosome 5 were extended to sporadic colorectal adenomas and carcinomas. Loss of genetic material on chromosome 5 appears to be a frequent early event in colorectal tumourigenesis.

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ABBREVIATIONS

The following abbreviations have been used in this thesis:

APC	*Adenomatous polyposis coli*
ATP	Adenosine-5'-triphosphate
BSA	Bovine serum albumin
CFS	Cancer family syndrome
dATP	Deoxyadenosine-5'-triphosphate
dCTP	Deoxycytidine-5'-triphosphate
dGTP	Deoxyguanosine-5'-triphosphate
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DNAase	Deoxyribonuclease
dTTP	Deoxythymidine-5'-triphosphate
E4	Dulbecco's modification of Eagle's minimal essential medium
EDTA	Ethylenediaminetetraacetic acid
FAP	Familial adenomatous polyposis
HAT	Hypoxanthine, methotrexate and thymidine
HSSCC	Hereditary site-specific colon cancer
PCR	Polymerase chain reaction
RH	Radiation hybrid
RNA	Ribonucleic acid
RNAase	Ribonuclease
SDS	Sodium dodecylsulphate
SSC	Saline sodium citrate
TE	Tris and EDTA
TEN	Tris, EDTA and NaCl
TES	Tris, EDTA and SDS
TK/tk	Thymidine kinase
Tris	Tris(hydroxymethyl)aminomethane

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CHAPTER ONE

INTRODUCTION

Introduction

Inherited mutations of the *Adenomatous Polyposis Coli (APC)* gene are responsible for familial adenomatous polyposis (FAP), a rare autosomal dominant predisposition to colorectal cancer. The *APC* gene has been mapped to human chromosome 5 by linkage studies in FAP families (Bodmer et al., 1987 and Leppert et al., 1987). There is evidence that somatic mutations of the *APC* are involved in the aetiology of non-FAP colorectal cancer (Solomon et al., 1987).

In order to clone and characterise the *APC* gene further DNA markers were required in the region; initially to construct a physical map and ultimately to isolate the gene itself. The mapping of further DNA markers to a region can be greatly simplified by the use of somatic cell hybrids. A panel of hybrids including one hybrid retaining the single human chromosome of interest and other hybrids retaining the same chromosome with a deletion or translocation in the region of interest allows the rapid mapping of probes.

The majority of colorectal carcinomas are thought to arise from pre-existing adenomatous polyps. This allows the investigation of the stage at which tumourigenic alterations of chromosome 5, involving the *APC* gene, occur in both FAP and non-FAP tumours. Further polymorphic markers on chromosome 5 would also allow the characterisation of the chromosomal mechanisms involved.

This thesis describes the production of somatic cell hybrids retaining human chromosome 5 and fragments of human chromosome 5 to construct a panel of hybrids to map probes into the *APC* gene region. The role of the *APC* gene in the aetiology of non-FAP colorectal cancer has also been investigated in studies of loss of constitutional heterozygosity in common colorectal adenomas and carcinomas.

Familial adenomatous polyposis

FAP is a rare inherited predisposition to colorectal cancer in which affected individuals develop benign adenomatous polyps of the large bowel during adolescence and almost inevitably go on to develop colorectal cancer at an early age (Bussey, 1975). The genetic locus responsible for this condition, designated *Adenomatous Polyposis Coli* (McAlpine et al., 1987), has been mapped to chromosome 5 by family linkage studies (Bodmer et al., 1987; Leppert et al., 1987).

Historical Review

Familial adenomatous polyposis was first clearly described by Sklifasowski (1881) in a 51 year old man with a long history of bloody diarrhoea in whom multiple rectal adenomatous polyps were present. Cripps (1882) described a further two cases of disseminated polyps of the rectum and Smith (1887) the familial nature of the disease when he reported three cases occurring in one family. Handford (1890) noted the potential of the adenomatous polyps to progress to adenocarcinoma. Cockayne (1927) classified the disease as a mendelian dominant and by 1934 early prophylactic colectomy with ileo-rectal anastomosis had been recognised as the treatment of choice (Lockhart-Mummery, 1934). The first extra-intestinal features of the condition, 'multiple soft and hard tumours', were described by Gardner (1951) and many other extra-intestinal features have subsequently been added.

Genetics and Prevalence

Polyposis registries have contributed a large proportion of the information available on the prevalence, genetics and clinical features of familial adenomatous polyposis. The population prevalence has been estimated at approximately 1 in 8,000 and there appears to be a fairly uniform world-wide distribution (Reed and Neel, 1955; Alm and Licznerski, 1973 and Bulow, 1987). A high proportion of cases (90/200 families at St Mark's) are isolated with

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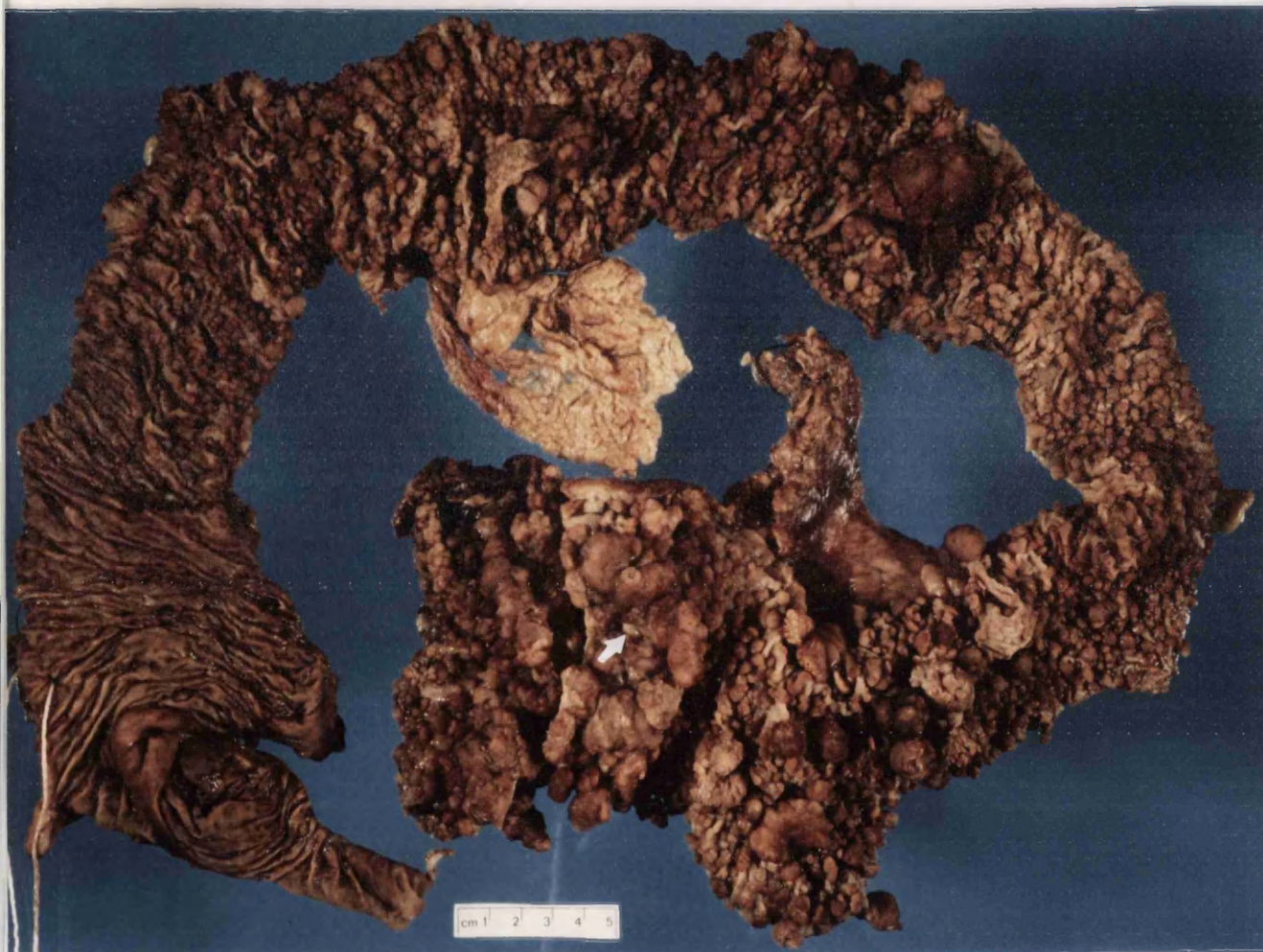
Figure 1

A surgical resection specimen of a familial adenomatous polyposis colon with multiple adenomatous polyps and a carcinoma (arrowed). The polyps are less prominent in the proximal colon.

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no known affected relatives at the time of diagnosis. These isolated cases have been shown to behave in exactly the same manner as familial cases in transmitting the disease to their descendants. It is uncertain whether these isolated cases represent new mutations or whether they represent incomplete ascertainment of the family history.

The condition is inherited as an autosomal dominant with a high penetrance. Ten cases of obligate carriers, who did not develop polyps, but who had siblings or parents with the disease and passed the disease on to their children have been recorded. However, these cases may be due to incomplete knowledge of the medical history or death before polyposis manifested itself (Bussey, 1975).

Clinical Features

In familial adenomatous polyposis multiple adenomatous polyps develop throughout the colon and rectum at a median age of 15 years (Murday and Slack, 1989). These polyps must be characterised histologically as adenomatous to distinguish them from the hamartomatous polyps of Peutz-Jeghers syndrome, the mucus retention polyps of juvenile polyposis and inflammatory polyps. More than a thousand visible polyps are usually distributed with increasing frequency from proximal to distal in the colon and the presence of in excess of a hundred differentiates between the polyposis and the non-polyposis syndromes (Bussey, 1975).

The regression and disappearance of rectal polyps following colectomy and ileo-rectal anastomosis has been observed in some patients and indicates the importance of non genetic factors in polyp development. However, the adenomas usually reappear within a few years (Bussey, 1975).

The mean age of development of colorectal cancer in polyposis patients at St Mark's series was 39 years of age and there were multiple carcinomas in 48% of patients. In contrast, non polyposis cancers had a mean age of onset of 60 years and were multiple in

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only 2.6% of patients. The distribution of the sites of carcinomas in the colon is the same for both types of tumours (Bussey, 1975).

Histology of Familial Adenomatous Polyposis

The first detectable histological abnormality in the progression to malignancy seen in the FAP colon is the single crypt adenoma (Bussey, 1975). However, others have investigated whether the genetic defect responsible for FAP results in a more generalised and subtle abnormality of the colonic epithelium. Cell kinetic studies have been reported to show a hyperproliferative normal-appearing colonic epithelium in FAP.

Two principal generalised abnormalities of the FAP mucosa have been described. In the Phase 1 lesion dividing cells, which incorporate tritiated thymidine, have been detected in the upper third of colonic crypts. In normal individuals these cells are usually confined to the lower and middle third of the crypt (Lipkin, 1974). In the Phase 2 lesion the major proliferative compartment is shifted towards the luminal surface of the crypt (Deschner and Lipkin, 1975). These abnormalities are not present in all FAP colonic mucosa and represent a widespread focal abnormality which precedes adenoma formation (Deschner and Raicht, 1981).

Similar proliferative changes have been detected in the colonic mucosa of patients with sporadic adenomas and carcinomas (Wilson et al., 1990).

Upper Gastrointestinal Neoplasia

Tumours of the upper gastrointestinal tract are now the most common malignant neoplasms in familial adenomatous polyposis patients who have had early prophylactic colectomies (Bussey, 1985).

Two principal types of upper gastrointestinal lesions have been described in FAP. Gastric polyps were first described by Hauser (1895) and were thought to be uncommon until the advent of

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flexible upper gastrointestinal endoscopy. In fundic gland polypsis there is a cystic dilatation of the fundic glands and this appears to be a benign lesion (Wanatabe et al., 1978). In contrast, duodenal adenomas have been found in a large proportion of polyposis patients and in a recent report were present in 100/102 patients in whom duodenal biopsies were taken for histology (Domizio et al, 1990). These adenomas appear to have a similar malignant potential to colonic adenomas and their periampullary distribution which has led to the suggestion that bile may be implicated in their development. Periampullary carcinomas are now the commonest fatal tumours in patients who have had a prophylactic colectomy (Jagelman et al., 1988).

Extra-intestinal features of familial adenomatous polyposis

Several extra-intestinal features have been described in familial adenomatous polyposis and have been proposed as early markers of the disease. Gardner and Richards (1953) described multiple subcutaneous cysts and osteomas in a family with polyposis. The subcutaneous lesions were later shown to be epidermoid cysts and are uncommon in unaffected young adults (Leppard and Bussey, 1975).

Utsunomiya and Nakamura (1975) detected mandibular osteomas in more than 90% of polyposis patients. However, mandibular osteomas are also common in the general population. Gardner syndrome, comprising the triad of adenomatous polyposis, epidermoid cysts and multiple osteomas is now considered to be part of the clinical spectrum of familial adenomatous polyposis as these features can be found in some members of many polyposis families.

Desmoid tumours develop in 4% of FAP patients (Bussey, 1975). They are band-like fibromatous intra-abdominal tumours that frequently arise following local trauma, such as surgery, in polyposis patients and although non malignant they may be rapidly growing and locally invasive.

Congenital hypertrophy of the retinal pigment epithelium (CHRPE) has been shown to be associated with polyposis in some families (Blaire and Trempe, 1980). A recent study of CHRPE in FAP included two cases of a parent with polyposis and CHRPE in whom an affected offspring had polyposis but did not have CHRPE. Thus CHRPE is not an entirely consistent marker of the inheritance of FAP in those families in which it is expressed (Polkinghorne et al, 1990).

Extra-intestinal Tumours

There is also an increased incidence of several extra-intestinal tumours in FAP patients. An increased incidence of primary brain tumours, particularly medulloblastomas, has been reported in FAP patients (Turcot et al, 1959; Kropilak et al, 1989). Papillary carcinoma of the thyroid has a 50-fold increased incidence in FAP patients and occurs at an earlier age than in the general population (Plail et al, 1985). There is also an increased incidence of hepatoblastoma in the children of FAP patients. There are reports of successfully treated hepatoblastoma patients from FAP families who have later developed adenomatous polyposis (Kingston et al, 1983; Li et al, 1987).

Hereditary Non-polyposis Colorectal Cancer

There are several other dominantly inherited predispositions to colorectal cancer that do not result in the development of hundreds of adenomatous polyps. Hereditary non-polyposis colorectal cancer is usually divided into two subcategories: cancer family syndrome (CFS or Lynch syndrome II) in which there is a predisposition to colorectal cancer, endometrial cancer, ovarian cancer and stomach cancer and hereditary site specific colon cancer (HSSCC or Lynch syndrome I) in which there is only a predisposition to colonic cancer (Lynch et al., 1985a; Mecklin et al., 1987).

Cancer Family Syndrome

Warthin (1913) reported a family in which there was a high incidence of colorectal carcinomas and also of adenocarcinomas of

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the uterus, breast and ovary. Lynch and Crush (1971) subsequently showed autosomal dominant inheritance of the predisposition over five generations. The colorectal carcinomas in the kindred had a mean age of onset of 45 years, twenty years earlier than the mean age of 65 years in common colorectal cancer. Two thirds of the tumours were right-sided as opposed to 15% in non-familial cases (Lynch et al, 1988).

Hereditary site specific colon cancer

Woolfe et al (1955) reported a family in which there appeared to be a dominant inheritance of colorectal cancer and an increased incidence of adenomatous polyps. However, there did not appear to be an increased incidence of other adenocarcinomas as in cancer family syndrome. A similar pedigree was subsequently reported by Lynch et al (1977) in which the mean age of onset of colorectal cancer was 45 years and there was a strong right-sided preponderance of carcinomas.

In HSSCC, unlike FAP the colon is not carpeted in adenomatous polyps and although adenomatous polyps are found in patients with this syndrome, their numbers may, in some cases, be less than in the general population (Mecklin and Jarvinen, 1986). Thus, if the adenoma is the precursor lesion, the feature of these syndromes is the high malignant potential of these adenomatous polyps.

Lynch et al (1985a) calculated that the nonpolyposis syndromes are responsible for approximately 5% of all colorectal cancers in the United States and similar estimates have come from Canada and Finland (Westlake et al., 1990; Mecklin, 1987).

Common colorectal cancer

Colorectal cancer is a major cause of mortality in the United Kingdom. In 1985 nearly 24,000 new cases were registeredand there were 17,000 deaths in England and Wales were registered

(Office Population Censuses and Surveys,1985a; Office Population Censuses and Surveys, 1985b).

A small proportion of colorectal carcinomas occur in individuals with highly penetrant autosomal dominant inherited disorders such as FAP, cancer family syndrome or site specific colon cancer and a few occur in individuals with predisposing conditions such as extensive ulcerative colitis (Lennard-Jones et al, 1983). In the vast majority of cases there has, until recently, been no evidence of any inherited predisposition.

The sex incidence of colorectal cancer is equal; although rectal tumours are slightly more common in men and colonic tumours slightly more common in women. The incidence increases with age and the average age of diagnosis is between 60 and 65 years of age.

Epidemiology of Colorectal Cancer

Epidemiological studies have indicated the important role of environmental factors in the aetiology of colorectal cancer. There is a high incidence in industrialised western countries such as the United Kingdom and the United States where there has been a gradual decline mortality (Cutler and Young, 1975; Boyle et al., 1985). In contrast, there is a lower incidence of colorectal cancer in South and Central America, Asia and Central and Eastern Europe where there has been an increase in mortality (Boyle et al., 1985). Migrants from areas of low incidence to areas of high incidence, such as Japanese immigrants to California, show an increase in the incidence of colorectal cancer (McMichael et al., 1980; Warshauer et al., 1986)

Diet has been implicated as the major environmental determinant of risk. A striking correlation has been noted between the national incidence rates of colon cancer and the per capita consumption of meat or animal fat. Case-control studies have shown a significant association between total fat, and also total energy, intake and the risk of colon cancer. It has been suggested that increased fat in the diet may increase the excretion of bile salts which in animal studies

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have been shown to act as tumour promoters. An inverse relationship between fibre intake and the rates of colon cancer has also been noted (Reviewed by Willett, 1989).

Willett et al. (1990) performed a prospective study of 120,000 U.S. women to examine the intake of fat and fibre in relation to the incidence of colon cancer over a six year follow up period. Total energy intake was not significantly associated with the incidence of colon cancer. However, there was a significant positive trend for animal fat after adjustment for total energy intake. This association was present for both saturated and monosaturated fat (the two primary components of animal fat) but not for fat from dairy sources. An inverse association between the energy-adjusted intake of dietary fibre and the risk of colon cancer was found which was most marked for fruit fibre but was not significant. The strongest association of individual foods with risk of colon cancer was seen with beef, lamb and pork intake.

They concluded that a high consumption of red meat and fat from animal sources increased the incidence of colon cancer independently of total energy intake.

Genetic Epidemiology of Colorectal Cancer

Despite the overwhelming evidence of the importance of dietary factors there is also strong evidence for the importance of hereditary factors in the aetiology of colorectal cancer.

Mortality Studies of Colorectal Cancer

The first studies of familial aspects of colorectal cancer involved mortality studies of the relatives of probands with colorectal cancer from which polyposis families were excluded. Woolf, in Utah, examined the death certificates of the parents and sibs of 242 probands whose death certificates mentioned colorectal cancer (Woolf, 1958). Controls were chosen to be of the same sex as the deceased relative and to have died in the same county at approximately the same age. Relatives of the cases were identified

through the genealogical records of the Church of Latter-Day Saints. Death certificates from these individuals showed 26 cases of colorectal cancer among the relatives of probands and only 8 among the relatives of controls (relative risk = 3.25). There were 81 other cancers among the relatives of probands and 83 among the controls indicating no statistical evidence for other substantial increased malignancy risk within these families.

Macklin, in Ohio, conducted a similar study except that the observed number of cases of mortality due to colorectal cancer among the relatives of cases was compared to the expected number estimated from a statewide examination of death certificates (Macklin, 1960). She found that for first degree relatives 31 of 392 had died of colorectal cancer as compared to 9.7 expected (relative risk = 3.2). The relative risk for parents was 3.3 (17 observed against 5.2 expected) and for siblings 3.1 (14 observed against 4.5 expected). When aunts, uncles and grandparents were considered 47 cases were found among relatives as compared to 17.5 expected (relative risk 2.7) indicating that the increased risk extends beyond first degree relatives. Again no evidence of increased risk for other malignancies was found in the relatives.

Finally, Lovett (1976) at St Mark's Hospital, London obtained family histories from 209 incident cases of cancer of the colon or rectum. Death certificates were obtained for all deceased parents and sibs. Overall, for first degree relatives, 41 deaths were due to colorectal cancer against 11.7 expected from mortality statistics (relative risk = 3.5). There was a slightly higher increased risk to siblings of probands (18 observed against 3.4, relative risk = 5.3) as compared to parents (23 observed against 8.8 expected, relative risk = 2.8). Increased risk to relatives was also associated with early age of onset and the presence of multiple lesions of the colon in the proband.

Incidence Studies of Colorectal Cancer

Following the rather consistent results of the mortality studies, further studies were performed to examine the family history of

colorectal cancer among the relatives of either cancer probands or adenoma probands. These studies were reviewed by Bishop and Burt (1990). These studies are consistent with the mortality studies in showing an increased risk to the relatives of colorectal cancer probands of between two and three times the population risk. The frequency of a positive family history is similar among the families of probands with colorectal cancer to that in adenoma probands. Studies of the prevalence of adenomas in the relatives of either colorectal cancer or adenoma probands has shown a two to three fold increase in incidence as compared to control families. These results are all consistent with the inherited predisposition being to adenoma formation.

Pedigree Studies

Burt et al (1985) studied the inheritance of colonic polyps and cancers in a large pedigree in which there were multiple cases of colorectal cancer but no clear inheritance pattern. They found a greatly increased incidence of polyps in the kindred as compared to spouse controls. Pedigree analysis suggested that this increased incidence was the result of the inheritance of a partially penetrant autosomal dominant predisposition to polyp formation.

This study of the familial clustering of colorectal cancers was extended to 34 further kindreds to determine how frequently colorectal adenomas resulted from an inherited susceptibility (Cannon-Albright et al, 1988).

Extensive pedigrees were screened for adenomas using 60cm fibreoptic endoscopy. These pedigrees were selected by three different criteria (i) multiple cases of colorectal cancer among close relatives (5 families), (ii) an adenoma larger than 5mm or multiple adenomas in a proband (19 families) and (iii) an asymptomatic adenoma in a proband (10 families). In all 670 individuals from 34 kindreds were examined for polyps. Polyps were found twice as frequently in the first degree relatives of probands as in those of spouses who served as controls. There did not appear to be systematic differences in the frequency of adenomas among family

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members implying that similar predisposing factors were important in all the pedigrees.

Pedigree analysis was used to examine single locus major gene models to explain the pattern of inherited susceptibility to adenomas within the families. A dominant model was significantly more likely to explain the observed familial occurrence than recessive inheritance or sporadic occurrence. The estimated gene frequency was 0.19 with a lifetime penetrance of 0.4 for adenomas or colorectal cancer in susceptible individuals.

Thus in addition to the well-known highly penetrant inherited predispositions to colorectal cancer, such as FAP, there is also familial clustering of common colorectal cancer. The increased familial incidence of common colorectal cancer may be due to a genetic predisposition to the development of colorectal adenomas which is inherited as partially penetrant autosomal dominant trait.

Adenoma-carcinoma sequence

The majority of colorectal carcinomas are thought to arise from preexisting benign adenomatous polyps (Muto et al., 1975).

An adenoma is an abnormal focus of epithelial proliferation where immature proliferating cells are no longer limited to the base of the colonic crypt, as in normal colonic mucosa, but occupy the entire crypt length and appear within the surface epithelium (Jass, 1989).

There are many different lines of evidence which support the adenoma-carcinoma sequence in colorectal neoplasia:

1.   In general, populations with a high incidence of colorectal cancer also have a high incidence of adenomas which are more often multiple, large and show epithelial dysplasia.

2   There is an increased frequency of adenomas in the colons of patients with colorectal cancer and many small carinomas are associated with adenomatous tissue.

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3   The peak incidence of symptomatic adenomas is ten years prior to the peak incidence of colorectal cancer (Morson et al, 1983).

Colorectal adenomas are common and most never become malignant but those of greater than 1cm in diameter with a pronounced villous component or with severe epithelial dysplasia have the greatest malignant potential (Morson et al, 1983).

Pathology of colorectal cancer

Carcinomas of the large intestine occur most frequently in the rectum and sigmoid colon. Most carcinomas are small ulcerating tumours with annular constricting growth. Tumours are classified according their grade (degree of differentiation) and stage (extent of spread).

Grade of colorectal carcinomas

Well differentiated tumours have a morphology similar to adult cells in the tissue of origin. In colorectal carcinomas well differentiated tumours form tubules and glands whereas poorly differentiated tumours grow as sheets of cells without identifyable glandular structures. The grade of a tumour is related to the prognosis with lymph node metastasis being more common in high grade (poorly differentiated) tumours and five year survival being worse (Morson and Dawson, 1979).

Stage of colorectal carcinomas

Colorectal carcinomas are staged according to the Dukes classification (Dukes, 1932; Dukes and Bussey, 1958). This was originally introduced as a classification of rectal carcinomas and subsequently extended to colonic cancers as well. Colorectal carcinomas tend to spread circumferentially in the wall of the large intestine and also through the wall to the serosa. Involvement of the paracolic lymph nodes may be an early event whereas spread to the liver and other organs occurs relatively late. In Dukes'

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classification stage A tumours are confined to the intestinal wall
and have neither extended beyond the muscularis mucosa nor
involved lymph nodes. Stage B tumours have spread beyond the
muscularis mucosa but have not involved lymph nodes. Finally
stage C tumours have spread to involve lymph nodes. The five year
survival of stage A tumours is almost 100%, that of stage B tumours
70-80% and that of stage C tumours 40% (Morson and Dawson,
1979).

Management of colorectal carcinomas

The management of colorectal cancer depends upon early detection
and resection of the tumour either by endoscopic or surgical
techniques. The prognosis is closely related to the grade and stage
of the tumour and if resection is unsuccessful radiotherapy and
chemotherapy have a very limited role to play.

The Genetic Basis of Cancer

Introduction

Early observations suggested that cancer was the result of genetic
damage and subsequent advances have confirmed this; in many
tumours the specific genetic alterations leading to neoplasia have
been characterised.

As long ago as 1914 Boveri proposed, after studying abnormal
mitoses during the development of sea urchin embryos, that
malignant cells were mutant clones that had acquired an
unbalanced chromosome complement (Boveri, 1914). At about this
time Tyzzer noted that tumours had different characteristics from
the soma in which they arose and showed that these were retained
during artificial propogation of the tumour. He concluded that this
stable alteration of phenotype was due to mutation of the soma and
that cancer was the result of somatic mutation (Tyzzer, 1916).

Cancer is now thought to arise from successive somatic mutations
leading to the stepwise release of a clone of cells from normal

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growth control. Evidence for the somatic mutation theory of cancer includes that chemical carcinogens and ionising radiation are mutagenic and that a group of recessively inherited diseases in which there is a failure to repair DNA damage, with a resulting increase in somatic mutations, is associated with an increased cancer incidence (Bishop, 1987).

The genetic basis of some tumours is clear from rare inherited predispositions to cancer. It has also been noted that every human cancer has been reported in both hereditary and nonhereditary form (Knudson et al, 1973). These typically have an autosomal dominant inheritance pattern and lead to the development of a specific type or group of tumours. The two most common hereditary conditions that strongly predispose to cancer are von Recklinghausen neurofibromatosis (NF1) and familial adenomatous polyposis (Knudson, 1986). There are also several examples of autosomal recessive conditions that predispose to the development of tumours owing to a failure of DNA repair (Lehman, 1982).

Inherited DNA repair defects

The first of these recessive conditions to be characterised at a biochemical level was xeroderma pigmentosum (XP). The condition is characterised by extreme sensitivity of the skin to sunlight which induces freckling and atrophy followed by benign growths and later malignant skin tumours. Degenerative neurological abnormalities of varying severity are also associated with it. Cells from patients with XP are unable to repair DNA damage induced by ultraviolet radiation. The mutation itself is not directly oncogenic but results in a high somatic mutation rate and, as a consequence, a high incidence of skin cancer, melanoma and some internal cancers (Cleaver, 1968).

Ataxia telangiectasia is an autosomal recessive condition in which there is increased sensitivity to ionising radiation which results in breakages and rearrangements of chromosomes. Affected individuals are at increased risk of lymphoid leukaemias and lymphomas, and primary carcinomas of the stomach, liver, ovary,

salivary glands, oral cavity, breast and pancreas. Individuals heterozygous for the ataxia telangiectasia gene have also been shown to have an excess risk of developing cancer (Swift et al, 1987). In this condition there is defective excision repair of irradiation-damaged DNA (Paterson et al., 1976).

Bloom syndrome is associated with excessive chromosome breakage and rearrangement and increased cancer incidence and has been shown to result from deficiency of DNA ligase I activity (Willis and Lindahl, 1987, Chan et al, 1987).

Metabolic polymorphisms in cancer susceptibility

Environmental chemicals, including mutagens in the diet, may be responsible for a large proportion of the somatic mutations that result in carcinogenesis (Ames, 1979). Inherited variations in chemical uptake, delivery, oxidation and conjugation would be expected to lead to variations in the incidence of cancer caused by environmental chemicals.

There are a number of steps which are important in the metabolism of carcinogens and these are frequently performed by members of multigene families. The accumulation of chemicals may be affected by drug transport systems, such as P-glycoprotein which transport chemicals out of the cell. Most chemicals are oxidised to more polar products before they are excreted and almost all these reactions are catalysed by the P450-dependent monooxygenase system. This may result in the conversion of some carcinogens to their mutagenic form. Polarised chemicals are subsequently processed by a variety of metabolic pathways prior to excretion and these pathways include conjugation with glutathione and acetylation (Wolf, In Press).

Inherited polymorphisms in several of the members of these multigene families have already been described. For example, the P450 CYPIA polymorphic variation in debrisoquine metabolism and slow and fast acetylators. It is anticipated that these genetic

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variants will have an important role modulating the toxic effects of environmental chemicals.

Tumour kinetics

Studies of the kinetics of carcinogenesis have attempted to determine the number of events required for the development of a carcinoma. Nordling (1953) noted that the incidence of cancer increased exponentially with age. He proposed that if the mutation rate is constant and successive mutations led to the development of cancer one would expect the incidence of tumours to accumulate according to a certain exponent of age. Conversely, if one event was sufficient to cause cancer the incidence would be expected to be equally common in all age groups and if two events were required the incidence would increase in direct proportion to age.

The incidence of all carcinomas in males increased according to the sixth power of age suggesting that approximately seven events are involved in carcinogenesis (Nordling, 1953). This hypothesis was tested in several different adult tumours and a good correlation found between the sixth power of age and cancer incidence (Armitage and Doll, 1954). In colorectal cancer an excess of early tumours was noted and this was attributed to familial adenomatous polyposis. It was later demonstrated that the incidence of cancer was compatible with a two step model of carcinogenesis if the first mutation led to an increase in the growth rate and hence the mutation rate (Armitage and Doll, 1957).

Ashley (1969a) compared these two models and concluded that in female gastric cancer the data fitted better with the multistage model than with a two stage model of carcinogenesis. He also compared the age of incidence of colorectal cancers in the general population with those in patients with familial polyposis coli and remarkably, in view of recent data, concluded that one or two less "hits" were required in carriers of the familial polyposis gene (Ashley, 1969b).

A statistical study of retinoblastoma, a childhood eye tumour, occurring in both a dominantly inherited and a sporadic (non-familial) form was published by Knudson (1971). He noted that the inherited predisposition itself was insufficient to lead to tumour formation, as some gene carriers never developed the tumour although they transmitted the trait to their offspring. The number of tumours in inherited cases followed a Poisson distribution and this indicated that the second event was random. Analysis of the age-specific incidence of retinoblastoma indicated that the kinetics were consistent with one event in the inherited cases and with two events in the sporadic cases.

This led Knudson to conclude that retinoblastoma is caused by two mutational events; the same genetic changes occuring in both the hereditary and the nonhereditary forms. In the dominantly inherited form one mutation is inherited in the germline and the second occurs in somatic cells and in the sporadic form both mutations occur in the same somatic cell.

Knudson later noted that hereditary forms exist for almost all cancers and proposed that the underlying genetic events in both forms were the same (Knudson, 1973).

Dominantly-acting oncogenes

Identification of the specific genes responsible for neoplastic transformation initially resulted, somewhat suprisingly, from the study of rapidly transforming retroviruses, a highly atypical group of RNA viruses (Varmus, 1982, Bishop, 1983). These agents, whose action had originally been observed by Rous (1911), are able to transform normal cells into malignant cells *in vitro* and to cause tumours at the site of inoculation after a short latency *in vivo*. Genetic studies of these viruses led to the identification of viral oncogenic loci and to the hypothesis that these might be the cause of most human cancer (Gross, 1974).

Molecular cloning and hybridization experiments demonstrated that each retroviral oncogene had a highly conserved homologue in

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normal vertebrate DNA. Viral oncogenes are now thought to have arisen by illegitimate transduction of these normal cellular proto-oncogenes into the viral genome (Bishop, 1981). Somatic mutations of proto-oncogenes is thought to play an important role in the aetiology of many human tumours and several different mechanisms leading to their activation have been demonstrated (Bishop, 1987).

Proto-oncogene activation

Several different mutational mechanisms may result in the activation of proto-oncogenes. These mechanisms include insertions, translocations, amplification and point mutations.

A number of malignancies are characterised by specific chromosomal translocations and some of these have been shown to result in proto-oncogene activation. In Burkitt's lymphoma the translocation (t8;14) results in the juxtaposition of the c-*myc* proto-oncogene and immunoglobulin sequences and leads to increased c-myc expression (Cory, 1986). In chronic myeloid leukaemia the translocation (t9;22) results in the fusion of the c-*abl* proto-oncogene and the *bcr* gene, the product of the fusion gene has altered tyrosine kinase activity (Konopka et al., 1984).

Amplification of proto-oncogenes has been demonstrated in many different tumours and results in increased expression of their gene products. These changes have generally been found in cells that have already taken some steps towards neoplastic growth and have been implicated in the progression rather than the initiation of the malignant phenotype (Alitalo et al., 1983).

Gene transfer experiments have demonstrated mis-sense point mutations in proto-oncogenes that result in a single amino acid substitution in the gene product and account for the transforming activity of the oncogene. In the k-*ras* oncogene, for example, specific mutations of codons 12 and 61 result in transforming activity (Forrester et al., 1987).

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Almost 100 proto-oncogenes have now been identified and all have been found to have a role in the regulation of normal cellular growth. They have been characterised as growth factors, growth factor receptors, protein kinases, signal transducers, nuclear oncogenes of unknown function and transcription factors.

Cooperation of oncogenes in tumourigenesis

The activation of a single proto-oncogene is insufficient to lead to the transformation of a normal cell. This is consistent with a the multi-step model of tumourigenesis. Transfection of an activated *ras* or a *myc* oncogene alone does not result in the transformation of rat embryo fibroblasts. However, when *ras* and *myc* are co-transfected they elicit a fully tumourigenic phenotype (Land et al, 1983; Ruley, 1983). The collaboration of these oncogenes indicates that each acts in a distinct complementary way and it has been proposed that the alteration of both a cytoplasmic and a nuclear oncogene is needed for neoplastic transformation (Weinberg, 1985).

Growth suppressor genes

In contrast to the activation of dominant oncogenes there is also considerable evidence for the existence of recessively acting growth suppressor genes. A recessive model of carcinogenesis was first proposed following a kinetic study of papilloma formation in mice (Charles and Luce-Clausen, 1942).

Suppression of Malignancy

In 1969 the experimental fusion of normal and neoplastic cells to form somatic cell hybrids was shown, in some cases, to suppress the neoplastic phenotype. Subsequent segregation of chromosomes from the hybrids sometimes resulted in a reversion to malignancy (Harris et al, 1969). In a wide variety of mouse tumours suppression of malignancy was associated with the retention of the mouse chromosome 4 derived from the normal parent cell. In human hybrids the reappearance of malignancy, which had been suppressed, was associated with the loss of chromosome 1 from the

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normal parental cell. It is of interest that there is considerable homology between mouse chromosome 4 and human chromosome 1 (reviewed in Harris, 1988).

Chromomsome 11 has also been shown to suppress malignancy in some human tumours. The loss of the normal human chromosome 11 from a fusion between human uterine carcinoma cells and a fibroblast line resulted in the reappearance of malignancy in hybrids in which it was initially suppressed (Stanbridge et al., 1981). There is evidence that a normal human chromosome 11 alone, in the absence of other chromosomes derived from the normal parent, can suppress malignancy. A single normal human chromosome 11 introduced by microcell fusion into cells derived from a human uterine carcinoma and a Wilms tumour resulted in the loss of the ability of the resulting hybrids to form tumours in nude mice (Saxton et al., 1986; Weissman et al., 1987).

Stanbridge and Ceredig (1981) showed that hybrids derived from uterine carcinoma cells in which malignancy had been suppressed behaved differently in vivo from segregants in which malignancy had reappeared. In nude mice malignant hybrids grew progressively as an undifferentiated epithelial tumour whereas non-malignant hybrids developed an increasingly fibrocytic morphology and gradually ceased to multiply.

These experiments provide strong evidence that carcinogenesis may result, in part, from the loss of functional genes and that their replacement leads to the suppression of the malignant phenotype and in some cases to a restoration of normal terminal differentiation.

Models of Growth Suppressor Gene Function

De Mars proposed that dominantly inherited predispositions to cancer might have an underlying recessive mechanism. In his model an affected individual would be heterozygous for a recessive gene and a subsequent somatic mutation of the normal allele would

lead to homozygosity for a neoplasm-causing allele within an individual cell (De Mars, 1969).

Comings (1973) proposed an integrated model of carcinogenesis with recessively acting regulatory genes controlling the expression of transforming genes. In his model tumourigenesis resulted from somatic mutations inactivating diploid pairs of recessively acting regulatory genes in a tissue specific manner. The loss of the function of both regulatory genes led to the uninhibited expression of the transforming gene and transformation of the cell. He proposed that in the case of inherited tumours, such as retinoblastoma, the germline inheritance of one inactivate regulatory gene and subsequent somatic mutation of the second would led to carcinogenesis.

Retinoblastoma

Retinoblastoma provides a general model for the role of tumour suppressor genes in inherited and sporadic tumours. As it was the first tumour in which these genetic events were characterised and the gene responsible cloned it is discussed below.

The prediction that loss of function led to the development of tumours led investigators to look for chromosomal deletions in cases of retinoblastoma. The location of the retinoblastoma gene was suggested by the observation that 3% of inherited cases of retinoblastoma show a constitutional deletion of one chromosome 13 (Franke,1976). Yunis and Ramsey (1979) provided the first evidence for the recessive nature of the change in retinoblastoma tumourigenesis when they observed occassional deletions of genetic material on chromosome 13q14.1 in retinoblastoma tumours. These observations defined the region that was likely to be involved in the development of retinoblastoma.

The activity of the enzyme esterase D was shown to be reduced in patients with constitutional deletions of chromosome 13 and in family linkage studies isozymic forms of esterase D were shown to be closely linked to the retinoblastoma gene on chromosome 13

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(Sparkes et al, 1983). Benedict et al. (1983) reported a case in which there was further evidence of loss of genetic material being associated with tumourigenesis in retinoblastoma. The patient had a constitutional esterase D activity half that found in the general population but had no evidence of a constitutional deletion on chromosome 13. In the tumour esterase D activity was absent and only one chromosome 13 was present on karyotypic analysis. This was interpreted as indicating that the normal chromosome 13 had been lost and only the chromosome 13 in which esterase D and, presumably the retinoblastoma locus, had been deleted remained in the tumour.

The same electrophoretic polymorphism was used to examine six retinoblastoma tumours in patients who were constitutionally heterozygous for esterase D. The tumour cells from four of the six patients expressed only one of the two esterase D alleles. As some of these were inherited cases this indicated that the second event in retinoblastoma was the somatic inactivation of genes near the esterase D locus probably including the remaining normal retinoblastoma locus (Godbout, 1983).

Cavenee et al (1983) proposed that a variety of mutational mechanisms could result in the loss of the wild-type retinoblastoma gene and expression of the recessive mutant allele. These mechanisms he proposed are illustrated in figure 2.

Initially an individual cell may have one mutant retinoblastoma allele either from the germline or from a somatic mutation. The loss of the remaining wild-type allele may then result from mitotic non-disjunction with loss of the normal chromosome, or mitotic non-disjunction with reduplication of the abnormal chromosome. Both of these mechanisms would result in heterozygous markers on chromosome 13 becoming hemi- or homo-zygous in the tumour. An alternative mechanism leading to loss of the wild-type gene is a mitotic recombination between homologous chromosomes with a breakpoint between the tumour locus and the centromere. This would result in loss of heterozygosity in markers distal to the breakpoint. Regional events such as interstitial deletions, gene

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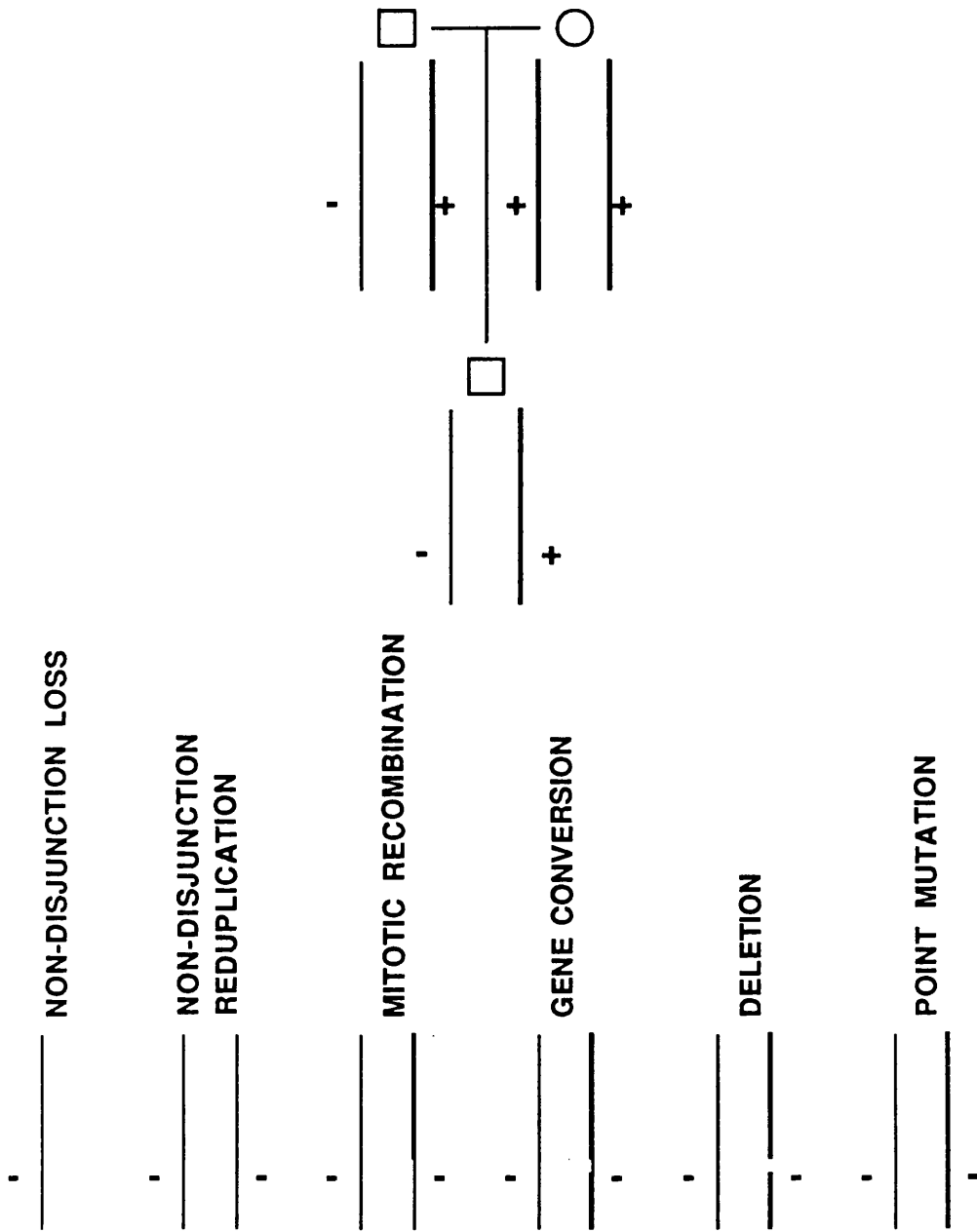
<u>Figure 2</u>

Chromosomal mechanisms that would reveal the presence of a recessive muatation, some of which would also result in the loss of constitutional heterozygosity (adapted from Cavenee et al., 1983).

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conversions and point mutations could also lead to the loss of the wild-type allele.

Cavenee et al. (1983) were able to demonstrate the loss of genetic material in retinoblastomas by the mechanisms they predicted. The loss of heterozygosity in tumours when compared to normal tissue has proved an extremely powerful method for demonstrating recessive changes in tumourigenesis. It has been used to detect the presence of tumour suppressor genes in a wide variety of tumours (see Table 1). Recent evidence suggests that other growth suppressor genes may not be entirely 'recessive' in the heterozygous state as was initially predicted in the retinoblastoma model (see Discussion Chapter).

Table 1 Summary by chromosome of allele loss in carcinomas

Chromosome

1 p	Phaeochromocytoma	
	Thryoid medullary carcinoma	(Mathew et al, 1987)
	Neuroblastoma	(Fong et al, 1989)
	Melanoma	(Dracopoli et al, 1989)
	Breast ductal carcinoma	(Genuardi et al, 1989)
	Colorectal carcinoma	(Leister et al, 1990)
1 q	Breast carcinoma	(Chen et al, 1989)
3	Lung small cell carcinoma	(Naylor et al, 1987)
		(Kok et al, 1987)
		(Yokota et al, 1987)
		(Braunch et al, 1987)
	Renal cell carcinoma	(Zbar et al, 1987)
	Ovarian carcinoma	(Ehlen et al, 1990)
	Cervical carcinoma	(Yotoka et al, 1989)
	Lung adenocarcinoma	(Yotoka et al, 1987)
4	Hepatocellular carcinoma	(Buetow et al, 1989)
5	Colorectal carcinoma	(Solomon et al,1987)
	Hepatocellular carcinoma	(Fujimori et al, 1991)
6	Ovarian carcinoma	(Ehlen et al, 1990)
9	Bladder carcinoma	(Tsai et al, 1990)
1 0	Glioblastoma multiforme	(James et al, 1988)
	Prostatic carcinoma	(Carter et al, 1990)
	Hepatocellular carcinoma	(Fujimori et al, 1991)
1 1	Wilms tumour	(Koufos et al, 1984)
		(Orkin et al, 1984)
		(Reeve et al, 1984)
		(Fearon et al, 1984)
	Hepatoblastoma	
	Rhabdomyosarcoma	(Koufos et al, 1985)
	Bladder carcinoma	(Fearon et al, 1985)
	Hepatocellular carcinoma	(Rogler et al, 1985)
	Breast carcinoma	(Ali et al, 1987)
	Insulinoma (MENI)	(Larsson et al, 1988)

	Parathyroid adenoma (MENI)	(Friedman et al,1989)
		(Thakker et al, 1989)
	Ovarian carcinoma	(Ehlen et al, 1990)
	Hepatocellular carcinoma	(Fujimori et al, 1991)
1 3	Retinoblastoma	(Cavenee et al, 1983)
	Osteosarcoma	(Hansen et al, 1985)
	Breast carcinoma	(Lundeberg et al, 1987
		(Varley et al, 1989)
	Gastric carcinoma	(Motomura et al, 1988)
	Lung small cell carcinoma	(Yokota et al, 1987)
1 6	Hepatocellular carcinoma	(Zhang et al, 1990)
	Prostatic carcinoma	(Carter et al, 1990)
	Hepatocellular carcinoma	(Fujimori et al, 1991)
1 7	Colorectal carcinoma	(Fearon et al, 1987)
		(Monpezat et al, 1987)
	Lung small cell carcinoma	(Yokota et al, 1987)
	Breast carcinoma	(MacKay et al, 1988)
	Gliomas	(James et al, 1988)
	Astrocytomas	(James et al, 1989)
	Bladder carcinoma	(Tsai et al, 1990)
	Hepatocellular carcinoma	(Fujimori et al, 1991)
1 8	Colorectal carcinoma	(Monpezat et al, 1987)
	Breast carcinoma	(Cropp et al., 1990)
2 2	Acoustic neuroma	(Seizinger et al, 1986)
	Phaeochromocytoma	
	Thyroid medullary carcinoma	(Takai et al, 1987)
	Meningioma	(Seizinger et al, 1987
		(Dumanski et al, 1987)
	Colorectal carcinoma	(Okamoto et al, 1988)

Table 1 (continued) Summary by chromosome of allele loss in carcinomas

The Retinoblastoma Gene

The retinoblastoma gene has now been cloned and encodes a M_r 105,000 phosphoprotein which is expressed in many tumour types. RNA transcripts are absent in both retinoblastomas and osteosarcomas (Friend et al, 1986; Lee et al, 1987a; Fung et al, 1987; Lee et al, 1987b).

A cloned wild-type retinoblastoma gene has been introduced, via retroviral-mediated gene transfer, into retinoblastoma cells that have an inactivated endogenous retinoblastoma gene. This resulted in the restitution of normal growth control (Huang et al, 1988). Inactivation of the gene has been reported in soft tissue- and osteosarcomas, small cell lung carcinomas, bladder carcinomas and breast carcinomas (Friend et al, 1987; Lee et al, 1988; Weichselbaum et al, 1988; Harbour et al, 1988; Horowitz et al, 1989).

The function of the retinoblastoma gene product

The cloning of the retinoblastoma gene has provided an unexpected insight into the action of transforming DNA viruses. It has been previously shown that the oncoprotein encoded by the E1A oncogene of the human adenovirus type 5 is associated with human host polypeptides in virus-transformed cells. Six host polypeptides of different molecular weights have been shown to complex E1A (Yee et al, 1985).

Harlow's group have demonstrated that one of these host proteins bound by E1A is the retinoblastoma gene product (RB). They were also able to demonstrate that mutations leading to the loss of transforming activity of E1A also prevented its binding to RB (Whyte et al, 1988). Thus one of the mechanisms of action of the oncoprotein E1A appears to be the binding of RB. Two other oncoproteins from DNA tumour viruses, SV40 large T (De Caprio et al, 1988) and Human papilloma virus Type 16 E7 (Dyson et al, 1989), have since been shown to bind RB. This suggests that these viruses have a common mechanism of transformation and indeed

an homologous transforming region is present in all three proteins (Figge et al, 1988).

Cyclical changes in the phosphorylation state of RB have been demonstrated during the cell cycle (Xu et al, 1989; DeCaprio et al, 1989; Buchlovitch et al, 1989; Chen et al, 1989). In G_0 RB is predominantly dephosphorylated whereas in cycling cells the majority of RB is phosphorylated. There appears to be a cyclical pattern of phosphorylation and dephosphorylation of RB during the cell cycle; RB being dephosphorylated after mitosis in G_1 and being rephosphorylated during S and G_2. Only dephosphorylated RB has been shown to co-precipitate with E1A and this has led to the hypothesis that phosphorylation regulates RB function (Ludlow et al 1990). In this model dephosphorylated RB in G_0 and G_1 would be active and inhibited by the oncoproteins E1A, large T and E7 whereas phosphorylated RB would be inactive. RB activity might either control the onset of S phase by blocking the onset of DNA synthesis or it might provide a window in which cell cycle exit is permitted (Cooper and Whyte, 1989). RB thus appears to have a central position in cell cycle control the details of which have yet to be determined.

It is of great interest that, despite the widespread expression of RB in all cell types and its apparently central role in the control of the cell cycle, germline mutations result in the development of one predominant tumour type. It is possible that the control of cell division in retinoblasts is sensitive to the absence of one gene product whereas in other tissues control of cell division is more complex. Similarly, the inactivation of the RB gene is only seen in selected adult tumours (see above).

Other Growth Suppressor Genes

The retinoblastoma model of growth suppressor gene function has been extended to many other inherited and sporadic tumours. Knudson et al. (1972) showed that the age-specific incidence of Wilms tumour, a childhood tumour of the kidney with inherited and sporadic forms, was also consistent with a two step model of

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tumourigenesis. A similar mechanism has also been proposed for inherited predispositions to adult tumours including von Recklinghausen neurofibromatosis, multiple endocrine neoplasia and FAP (see Discussion Chapter).

Human Gene Mapping

Introduction

In the past ten years there have been great advances in the techniques available for the mapping and cloning of human genes. These have largely arisen owing to developments in molecular biology which allow the cloning of DNA segments and the detection of polymorphic DNA sequences, known as restriction fragment length polymorphisms (RFLPs), in human DNA by restriction enzymes (Kan and Dozy, 1978). These DNA markers provide an almost unlimited source of polymorphic information. Solomon and Bodmer (1979) proposed that these would enable the construction of a linkage map of the human genome and Botstein et al. (1980) later enlarged upon the methods that would be required. Hitherto human gene mapping had been severely limited by the lack of polymorphic markers and dependent on chromosome analysis and the expression of phenotypes in somatic cell hybrids.

Chromosome Analysis

The human chromosome complement was first correctly described in 1956 (Tjio and Levan, 1956). High resolution banding of human chromosomes allowed the detection of interstitial deletions and translocations (Harnden and Klinger, 1985). In clinical genetics the association of karyotypic abnormalities with particular inherited diseases was sought (for example, triploidy of chromosome 21 in Down syndrome). Whereas in cancer research the association of karyotypic alterations with the development of malignancy were studied (for example, the Philadelphia chromosome).

Since the realisation that mutations of the same genes are involved in the development of both inherited and sporadic tumours constitutional karyotypic abnormalities associated with inherited

predispositions to malignancy have been sought. These chromosomal abnormalities may provide an indication as to the location of the disease genes.

Family Linkage Studies

Mapping by meiotic linkage was developed in Drosophila (Morgan, 1911). In humans its use was initially severely limited by small families, long generation time and the lack of polymorphic markers. These early markers depended upon the expression of protein polymorphisms, such as cell surface antigens and isozymes, and upon the expression of hereditary diseases. However, RFLPs have enabled the construction of a linkage map of the human genome. In practice it remains difficult to use linkage analysis to define a chromosomal position with an accuracy of more than a few centimorgans. This may represent a physical distance of several million base pairs.

Somatic Cell Genetics

The formation of interspecies hybrids allows the segregation of human chromosomes from one another. Barski and Cornefert (1962) demonstrated that fusion can occur between cultured mouse cells. Human and rodent cells will fuse more efficiently in the presence of fusogenic agents such as inactivated sendai virus (Harris and Watkins, 1965) or polyethylene glycol (Pontecorvo, 1975) to form non-dividing heterokaryons. A small proportion of the heterokaryons will reform a single nucleus and become a proliferating hybrid cell. Human chromosomes are segregated from rodent-human hybrid cells in an almost random manner (Weiss and Green, 1967). This results in clones retaining different human chromosomes. In a panel of rodent-human somatic cell hybrids the presence of a particular phenotype could be correlated with the presence of a particular human chromosome.

Selection of chromosomes in somatic cell hybrids

The development of drug-resistant and auxotroph (with special nutritional requirements) rodent cell lines has enabled selection for particular human chromosomes in somatic cell hybrids. 8-azoguanine and 5-bromodeoxyuridine resistant cell lines were first described by Szybalski et al. (1962).

Littlefield (1964; 1966) grew 8-azoguanine and 5-bromodeoxyuridine resistant cell lines together in the presence of hypoxanthine, thymidine and aminopterin (HAT). Aminopterin blocks the endogenous biosynthesis of purines and pyrimidines. 8-azoguanine and 5-bromodeoxyuridine resistant cells are deficient of hypoxanthine phosphoribosyl transferase (HPRT) and thymidine kinase (TK) respectively and are unable to synthetise purines and pyrimidines via a salvage pathway. Resistant colonies grew as a result of "mating of fibroblasts" from the two cell lines *in vitro* to form somatic cell hybrids.

The TK gene was mapped to human chromosome 17 by the demonstration of the selective retention of this human chromosome in human-rodent somatic cell hybrids made between thymidine kinase deficient rodent cells and human lymphocytes grown in HAT medium (Migeon and Miller, 1968).

Various other methods have been developed to allow the the selective retention of other human chromosomes in human-rodent hybrids. These methods include auxotroph mutants (Puck and Kao, 1967) and the selection by fluorescein-labelled monoclonal antibodies of hybrids expressing particular human cell surface markers. Since the advent of DNA polymorphisms mapping in somatic cell hybrids is no longer dependent on gene expression.

If mutant rodent lines are not available to select for the retention of a particular human chromosome a dominantly selectable marker may be used. Selectable markers may be transfected randomly into the donor cells, the human chromosome selectively retained in the resulting hybrids indicating the chromosome into which the marker

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has integrated (Tunnecliffe et al., 1983; Koi et al., 1989). More recently homologous recombination has been used to direct the site of integration of a dominantly selectable marker (Dorin et al., 1989).

Sub-chromosomal mapping of Genes

The use of cell lines with deletions or translocations to produce somatic cell hybrids allows the mapping of probes to particular regions of a chromosome. However, there are a limited number of deletions and translocations available and various methods have been devised to allow the segregation of chromosomal fragments within somatic cell hybrids.

In chromosome mediated gene transfer metaphase human chromosomes are transfected into rodent cells and selection applied for a dominantly selectable marker (McBride and Ozer, 1973). This results in the retention of human chromosomal fragments and allows the construction of panels with fragments of the selected chromosome. Unfortunately the process results in frequent rearrangements within the chromosomal fragments (Goodfellow and Pritchard, 1988).

Radiation Hybrids

An alternative method of mapping genes within human chromosomes was devised by Goss and Harris (1975). The donor cells were irradiated to induce double-stranded breaks in the chromosomes. The irradiated cells were then rescued by fusion to an HPRT deficient rodent cell line and HAT selection instituted. A panel of clones from the fusion was then analysed for the presence of markers on the human X chromosome. From the co-transfer of markers it was possible to determine the linear order of genes and to estimate the distance between them. The further apart markers are the less likely they are to be retained together since there is more likely to have been an irradiation induced break between them producing two separate fragments (Goss and Harris, 1977).

A somatic cell hybrid retaining a single human chromosome was later used as the irradiated donor cell in order that the resulting hybrids would only retain human chromosomal fragments from one human chromosome (Graw et al., 1988)

Cox et al. (1989) further modified the technique using a somatic cell hybrid retaining a single human chromosome as the irradiated donor cell but selecting for a hamster gene product and relying in the random co-transfection of human chromosomal fragments with hamster fragments into the recipient hamster cells. A panel of radiation hybrids was then screened for the presence or absence of specific human DNA markers. This allowed the ordering of DNA markers spanning millions of base pairs at the 500kb resolution level. Benham et al. (1989) used the same technique with three different dosages of radiation. It was anticipated that the smaller chromosomal fragments produced by higher radiation dosages might provide a better source of DNA for cloning.

Alternative methods of sub-chromosomal localisation

The availability of large numbers of polymorphic DNA markers has allowed family linkage studies to map the chromosomal position of probes to within a few centimorgans, although this is a time consuming method. *In situ* hybridisation using fluoroscein-labelled probes now also allows probes to be mapped to within 1-2% of the chromosome length (Landegend et al., 1987). More recently it has become possible to clone defined chromosomal regions by microdissection of banded chromosomes and enzymatic amplification (Ludecke et al., 1989).

Genomic Libraries

Genomic libraries contain a set of cloned DNA fragments which, as far as possible, are retained randomly, so that all fragments have an equal probability of being represented, and represent all the genomic DNA. These libraries are most commonly constructed in lambda phage and cosmid vectors introduced into E. coli. These allow insert DNA of between 7 and 22kb in lambda phage and of

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approximately 45kb in cosmids. Cosmid libraries, because of their larger insert size, are well-suited to isolating polymorphic probes and for chromosome walking whereas lambda phage libraries require less DNA and are easier to construct and simpler to screen.

Physical Mapping

The presence of CpG islands in mammalian DNA which are sensitive to digestion by rare-cutting methylation-sensitive enzymes allows the separation of DNA of several million base pairs in size in agarose gels using pulsed field electrophoresis (Smith et al., 1988). This mapping technique is limited by the non-random distribution of CpG islands in the genome.

CpG islands have been shown to be frequently associated with the 5' ends of transcribed genes (Bird, 1986). These regions may be selectively cloned in linking or end clone libraries to provided a series of spaced probes that are associated with transcribed genes (Frischauf, 1989). Large fragments of DNA of up to a million base pairs may also be cloned in yeast artificial chromosomes (Green and Olson,1990).

Deletion mapping, in which the common region of loss of heterozygosity in tumours is defined, may be used to map growth suppressor genes (Baker et al., 1989). Regional mutations around the disease gene, such as interstitial deletions, are found with increasing frequency with increasing proximity to the gene. The percentage of tumours showing allele loss will therefore increase with markers close to the disease gene.

Candidate Genes

The presence of a candidate gene is suggested by the identification of conserved non-repetitive sequence in the region where the disease locus has been mapped. Conserved sequences will hybridise to DNA from other species in a zoo blot. Transcribed sequences can be detectable on northern blots and the same probe may be used to screen cDNA libraries. Confirmation that the candidate gene is

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indeed the disease gene requires the demonstration of expression in apppropriate tissues and of mutations that either affect its level of expression or alter its sequence. It is particularly important to demonstrate a new mutation in an individual with no family history of the disease in whom the parents do not have the mutation to exclude the possibility of the mutation being a polymorphism. A truely recessive tumour suppressor gene would not be expected to express a functional product in the tumour.

Mapping of the APC gene

In the absence of any consistent karyotypic abnormality associated with FAP there was no clue as to the location of the APC gene. Herrera et al. (1986) published a case report of an individual with FAP and multiple other developmental abnormalities who had a constitutional interstitial deletion of the long arm of chromosome 5.

Bodmer et al. (1987) reported linkage between C11p11, a probe mapped to chromosome 5q21-q22, and APC. This linkage has since been confirmed by a number of other investigators including Leppert et al. (1987) and Meera Khan et al. (1988). Leppert et al. (1987) found no evidence of genetic heterogeneity between FAP and Gardner syndrome (FAP with extra-intestinal features) and this suggests that they are allelic.

Solomon et al. (1987) investigated whether the APC locus was involved in the aetiology of common colorectal cancer by looking for loss of heterozygosity in paired normal and tumour tissue. They found allele loss in between 20% and 40% of colorectal carcinomas. This suggested that somatic mutations of the APC gene were an important step in colorectal tumourigenesis.

Aims of Project

The papers of Bodmer et al. (1987) and Solomon et al (1987) suggest that the *APC* gene is a recessively-acting tumour suppressor gene on the long arm of chromosome 5.

The cloning and characterisation of the gene requires the isolation of further DNA markers in the region. It was anticipated that this would be greatly aided by a somatic cell hybrid which retained chromosome 5 as its only human material and by somatic cell hybrids containing human chromosome 5s with interstitial deletions in the *APC* gene region in the absence of the normal chromosome 5 homologue.

The proportion of colorectal tumours showing loss of heterozygosity and the stage at which this occurs could also be investigated further. This would require non-FAP adenomas, a larger panel of carcinomas and further polymorphic DNA markers on chromosome 5.

The project had three principal aims:

1   The construction of a panel of somatic cell hybrids that would allow DNA probes to be mapped into the *APC* region. This required a hybrid in which a normal chromosome 5 was the only human material and a series of hybrids in which chromosome 5s with deletions in the *APC* region were retained in the absence of a normal chromosome 5. The presence of a human band in the hybrid retaining the normal human chromosome 5 would map a probe to that chromosome and the absence of a human band in the hybrids retaining human chromosomes 5s with deletions in the *APC* region would map the probe to that region.

2.   The construction of a panel of radiation hybrids containing random fragments of human chromosome 5. The retention pattern of probes in these hybrids would allow the order of probes on chromosome 5 to be determined (Goss and Harris, 1977) and hybrids retaining DNA from the *APC* region could also be used as a

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source of DNA for cloning. A somatic cell hybrid in which only human chromosome 5 was retained could be used to produce the panel of radiation hybrids.

3.   The investigation of a larger panel of paired samples of normal and common colorectal carcinomas DNA for evidence of allele loss using further polymorphic DNA markers on chromosome 5. The common area of allele loss in these tumours would provide additional information as to the location of the *APC* gene. It was also intended to investigation non-FAP adenomas for evidence of allele loss on chromosome 5 to determine if genetic changes on chromosome 5 were also an early event in common colorectal tumourigenesis.

CHAPTER TWO

MATERIALS AND METHODS

Tissue Culture

Blood Collection

A 50ml tube (Falcon) containing 25ml of blood collection media was brought to room temperature and 25ml fresh blood added. The tube was inverted to mix. The sample was then maintained at room temperature.

Sterile Separation of Lymphocytes

The contents of each blood collection bottle were poured into a 250ml flask and the blood bottle was rinsed with 4ml of RPMI/HEPES. 20 sterile glass beads were added to the flask and then 0.6ml of sterile 1M calcium chloride was added through the foil top of the flask and the blood defibrinated for 15 minutes at 250 rpm on a gyratory shaker.

20ml RPMI/HEPES was then added to the flask and the defibrinated blood was carefully overlayed on two 50ml tubes each containing 14mls of 'Lymphoprep' (Nyegaard). The cells were separated by centrifugation at 700g for 20 minutes. The interface between the 'Lymphoprep' and the serum was removed and diluted 1:1 with RPMI/HEPES. The cells were counted in a haemocytometer. The cells were then spun at 1000g for 10 minutes and the supernatant aspirated. The cell pellets were then frozen in 1 ml foetal calf serum plus 10% Dimethyl sulphoxide at
-70^{0}C.

Epstein Barr Virus Transformation of Lymphocytes

This was performed by Miss Cynthia Dixon (Director's Laboratory, ICRF) according to the protocol of Pelloquin et al. (1986).

Cell Culture

The lymphoid cell lines were grown in suspension at 37^0C buffered in 5% CO_2 in RPMI1640 medium with 100 units/ml penicillin and 100μg/ml streptomycin supplemented with 10% foetal calf serum .

The Chinese hamster ovary cell line tsH1 (Thompson et al., 1973) was grown at 33^0C buffered in 10% CO_2 in E4 (Dulbecco's modified minimal essential medium) with 100 units/ml penicillin and 100μg/ml streptomycin supplemented with 10% foetal calf serum and L-proline $(10^{-4}M)$.

The chinese hamster ovary cell line A23 tk⁻ (Westerveld et al., 1971), which is thymidine kinase deficient, was grown at 37^0C in 10% CO_2 in E4 (Dulbecco's modification of Eagle's minimal essential medium) with 100 units/ml penicillin and 100μg/ml streptomycin supplemented with 10% foetal calf serum. HAT medium was made by adding hypoxanthine $(10^{-4}M)$, thymidine $(1.6 \times 10^{-6}M)$ and methotrexate $(10^{-5}M)$.

Suspension cultures were harvested by centrifugation at 1000g in 50ml tubes. Monolayer cultures were either trypsinised after washing with phosphate buffered saline and versine or were physically removed from the plate with a rubber policeman.

Cell Fusions

Interspecific hybrids were produced according to the protocol of Pontecorvo (1975). 2gm of polyethylene glycol 4000 was autoclaved in a glass universal and a bottle of serum-free RPMI 1640 was prewarmed to 45^0C. 2ml of prewarmed RPMI 1640 was slowly added to dissolve the polyethylene glycol. The resulting solution was equilibrated in a 5% CO_2 incubator at 37^0C overnight.

5×10^6 cells from each parental cell line were harvested and washed in serum-free E4 and then separated by centrifugation at 1000g into a common pellet and the supernatant aspirated. 1ml 50% v/v polyethylene glycol, prewarmed to 37^0C, was slowly added

to the pellet over 1 minute while gently stirring. The pellet was then incubated for 90 seconds at 37^0C. 1ml of serum-free E4 was added slowly over 1 minute while gently stirring, a further 5mls was then added over the following minute and finally 10mls added over a third minute. The suspended cells were then separated by centrifugation at 1000g for 5 minutes, the supernatant aspirated and the cells resuspended in growth medium.

The hybrid cells were divided between ten 90 mm petri dishes to which 10ml growth medium was added. The temperature was raised to 39^0C to select for the hybrid cells and 10 μM Oubain added to select against human cells (Kucherlapati et al, 1975). The medium was changed twice weekly and colonies were ready for picking after two to three weeks and were expanded.

Radiation Hybrids

Radiation hybrids were produced according to the protocol of Benham et al. (1988). 5 x 10^6 cells of the donor cell line (PN/TS-1) were harvested and suspended in growth medium. They were then placed on ice and irradiated using an industrial X-ray unit (HF320 SR, Pantak). After irradiation the cells were washed in serum-free medium and combined with 5 x 10^6 recipient cells. The cells were then fused according to the protocol described above. After fusion the cells were plated in twenty 90mm petri dishes and HAT selection applied after 24 hours. Colonies were ready to be picked after ten days and were expanded.

Lysis of cells for Polymerase Chain Reaction

Four different methods of cell lysis were used, in which approximately one thousand cells from each hybrid clone and from both parental cell lines were harvested.

Method I. The cells were lysed by heating in distilled water for 3 minutes at 95^0C and spun before being added to the PCR reaction mixture (Jeffreys et al., 1988)

Method II. The cells were digested with 50μg/ml proteinase K in 5mM Tris-HCl pH7.5 and 3.4μM SDS for 45minutes at 37^{0}C and then for 3minutes at 95^{0}C before being added to the PCR reaction mixture (Jeffreys et al., 1988).

Method III. The cells were digested with proteinase K as above and the DNA was extracted once with phenol: chloroform: isoamylalcohol (50:48:2), precipitated with ethanol and air-dried before being dissolved in the PCR reaction mixture (Lench et al., 1988).

Method IV. The cells were resuspended in 1ml 1% SDS and incubated for 1-2 hours at 55^{0}C, the DNA was extracted once with phenol: chloroform: isoamylalcohol (50:48:2), precipitated with ethanol and air-dried before being dissolved in the PCR reaction mixture (Lench et al., 1988).

Chromosome Analysis

Chromosome analysis was performed by Dr Sarah Williams (Human Cytogenetics Laboratory, ICRF). Chromosome preparations were made from the hybrids by standard techniques (Bobrow and Cross, 1974; Caspersson et al., 1971). Slides were prebanded by incubation in 2x SSC at 60^{0}C followed by staining with Wright's stain. After photographs had been taken the slides were destained and hybridised in situ with biotinylated total human DNA (Pinkel et al., 1986). Extended banding following BrdU incorporation was used to confirm which chromosome 5 was present in each hybrid. Isoenzyme analysis was sometimes performed by Dr M.S. Povey's laboratory (Galton Laboratory, University College London) to establish whether a particular human chromosome was retained in a hybrid clone (Harris and Hopkinson, 1977).

DNA Methods

DNA manipulations were usually performed according to the protocols of Sambrook et al. (1989)

Extraction of Genomic DNA

10^8 mammalian cells were harvested in 1ml 10mM Tris/HCl pH 8.0 and 9ml of 10mM Tris/HCl pH 8.0, 10mM EDTA, 10mM NaCl, 0.5% SDS added. To this was added proteinase K to 100μg/ml and the mixture incubated at 50°C overnight on a rocking platform. Phenol: chloroform: isoamylalcohol (50:48:2) extractions were performed until no protein remained at the interface and then one chloroform extraction was performed after which RNAase A was added to 50μg/ml and incubated at 37°C for one hour. Proteinase K (100μg/ml) and SDS (1%) were then added and incubated for a further hour. A second set of phenol:chloroform and chloroform extractions were performed and the DNA ethanol precipitated. The DNA was finally resuspended in 1-2ml of TE. High molecular weight DNA for the construction of libraries was not precipitated with ethanol but was dialysed against 5L TEN at 4°C. The DNA concentration was measured spectrophotometrically.

Restriction endonuclease digestions

These were carried out in the buffers and at the temperatures recommended by the suppliers of the restriction endonucleases. The amount of enzyme used was in each case 1-6 fold over that required to complete the digestion in one hour under optimal conditions. Due to the inhibitory effect of the glycerol present within restriction enzyme storage buffers, on no occassion was this component allowed to constitute more than 10% of the reaction volume. Reactions were incubated for 1-2 hours for cloned DNA and for at least 3 hours for genomic DNA. Incomplete digestions after this time were phenol/chloroform extracted and precipitated prior to a second digestion.

Multiple digestions were conducted simultaneously if the reaction conditions required by each enzyme concerned were similar. If not the DNA was ethanol precipitated after each restriction and resuspended in an appropriate buffer for a subsequent digestion.

Aliquots of all digestions were separated on appropriate percentage agarose gels to determine the completeness of the digestion. If final removal of the enzyme activity was required, susceptible enzymes were inactivated by heating at 65ºC for 5-10 minutes or alternatively the samples were phenol/chloroform extracted and ethanol precipitated.

DNA Sonication

DNA for sonication was made up to 20-200 μg/ml in H$_2$0 and sonicated at full power using 20-30 second bursts with a Kontes sonicator obtained from Burkard Scientific Instruments. After each burst an aliquot was examined by agarose gel electrophoresis. Sonication was continued in this way until sufficient degradation had been achieved (0.4-2kb).

Agarose gel electrophoresis

This was performed using 0.7-1.4% agarose gels of 200 or 300 ml volume poured upon 20 x15 cm or 25 x 15 cm perspex trays respectively. Various plastic well-formers were employed to make gel slots. The gels were submerged under a 1 cm depth of electrophoresis buffer and after loading the DNA in 20% (v/v) loading buffer a constant voltage of 0.5-3 V/cm was applied. Ethidium bromide at 0.5 μg/ml was usually included in both the gel and the the electrophoresis buffer to permit visualisation of the nucleic acids under ultraviolet light.

Southern blots

These were performed using nylon filters (Hybond N or Hybond N Plus, Amersham) according to the manufacturer's recommendations.

Filter Hybridisations

Filters were prehybridised in plastic bags containing hybridisation buffer plus heat denatured competitor DNA as required at 65ºC for 2-4 hours.

Denatured double-stranded DNA probes were radio-labelled according to the oligo-labelling method of Feinberg and Vogelstein (1984) using (α^{32}P) dCTP. To remove unincorporated nucleotides probes in 250μl TES were passed through a Sephadex G-50 (fine) column prepared in a 1 ml syringe by centrifugation of the column for 5 minutes at 1000g . The degree of radionucleotide incorporatation was assessed with a 1μl aliquot. After denaturation at 100°C for 5-8 minutes probes were injected into the prehybidisation bags and, after careful resealing, the hybridisation was allowed to proceed at 65°C for 16 hours in a gently shaking water bath.

Removal of the nonspecifically bound probe was achieved by successive washes at 65°C in 0.1% SDS buffers containing 2x SSC, 1x SSC and 0.1x SSC. Several further washes at this final stringency were applied. Filters were exposed to X ray film XAR 5 (Kodak) with intensifying screens at -70^{0}C.

Hybridisation with Competitor DNA

Repetitive probes were incubated with sheared total human or hamster competitor DNA prior to hybridisation. 200μg of sonicated competitor DNA was added to the probe and heated at 100^{0}C for ten minutes, the solution was then incubated for two hours at 65^{0}C before being added to the hybridisation mixture (Sealey et al., 1985; Litt and White, 1985).

Stripping of Filters

To enable re-examination of the filters by other probes the filters were stripped of hybridised probe according to the manufacturer's recommendations.

Densitometric Scanning of Autoradiographs

To quantitate allele loss in paired samples of tumour and normal tissue, the normal and tumour alleles on the autoradiograph were

scanned with a densitometer (LKB Enhanced Laser Densitometer). From the scans the ratios of the two alleles in tumour material to those in normal matched tissue were estimated (T1/T2 divided by N1/N2) (Solomon et al., 1987).

Oligonucleotide Synthesis

Oligonucleotides were synthetised by the Human Genetic Resources Laboratory (ICRF, Clare Hall). The melting temperature (T_m) of the oligonucleotides were estimated by multiplying the number of A + T residues by 2^0C and the number of G + C residues by 4^0C and adding the two numbers (Itakura et al., 1984).

Polymerase Chain Reaction

100ng DNA or approximately 1000 lysed cells were added to a 100μl reaction mix containing: 10μl 10x PCR buffer, 10μl DMSO, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 200 μM dTTP and 50pM of each oligonucleotide primer. This was heated to 96^0 for 5 minutes. 2 units Taq polymerase (Amplitaq, Cetus) were added and the reaction mix covered with paraffin oil (Saiki et al., 1988; S. Povey, personal communication).

The polymerase chain reaction was performed on a programmable heating block (Programmable Dri-block, Techne). The reaction was denatured at 94^0C and reannealed at 8^0C less than the calculated Tm of the oligonucleotide primers, the polymerisation temperature was at 72^0C. Extension times were between 0.5 and 4 minutes, depending on the size of the PCR product, and 30 cycles were performed. After completion of the reaction a 10μl aliquot was run out on an agarose gel and a photograph taken of the ethidium-stained gel.

Construction of Lambda Library

The library was constructed according to the protocol of Frischauf (1988).

Preparation of Lambda Vector

10μg of λEMBL3 DNA (Stratagene) were digested with 30 units *Bam*HI in 50μl high salt buffer for 1 hour at 37⁰C and then placed on ice. An aliquot was heated for 2 minutes at 68⁰C, to denature the sticky ends, chilled and run out on an agarose gel to test for the completeness of digestion. An aliquot was taken and incubated for 10 minutes at 68⁰C and then frozen as a control for ligation and packaging. To the remainder 100 units *Eco*RI was added and digested for a further 30 minutes at 37⁰C. EDTA was added to a final concentration of 15mM and then incubated for 10mins at 68⁰C. This was then extracted once with phenol: chloroform: isoamylalcohol (50:48:2) and the organic phase reextracted with TE containing 100mM NaCl. The salt concentration in the combined aqueous phase was adjusted to 0.45M with 3M sodium acetate pH6 and 0.6 volumes isopropanol added and placed on ice for 10 mins. The tube was then centrifuged and the supernatant carefully removed, the pellet was the washed once with 70% ethanol and the tube left open at room temperature for 10 minutes to dry. Finally the pellet of DNA was redissolved in 18μl TE to a final concentration of 0.5μg DNA/μl.

Preparation of Insert DNA

An analytical digestion of the insert DNA was performed to calculate the appropriate digestion time and enzyme concentration. 1μg of high molecular weight insert DNA was dissolved in 30μl of medium salt buffer. A 5μl aliquot was taken at time zero and then 0.2 units of *Mbo*I was added and incubated at 37⁰C. Further 5μl aliquots were taken at 5, 10, 20, 40, and 80 minutes. 1μl of 0.5M EDTA pH 7 was added to each aliquot and incubated for 10 minutes at 68⁰C to stop the digestion. When all the aliquots had been collected they were loaded on a 0.35% agarose gel with uncut λ DNA and λ cut with *Hind*III as size markers and run overnight at

0.5V/cm. The optimal enzyme concentration and digestion time were estimated from a photograph of the ethidium-stained gel.

For the preparative digestion 10μg high molecular weight DNA was dissolved in 150μl medium buffer at 37^0C. A 3μl aliquot was taken before the calculated amount of *Mbo*I was added. A 70μl aliquot was taken at the two estimated digestion times and 10μl 0.1M EDTA pH7 added to each and incubated for 15 minutes at 68^0C. A 3μl aliquot from each time point was run on a gel as described above. To the remainder of each aliquot 1.5 units alkaline phosphatase was added and incubated for 30 minutes at 37^0C. 8μl 100mM nitrilotriacetic acid pH7 was then added and incubated for 15 minutes at 68^0C. The DNA was extracted three times with phenol: chloroform: isoamylalcohol (50:48:2) and the organic phase re-extracted with 10mM Tris pH7.6, 100mM NaCl, 1mM EDTA. It was then extracted twice with water-saturated ether and after adjusting the salt concentration in the combined aqueous phase to 0.45M with 3M sodium acetate pH6 precipitated with 2.1 volumes ethanol. The precipitate was separated by centrifugation for 5 minutes at 1000g. The supernatant was carefully removed and the pellet was the washed once with 70% ethanol and the tube left open at room temperature for 15 minutes to dry. The DNA was taken up into TE at 0.5μg/μl and the concentration checked by running out 1 μl on a gel.

Dephosphorylation Control

0.25μg of DNA pre- and post- phosphatase treatment were incubated overnight at 15^0C in ligation buffer with 200 units of T4 DNA ligase. A further sample of dephosphorylated insert DNA was incubated in ligation buffer without adding ligase. The samples were then run out on a 0.35% agarose gel together with size markers.

Ligation of Vector to Insert DNA

5μg of λEMBL3 DNA cut with *Bam*HI and *Eco*RI and 2.5μg dephosphorylated insert DNA in 25μl ligation buffer with 0.5mM ATP were incubated overnight at 15⁰C.

Packaging

The recombinant phage DNA was packaged using Gigapack II Gold packaging extract (Stratagene). The freeze/thaw extract was quickly thawed and 1μg recombinant phage DNA added and the tube placed on ice. 15μl of freshly thawed sonic extract was then added and gently mixed. The tube was briefly spun and incubated at room temperature for two hours. 500μl of phage dilution buffer and 20μl chloroform were added and the tube spun briefly. The plating efficiency of the supernatant was then titered.

Plating Cells

Plating cells were streaked out on an L agar plate and grown overnight at 37⁰C. A single colony was picked into L broth and grown overnight at 37⁰C with vigorous aeration. The saturated culture was chilled and the cells precipitated by centrifugation. The supernatant poured off and the cells resuspended in 10mM MgSO₄.

Test Plating

Dilutions of the packaged ligation mixture were made in dilution buffer and plated on three hosts, NM 646, a selective host with P lysogen, NM 621, a non-selective host and ED 8767, a RecA⁻ host on which only uncut vector will plate. The packaged ligation mixture was added to 100μl of plating cells and incubated for 15 minutes at 37⁰C. The mixture was then added to 3ml BBL top agar at 42⁰C and plated on a BBL agar plate. The plates were incubated overnight at 30 or 37⁰C and the plaques counted.

Plating

An aliquot of titrated packaged ligation mix containing approximately 100,000 to 150,000 plaque forming units were added to 2ml NM 646 plating cells and absorbed for 15 minutes at 37^0C. They were then added to 30ml BBL top agarose with 10mM $MgSO_4$ at 42^0C and poured onto prewarmed 23 x 23cm BBL agar plates and incubated overnight at 37^0C.

Plaque Lifts and Screening

These were taken with nylon membranes (Hybond-N, Amersham) according to the manufacturer's recommendations. The libraries were probed with 50ng ^{32}P-labelled sheared total human DNA competed with 200μg sheared hamster DNA for two hours at 65^0C. Positive plaques were picked into 200μl phage dilution buffer and stored at 4^0C overnight. The recombinant phage were then replated at various dilutions as a secondary screen.

Minipreparations of Bacteriophage λ

A positive plaque was picked as an agar plug into 3ml L-broth with 10mM $MgSO_4$ in a chloroform-resistant tube and incubated overnight at 37^0C while being vigorously aerated. 30μl chloroform was then added and the culture shaken for a further 5 minutes. The cells were separated by centrifugation for 15 minutes at 2000g and the supernatant carefully transferred to a fresh tube. 0.5ml was stored as a stock at 4^0C·

3μl of both RNAase and DNAase (10mg/ml) were added to the remainder and incubated for 30 minutes at 37^0C. The NaCl concentration was then adjusted to 0.3M (210μl of 5M NaCl), the EDTA concentration to 10mM (70μl of 500mM EDTA) and the Tris concentration to 100mM (350μl of 1M Tris pH 7.5). 30μl of proteinase K (10mg/ml) was added and incubated for 30 minutes at 50^0C.

0.6 volumes of isopropanol were added and the mixture placed on ice for 10 minutes then spun for 20 minutes at 2000g. The supernatant was poured off and the pellet redissolved in 200μl TE. This was extracted once with phenol: chloroform: isoamylalcohol (50:48:2) and once with ether. The DNA was precipitated by adding 3M sodium acetate to a final concentration of 0.3M and 0.6 volumes isopropanol, spun, washed in 70% ethanol and allowed to dry before being dissolved in 100μ TE. Approximately 10μl were used as probe for each labelling reaction.

Table 2 Buffers and Solutions

50x Electrophoresis Buffer	Tris base	242g
	Sodium acetate	20.5g
	EDTA	18.6g
	Glacial acetic acid to	
	pH 7.8 to 1L with H_2O	
Hybridisation Buffer	Dextran Sulphate	10%
	Denhardt's solution	5x
	SDS	0.5%
	SSC	6x
	Tarula Yeast RNA	1mg/ml
Denhardt's Solution(50x)	Bovine serum albumin	5g
	Ficoll	5g
	Polyvinyl pyrrolidine	5g
	made to 500ml with H_2O	
Loading Buffer	Bromophenol blue	0.25%
	Xylene cyanol	0.25%
	30% Glycerol in H_2O	
Phenol	Phenol	500g
	plus 300ml of:	
	Tris-HCl pH 8.0	500mM
	$Na_2EDTA.2H_2O$	10mM
	NaCl	10mM
	Hydroxyquinoline	800mg
Chloroform	Chloroform	96ml
	Iso amyl alcohol	4ml
SSC (20x)	NaCl	175.3g
	Sodium Citrate	88.2g
	adjusted to pH7.0	
	with NaOH and autoclaved	
TE	Tris-HCl pH 7.4	10mM
	Na_2 EDTA.$2H_2O$	1mM
	autoclaved	
TES	Tris-HCl pH 7.4	10mM

	Na_2 EDTA.$2H_2O$	1mM
	SDS	0.1%
TEN	NaCl	0.1M
	Tris-HCl pH 7.4	10mM
	Na_2 EDTA.$2H_2O$	1mM
PBS	NaCl	10g
	KCl	0.25g
	KH_2PO_4	0.25g
	Na_2HPO_4	1.43g
	$CaCl_2$	1.334g
	$MgCl_2$	1.0g
	made up to 1L with H_2O	
PCR Buffer (10x)	$(NH_4)_2 SO_4$	166mM
	Tris-HClpH 8.8	0.67M
	$MgCl_2$	67mM
	2 Mercaptoethanol	100mM
	Na_2 EDTA.$2H_2O$	67μM
	BSA	1.7mg/ml
High Salt Buffer	Tris-HCl pH7.5	10mM
	$MgCl_2$	10mM
	NaCl	100mM
Medium Salt Buffer	Tris-HCl pH7.5	10mM
	$MgCl_2$	10mM
	NaCl	50mM
	Dithiothreitol	1mM
Ligation Buffer	Tris-HCl pH7.5	40mM
	$MgCl_2$	10mM
	Dithiothreitol	1mM
	ATP	0.5mM
Phage Dilution Buffer	Tris-HCl pH7.5	10mM
	$MgSO_4$	10mM
	EDTA	1mM

Oligonucleotide Primers

Complement Factor 9   TAGATACATTGAGTCTCTCCTGATT
             CAGTCTATCACAATGAGAGAGATGG
             (Abbott et al., 1989)

ECB 27   AGTTATTGAACTTAACGAGGAGG
             TTGATTTGGGTTAAATCTGC
             (Varesco, personal communication)

C11P11   CATAAACAGAAGCTTATAATGCT
             CATATGTTCTATATCAGTGC
             (Khan, personal communication)

TC 65 (Alu 559)   AAGTCGCGGCCGCTTGCAGTGAGCCGAGAT
             (Nelson et al., 1989)

Media

L Broth	Bacto Tryptone	10gms
	Yeast Extract	5gms
	NaCl	10gms
	pH7.2 in 1L H_2O	

BBL Agar	Trypticase peptone	10gms
	NaCl	5gms
	Agar pH7.2	10gms
	in 1L H_2O	

BBL Top Agar/Agarose	Trypticase peptone	10gms
	NaCl	5gms
	$MgSO_4$	10mM
	Agar pH7.2 or Agarose	6.5gms
	in 1L H_2O	

Bacterial Strains

NM621   *recD thy*[+] P1 transductant of SB204
             (Whittaker et al.,1988)

NM646   P2*cox*3 lysogen of K802
             (Whittaker et al.,1988)

ED8767   *supE, supF, hsdS⁻, met⁻, recA56*
                                (ATCC)

Materials

Nitrilotriacetic acid   Merck

Ligase   New England Biolabs

Restriction Enzymes   New England Biolabs and
                               Boehringer Mannheim

Phosphatase (Calf Intestine) Boehringer Mannheim

Blood Collection Media

                    RPMI 1640 HEPES Buffered Medium   200ml
                    3.3% Trisodium Citrate   40ml
                    5µM Mercaptoethanol   2ml
                    25ml aliquotted into 50ml sterile blood bottles
                    (Falcon)

E4   Dulbecco's modification of Eagle's minimal
                    essential medium (ICRF)

RPMI 1640   (Flow Laboratories) with 300mg Glutamine/L.

Versine   EDTA .002% in PBS A (without Ca and Mg)

Trypsin   2.5% in Tris saline

Penicillin   5000 units/ml (Flow Laboratories)

HAT   Hypoxanthine 10^{-4} M
                    Thymidine 1.6×10^{-6} M
                    Methotrexate 10^{-5} M

Streptomycin   5000µg/ml (Flow Laboratories)

5-bromodeoxyuridine   (Sigma)

Table 3 Cell Lines

Epstein-Barr Virus Transformed Lymphoblastoid Lines

FPC-3F	(Gift of J.D.A. Delhanty, Galton Laboratory, University College London)
Del 5	(Gift of S.J. Fennell, Royal Manchester Children's Hospital)
Mother Del 5	(Gift of S.J. Fennell, Royal Manchester Children's Hospital)
Father Del 5	(Gift of S.J. Fennell, Royal Manchester Children's Hospital)
Sister Del 5	(Gift of S.J. Fennell, Royal Manchester Children's Hospital)
Brother Del 5	(Gift of S.J. Fennell, Royal Manchester Children's Hospital)
PW	(Hockey et al., 1989)
ND	(Hockey et al., 1989)
Father PW/ND	(Hockey et al., 1989)
GM03314	(Human Genetic Mutant Cell Repository)
JT	(Gift of M. Chiba, University of Akita, Japan)
PS	(Gift of J. Burn, Royal Victoria Infirmary, Newcastle)

C 0501   (Gift of B.S. Emanuel, The Children's
                       Hospital of Philadelphia, Pennsylvania)

L 3214   (Gift of R.L. White, Howard Hughes Medical
                       Institute, University of Utah)

Chinese Hamster Ovary

Ts H1   (Thompson et al., 1973)

A23 tk⁻   (Westerveld et al., 1971)

Mouse

LMtk⁻   (Kit et al., 1963)

Table 4 Somatic Cell Hybrids

Somatic Cell Hybrids

Parental Line Del 5:

    MD/TS-1

    HT 991

Parental Line PW:

    PD/TS-1

    PN/TS-1

Parental Line JT:

    JT/TS-01

    JT/TS-3

Parental line L3214:

    HHW1155 (Gift of J.J. Wasmuth, University of California, Irvine)

    HHW1159 (Gift of J.J. Wasmuth, University of California, Irvine)

Radiation Hybrids

    IFGT 1 through to 172 (See table)

CHAPTER THREE

SOMATIC CELL HYBRIDS

Introduction

The aim of these experiments was to segregate a human chromosome 5 from the other human chromosomes within a human: hamster somatic cell hybrid. An additional aim was to segregate human chromosomes 5 with deletions in the region of the *APC* gene from their normal homologues. This process is greatly facilitated by the availability of a selection system specific to chromosome 5. The original description of a selection system for the production of somatic cell hybrids was that of using thymidine kinase (TK) or hypoxanthine phosphoribosyl transferase (HPRT) deficiency and HAT medium. This selected for hybrids in which the wild-type human TK or HPRT genes complemented for the defective hamster genes leading to the retention of human chromosomes 17 and X respectively (Littlefield, 1964 and 1966).

Two selection systems have been described that are specific to human chromosome 5. The first of these involves the leucyl t-RNA synthetase gene which maps to chromosome 5 and is temperature-sensitive in the Chinese hamster ovary cell line tsH1. These cells will grow at 33^0 (permissive temperature) but will not at 39^0 (non-permissive temperature) (Thompson et al., 1973). When human lymphocytes are fused with tsH1 and the resulting hybrids grown at the non-permissive temperature human chromosome 5 is selectively retained in hybrids (Giles et al., 1980).

The second selection system specific to chromosome 5 involves the Chinese hamster ovary cell line Mev-1 which is deficient of the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthetase (Schnitzer-Polokoff et al., 1982). This cell line will only grow in the presence of mevalonic acid. In somatic cell hybrids between Mev-1 and human lymphocytes grown in the absence of mevalonic acid the mutant defect is complemented by the selective retention of human chromosome 5. The location of the human HMG-CoA synthetase gene is clearly on human chromosome 5 (Leonard et al., 1986).

Human Cell Lines

Several different human lymphoblastoid cell lines and a fibroblast cell line were used in the construction of chromosome 5 somatic cell hybrids (see Table 3).

FPC-3F

This fibroblast cell line from a FAP patient developed a translocation between chromosomes 5 and 17 while in culture. The region of chromosome 17 which was translocated onto the long arm of chromosome 5 below the APC gene region t(5;17) 5q22:17q26 included the thymidine kinase gene (S. Rider, personal communication). This made it possible to select for the retention of the translocation chromosome following fusion to thymidine kinase deficient rodent cell line and HAT selection.

Manchester Del-5

Patient M was investigated for developmental delay and found to have a constitutional interstitial deletion of chromosome 5 (Figure 3) similar to that described by Herrera et al. (1986). The patient was a ten year old girl with mildly dysmorphic features and mild intellectual handicap. She had no features of Gardner syndrome and no evidence of congenital hypertrophy of retinal pigment epithelium (CHRPE), another extra-intestinal feature of FAP. Both parents were normal as were her brother and sister. She was too young to be screened for the presence of colonic polyps as these usually develop during adolescence (S.J. Fennell, personal communication).

P.W. and N.D.

These two brothers were reported by Hockey et al. (1989) following the publication of linkage between FAP and markers on chromosome 5 (Bodmer et al., 1987). Their mother, who was mentally retarded, died in her forties of disseminated colorectal cancer and at autopsy was found to have FAP. She had two

5   del5   5   del5

Figure 3
Partial karyotype of Patient M with the normal chromosome 5 on the left and the deletion chromosome 5 (del 5q15-q21) on the right.

5   del5   5   del5   5   del5

Figure 4
Partial karyotype of Patient PW with the normal chromosome 5 on the left and the deletion chromosome 5 (del 5q15-q21) on the right.

π 227	1 ?	3 2
C11P11	2 ?	2 2
MS8	3 ?	2 1

†40 Ca Colon

π 227	1 2		1 2	
C11P11	2 2		2 2	
MS8	3 1		3 1	

† ‹1
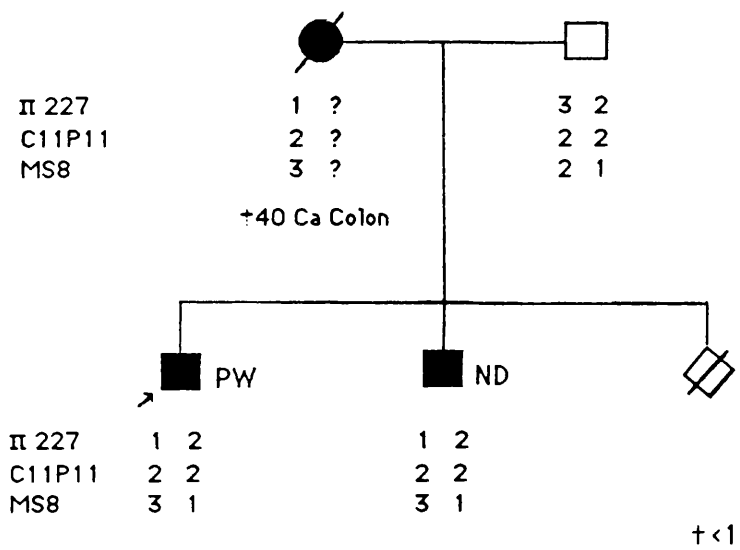
Figure 5

Pedigree of a family with familial adenomatous polyposis and a constitutional interstitial deletion of chromosome 5q. The haplotypes for three polymorphic markers on chromosome 5 are included.

75

surviving sons P.W. and N.D. A third child had died in infancy (see Figure 5). Both brothers were intellectually handicapped, had dysmorphic features and on screening were found to have FAP. It was initially unknown whether they had the same father. On karyotyping both were found to have an interstitial deletion 5q13-q15 or 5q15-q21 (see figure 4) similar to that reported by Herrera et al. (1986). Polymorphic probes in the *APC* region demonstrated that both brothers had inherited one haplotype from E.D. their common father and the other haplotype from their mother, which was later shown to be that in the deletion hybrid PD/TS-1.

GM03314

This patient, who came from a large kindred with FAP, was found to be mosaic, in 60% of metaphases, for a balanced translocation t(5;15) (5q22:15q26) (Human Genetic Mutant Cell Repository). The other affected members of the family had a normal karyotype. The position of the translocation is interesting in that it is close to the region where the *APC* gene has been mapped and might helpfully subdivide the region.

J.T.

This patient with FAP is also intellectually handicapped (Chiba, personal communication). Unlike the cases described above with FAP and mental retardation he does not have a visible chromosome 5 deletion. This raises the possibility that a submicroscopic deletion may be present in the *APC* gene region which would be extremely useful in localising markers to that region.

CH0501

This patient with FAP has a large interstitial deletion on the long arm of chromosome 5 (5q15-q23.1) (Figure 6). In situ hybridisation with probes mapped in the *APC* region indicated that the proximal extent of the deletion included the known proximal flanking markers ECB27 and YN564 and thus did not helpfully subdivide the region (S.Williams, personal communication). The distal extent of
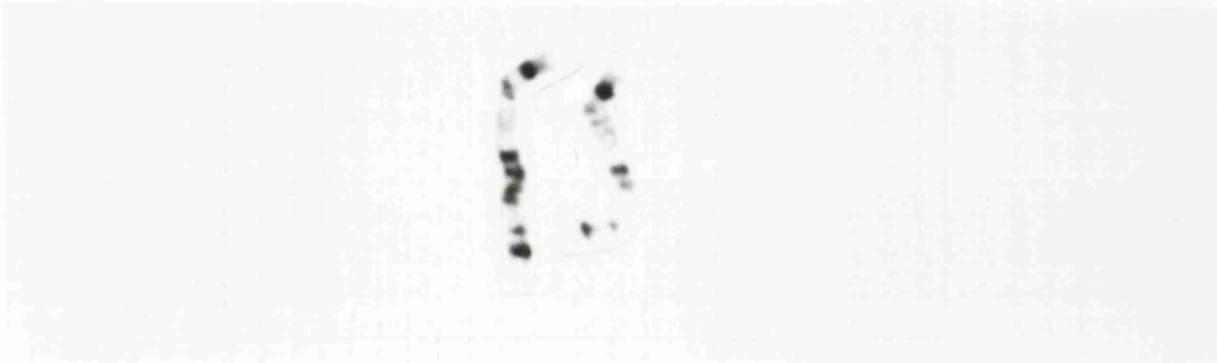
Figure 6

Partial karyotype of Patient CH0501 with the normal chromosome 5 on the left and the deletion chromosome 5 (del 5q15-q23.1) on the right.

Figure 7

Partial karyotype of Patient PS with the normal chromosome 5 on the left and the deletion chromosome 5 (del 5q22.1-q23.2) on the right.

the deletion was clearly considerably telomeric to the deletions described above.

P.S.

This mentally retarded 25 year old man was diagnosed as having FAP with the extra-intestinal features of osteomas, epidermoid cysts, lipomas and congenital hypertrophy of the retinal pigment epithelium (Burn, personal communication). His mother had died of a cerebral tumour, a known association with FAP (Turcot et al., 1959; Kropilak et al., 1989), and his aunt also has polyposis and is mentally retarded. Karyotypic analysis demonstrated a constitutional interstitial deletion of chromosome 5 (5q22.1-q23.2) in the proband and in his aunt (Figure 7). This is of great interest as the deletion's proximal margin is more distal than in those FAP deletions described above and thus further subdivides the *APC* gene region.

Elimination of Revertants in Recipient Rodent Cell Lines

Thymidine kinase (TK) deficient cell lines were grown in 100µg/ml bromodeoxyuridine (Westerveld et al., 1971). TK⁻ cells are resistant to this drug which is incorporated into DNA by wild-type cells causing lethal genetic changes (Szybalski et al., 1962).

The tsH1 cell line during passage acquired a high proportion of revertants that grew at the non-permissive temperature of 39°C. It was not clear whether this was due to a high spontaneous reversion rate or whether the revertants increased in numbers by chance as the number of passages increased. Two different strategies were used to selectively remove the revertants.

Thompson et al. (1973) originally produced the tsH1 line by exposing wild-type Chinese hamster ovary cells to a mutagen and allowing them to recover while growing 34°C. The cells were then moved to a 38.5°C incubator and exposed to ³H thymidine for 24 hours. Cells capable of DNA synthesis at this temperature incorporated ³H thymidine and died. The surviving temperature-

sensitive cells which were unable to grow at 39°C were then cloned at 34°C (Thompson et al., 1973). To select against dividing cells as in the original experiment would have involved considerable exposure to ^{3}H thymidine. However, the original proline-dependent Chinese hamster ovary cell line from which tsH1 was derived was selected using 5-bromodeoxyuridine to kill dividing cells (Puck and Kao,1967). Thus to select against revertants in these experiments tsH1 cells were incubated at the non permissive temperature of 39°C in the presence of 5 x 10^{-6}M 5-bromodeoxyuridine to eliminate dividing revertant cells. These experiments were not successful, as described below.

5-bromodeoxyuridine-elimination of revertants

Experiment 1

TsH1 cells were plated out at 5 x 10^6 cells per tissue culture flask in growth medium and incubated for 24 hours at 33°C, their permissive temperature. The flasks were then wrapped in aluminium foil, to exclude all visible light, and transferred to 39°C, the non permissive temperature, for 24 hours to allow the growth of revertants. 5-bromodeoxyuridine was then added to a final concentration of 5 x 10^{-6}M and the cells were incubated for a further 24 hours at 39°C. The medium containing 5-bromodeoxyuridine was then aspirated and the cells washed in PBS before growth medium was added. The cells were incubated for a further two hours at 39°C and then exposed to visible light for two hours 40cm from the light source (60W fluorescent tube) in the tissue culture cabinet. Finally the cells were returned to 33°C and allowed to grow at their permissive temperature. During the following two weeks no viable cells were seen.

Experiment 2

To determine the cause of the cells dying in the previous experiment and to select against revertants a further experiment was undertaken. The length of time at the non-permissive temperature and of exposure to 5-bromodeoxyuridine was reduced and additional controls added. TsH1 cells were again plated out at 5 x 10^6 per flask and grown overnight at 33°C. The flasks were then

wrapped in foil and transferred to 39^0C for three hours. 5-bromodeoxyuridine was then added to 5 x 10^{-6}M and the cells incubated overnight at 39^0C. The cells were then washed in PBS and growth medium added. They were incubated for a further two hours at 39^0C and then exposed to visible light for 30 minutes as described above. The cells were then cultured at their permissive temperature of 33^0C.

Control flasks included cells 1) grown at 33^0C throughout, 2) grown at 39^0C with no 5-bromodeoxyuridine added and 3) grown at 39^0C with 5-bromodeoxyuridine but not exposed to visible light. All the cells in the controls remained viable.

When confluent the cells exposed to 5-bromodeoxyuridine and the controls were harvested and counted. 5 x 10^6 cells were plated in growth medium and and incubated at 39^0C to test for revertants, the remainder were frozen in three aliquots. The number of colonies growing in each flask were counted after a week (Table 5).

Table 5 Number of revertant colonies in Experiment 2

Flask Number	Colonies
TsH1 (1)	1
TsH1 (2)	2
TsH1 (3)	0
TsH1 (4)	0
TsH1 (5)	0
TsH1 33^0 control	1
TsH1 33^0 control	1

This experiment failed to eliminate the revertants. However the cells remained viable, unlike the previous experiment.

Experiment 3
The experiment above was repeated varying the length of time of exposure to 5-bromodeoxyuridine and varying the exposure to light. Cells were grown at 39^0C in 5 x 10^{-6}M 5-bromodeoxyuridine for either 24 or 48 hours and were then exposed to light for either

30 or 60 minutes, controls were included at each step. After completion of the protocol the cells were again harvested and counted and 10^6 cells incubated in growth medium at 39^0C. Following this protocol all the flasks contained multiple revertant colonies.

Conclusion

The likely explanation for the failure to eliminate revertants in these experiments appeared to be that the concentration of 5-bromodeoxyuridine used was too low and that this allowed the proliferation of revertants at the non permissive temperature without leading to cell death. It is of note that the concentration of 5-bromodeoxyuridine used to select against thymidine kinase revertants by Westervelt et al. (1971) is thirty times greater ($100\mu g/ml$ is equivalent to approximately $1.5 \times 10^{-4}M$) than that used by Puck and Kao (1967) and in these experiments.

Dilution Experiments

A simpler alternative strategy to eliminate the revertants was to dilute the cells to a number which statistically was unlikely to contain revertants. The success of this depended on there not being a high spontaneous reversion rate.

Experiment 1

A frozen aliquot of tsH1, known to have a high proportion of revertants, was thawed and grown up. The cells were harvested and counted and plated on 24 well dishes (Falcon) in aliquots of approximately 100 cells. These were grown at 33^0C and ten clones expanded on to $25cm^2$ flasks and then on to two $75cm^2$ flasks. The cells were then harvested and counted. 10^6 cells from each clone were plated in a $75cm^2$ flask and grown at 39^0C to test for revertants (Table 6) and the remainder for each clone divided and frozen in three aliquots.

Table 6 The number of revertant colonies in Dilution Experiment 1

Flask Number	Revertant Colonies
11	Confluent
12	Multiple
13	Multiple
14	Multiple
15	Several
16	Few (<10)
17	Multiple
18	Multiple
19	Multiple
20	Confluent

Experiment 2

Culture 16 appeared to have considerably fewer revertants than the other subclones. A frozen vial was thawed, plated in growth medium and incubated at 33^0C. The cells were then harvested and counted and approximately 100 cells plated in each well of a 24 well dish for expansion. The same protocol was followed as in the experiment above. The results are given in the Table 7.

Table 7 The number of revertant colonies in Dilution Experiment 2

Flask Number	Colonies
21	None
22	None
23	None
24	None
25	None
26	None
27	None
28	None
29	None
30	None

Thus two rounds of dilution had produced tsH1 clones in which no revertants were present in 10^7 cells plated. These cultures were used in all subsequent tsH1 fusions.

Conclusion

The success of the dilution experiment in eliminating revertant cells suggested that their presence was not due to a high mutation rate but to the chance increase in number of revertants already present in the cell population. The revertant-free tsH1 cells were only available for use in the fusions with J.T. and the earlier fusions were performed with tsH1 cells that contained revertants.

Somatic Cell Fusions

The revertant-free cultures of TsH1 were only available for the later fusions and hence a high number of revertants clones were picked and expanded in the earlier fusions.

Fusion 1

Parental cells: FPC-3F and LMTK⁻.

A polyethylene glycol fusion was performed, as described in the methods section, between 10^7 FPC-3F [t(5;17)] cells and 10^7 LMTK⁻ cells. The fusion products were grown in HAT medium in the presence of 10^{-5}M oubain to select for TK positive hybrid cells. One colony grew and this was ring cloned 28 days after the fusion and expanded. A karyotype demonstrated that this contained a normal human chromosome 17 and not the t(5:17) chromosome.

Fusion 2

Parental cells: Del 5 and TsH1

A polyethylene glycol fusion was performed between 5×10^6 cells of Del 5 and 5×10^6 cells of tsH1. The cells were then incubated in growth medium at 39^0C with 10^{-5}M oubain to select for human: hamster hybrids. No revertants were detected in 10^6 tsH1 cells grown as a control at 39^0C. At 28 days colonies were present on

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three plates. The colonies were ring cloned and the cells from one confluent plate were single cell cloned. The clones were expanded and aliquots frozen in liquid nitrogen.

DNA was prepared from each clone and from the parental cells, digested with *Hinf*I and separated on an agarose gel before being transferred to a nylon membrane. The filter was then hybridised with ^{32}P-labelled MS8, a highly polymorphic minisatellite probe mapped to chromosome 5 (Wong et al., 1987). All the hybrids contained the smaller human parental MS8 allele. The hybrids clones were then karyotyped and the percentage of cells retaining each human chromosome determined as tabulated below (Table 8).

Table 8 Fusion 2 clones karyotypes and MS8 alleles

Clone	MS8 allele	Percentage retention of each human chromosome
C2/7	a2	5(80%);10(5%);18(70%);21(5%)
C2/11	a2	5(100%);18(53%)
C3/15	a2	5(82%);18(65%);21(6%)
C3/22	a2	5(80%);10(25%);18(75%)
RC4	a2	5(54%);10(38%);18(77%);21(15)
RC5	a2	5(90%);18(65%)
RC6	a2	5(95%);10(20%);18(75%)
RC7	a2	5(80%);10(65%);18(75%)

C2/11 contained human chromosome 5 in all metaphases and chromosome 18 in 53% of metaphases. It was single cell cloned and ten clones expanded. Isoenzyme analysis of these clones was then performed by Dr M.S. Povey (Galton Laboratory, University College London) to determine in which hybrids human chromosomes 5 and 18 had been retained. All ten clones retained chromosome 5 but three (Subclones 3, 5 and 7) did not contain chromosome 18. These three clones were expanded and karyotyped. Extended chromosomal banding demonstrated that the deleted human chromosome 5 was retained and that no other human chromosomes were present (Figure 8).

Patient M   Hybrid MD/TS-1

5   del(5)   5   del(5)   del(5)   del(5)

Figure 8

Partial karyotype of the human-hamster somatic cell hybrid MD/TS-1 in which only the deletion human chromosome 5 is retained.

Patient P   Hybrid PD/TS-1

5 del(5)   5 del(5)   del(5)   del(5)
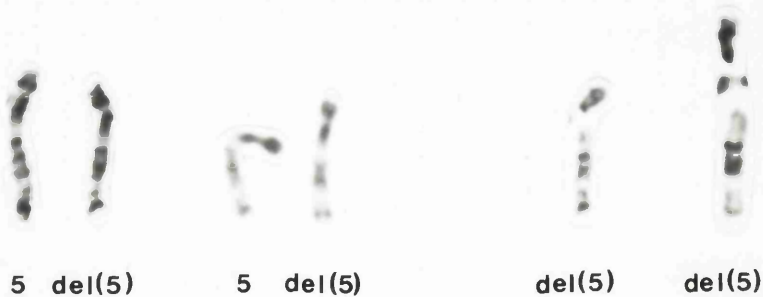
Figure 9

Partial karyotype of the human-hamster somatic cell hybrid PD/TS-1 in which the deletion human chromosome 5 is retained.

Subclone 3 of C2/11 was named MD/TS-1 to represent Manchester patient (M), deletion chromosome 5 (D), temperature-sensitive selection (TS) and clone one (1).

C2/11 was later used by Dr David Markie (Director's Laboratory, ICRF) to produce a hybrid clone in which the only human material was chromosome 18. C2/11 was incubated at the non-selective temperature of 33^0C to allow the segregation of chromosome 5 from chromosome 18. Single cell clones were then analysed for the presence of chromosome 5 using the polymerase chain reaction (PCR) with oligonucleotides specific to human complement factor nine, which has been mapped to chromosome 5 (Abbott et al., 1989). A hybrid clone was identified in which chromosome 5 was not retained. On chromosomal analysis the only human chromosome present in the hybrid cells was 18. This clone was named DL 18 *ts*.

Fusion 7

Parental cells: P.W. and TsH1/5

A polyethylene glycol fusion was performed between 5×10^6 cells of each parental cell line and the products incubated at 39^0 in growth medium with 10^{-5}M oubain. Multiple revertant colonies grew in the control tsH1 cells incubated at 39^0C. Colonies were ring cloned on day 21 and expanded. Aliquots of each clone were frozen down in liquid nitrogen.

DNA was then prepared from the cells of each clone and from the parental cells, digested with *Hinf* I and separated on an agarose gel before being transferred to a nylon membrane. The filter was then hybridised with ^{32}P-labelled MS8. Three of the ten hybrids contained the smaller human parental MS8 allele and the remainder gave no signal with MS8 and were presumably revertants. As all the hybrids had retained the same MS8 allele one hybrid clone (781) was karyotyped. The hybrid contained human chromosome 5 in 78% of metaphases, chromosome 18 in 83% of metaphases and three marker chromosomes in between 43%, 57% and 91% of metaphases. Extended banding studies demonstrated

that it was the deletion chromosome 5 that was retained in this hybrid clone (Figure 9).

Hybrid clone 781 was named PD/TS-1 to represent P.W. parent cells (P), deletion chromosome 5 (D), temperature-sensitive selection (TS) and clone one (1).

Fusion 8

Parental cells: GMO3314 and TsH1/5

A polyethylene glycol fusion was performed between 5 x 10^6 cells of each parental cell line. There were multiple revertants on the control plates of tsH1 grown at 39^0C. Eight colonies were picked from different plates and expanded. On southern analysis none of the 'hybrid' clones gave a signal with MS8 and they were all presumed to be revertants.

Fusion 9

Parental cells: Del 5 and TsH1/5

A fusion was performed between 5 x 10^6 cells of each parental line. Multiple revertants colonies grew on the control plates incubated at 39^0C. Five colonies were picked from the fusion plates, expanded, aliquots frozen down and DNA prepared. On southern analysis two of the clones gave a signal with MS8. Clone 972 retained the smaller MS8 allele from the parental line Del 5 which had previously been detected in association with the deletion chromosome 5 in MD/TS-1 (Clone 781). Clone 991 retained the larger MS8 allele and thus would be expected to contain the normal chromosome 5 of Del 5. On chromosome analysis 991 was found to retain multiple human chromosomes, including chromosome 5. The retention of multiple human chromosomes made 991 unsuitable for the production of a subclone in which only the normal human chromosome 5 was retained.

Fusion 10

Parental cells: P.W. and TsH1/5

5 x 10⁶ cells from each parental line were fused and incubated at the selective temperature of 39°C. 22 days after the fusion six colonies were picked from three different plates and expanded. Again aliquots were frozen and DNA prepared. Southern analysis with MS8 showed the presence of the larger human parental allele in one clone, 1011, the other colonies representing revertants. The retention of the deletion chromosome 5 in PD/TS-1 (781) had been associated with the retention of the the smaller MS8 allele and therefore the larger allele in 1011 would be expected to demonstrate the retention of the normal chromosome 5. Karyotypic analysis showed the presence of multiple human chromosomes in addition to chromosome 5.

Fusions 11 and 12

Parental cells: P.W. and TsH1/4

In fusion 11 equal numbers of each parental cell line were fused (5 x 10⁶) and in fusion 12 the cells were fused in a ratio of 10: 1 (10⁷ P.W.: 10⁶ tsH1). The products of each fusion were incubated in growth medium at 39°C. Multiple revertant colonies grew on the control plates at 39°C. Nine colonies were picked from different plates of fusion 11 and eight colonies were picked from different plates of fusion 12. The clones were expanded, aliquots frozen and DNA prepared. Each clone was assessed for the presence of human chromosome 5 using the polymerase chain reaction (PCR) with oligonucleotides specific to human complement factor nine, which has been mapped to chromosome 5 (Abbott et al., 1989). Southern analysis was then performed on each clone with the probe MS8. In four of seventeen clones an amplification product was detected on an ethidium-stained gel after PCR and the same four clones retained the larger parental allele of MS8. This allele would be expected to be present on the normal chromosome 5 of P.W. The four clones were karyotyped and the results are tabulated below.

Table 9 Fusion 11 and 12 clones karyotypes and MS8 alleles

Clone	MS8 allele	Percentage retention of each human chromosome
1142	a 1	5(70%); 10(65%);
1222	a 1	Several chromosomes
1241	a 1	Several chromosomes
1251	a 1	Several chromosomes

Detailed karyotyping of Clone 1142 was performed to detect all human DNA present in the metaphases using *in situ* hybridisation with biotinylated total human DNA and detection with fluoroscein-labelled avidin (Pinkel et al., 1986). This showed that only half the metaphases contained human chromosomes and that 30% of these only contained chromosome 5 and the remainder contained either chromosome 10 alone or chromosomes 5 and 10. Extended banding studies demonstrated that the retained chromosome 5 was normal with no interstitial deletion.

Single cells of 1142 were picked and cloned on to a 96 well plate. The plates were inspected to check which wells contained single cells and these clones were expanded. Aliquots of 25 single cell clones were frozen and a small cell pellet retained for PCR.

The cells were lysed as described in method II of the lysis of cells for the polymerase chain reaction. A PCR was performed using complement factor nine oligonucleotides. An amplification product was detected in 14 of 25 clones. Cells from these 14 clones, which had retained chromosome 5, were sent to Dr M.S. Povey for isoenzyme analysis to see which ones had also retained chromosome 10. Four single cell clones (BB3, BC4, BE6 and BF11) were clearly negative for the human isoenzyme serum glutamate oxaloacetate transaminase (GOT$_S$) on chromosome 10.

Two single cell clones, BC4 and BB3, were karyotyped. BC4 contained human chromosome 5 in 93% of metaphases and no other human genetic material was detected on *in situ* hybridisation with total human DNA (figures 10 and 11). BB3 contained a marker
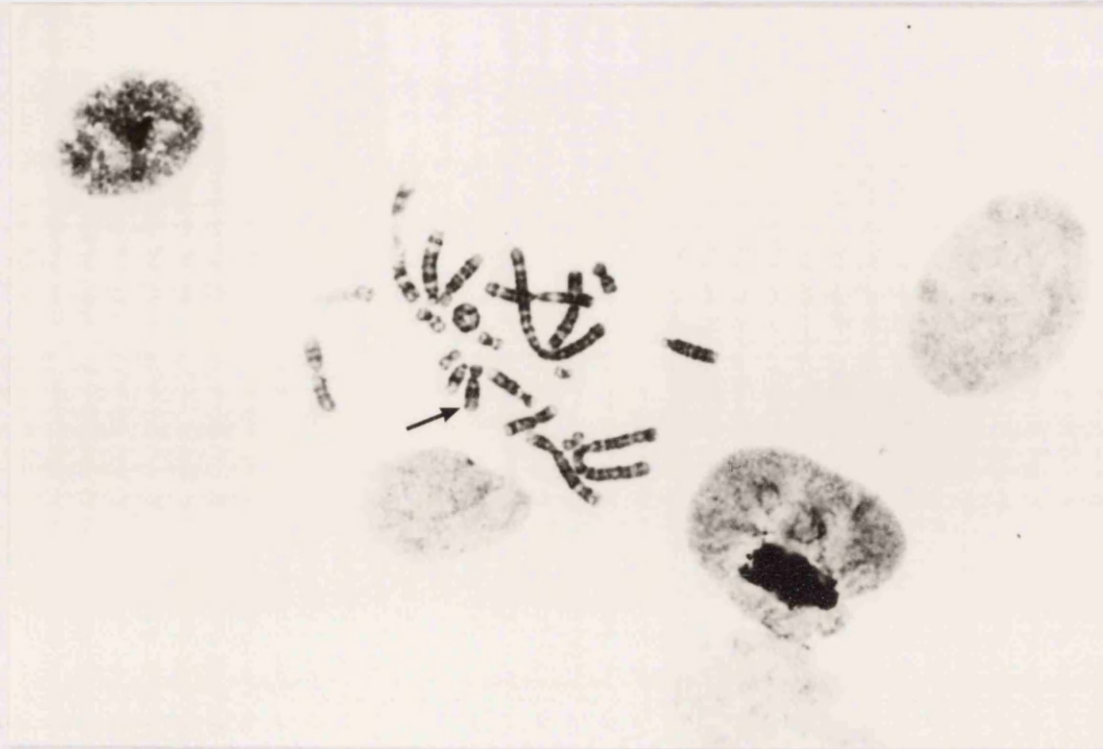
Figure 10

Karyotype of the human-rodent somatic cell hybrid PN/TS-1. The arrowed chromosome has a banding pattern characteristic of human chromosome 5.

Figure 11

In situ hybridisation of the same metaphase spread of PN/TS-1 as in Figure 8 with fluoroscein-labelled human genomic DNA. The only chromosome to fluoresce is that with the human chromosome 5 banding pattern.

chromosome, possibly derived from chromosome 5, in 69% of metaphases and chromosome 5 in 7% of metaphases.

Subclone BC4 of 1142 was named PN/TS-1 to represent P.W. parent cells (P), normal chromosome 5 (N), temperature-sensitive selection (TS) and clone one (1).

Fusion 14

Parental cells: J.T. and TsH1/22

10^7 cells from each parental cell line were fused, plated on to 20 dishes and incubated at 39^0C in growth medium. Two control plates of 10^6 tsH1/22 cells, a revertant-free subclone of tsH1, were incubated at 39^0C and no colonies grew. Between three and four weeks after the fusion 11 clones were picked from 10 different plates. These clones were expanded, aliquots frozen and DNA extracted from seven clones from different plates.

On southern analysis J.T., the parental cell line, was homozygous for one *Hinf* I allele detected by the probe MS8. Two probes mapped to the *APC* gene region and detecting polymorphisms, ECB 27 and YN548, were used instead (Nakamura et al, 1988; Varesco et al., 1989). J.T. was heterozygous for the *Bgl* II polymorphism detected by ECB 27 However, the human alleles and the hamster bands overlapped making interpretation of the autoradiograph difficult. J.T. was heterozygous for the *Taq* I polymorphism of YN548. Human bands were present in all the clones indicating that there were no revertants. There were hybrids retaining each chromosome 5 allele (see Table 10).

Table 10 Fusion 14 clones ECB27 and YN5.48 alleles

Clone	ECB27 allele	YN5.48 allele
JT/TS-01	a 2	a 2
JT/TS-3	a 1	a 1
JT/TS-6	a 2	a 2
JT/TS-9	a 1	a 1
JT/TS-10	a 2	a 2
JT/TS-12A	a 2	a 2
JT/TS-15	a 2	a 2

JT/TS-01 and JT/TS-3, each of which contained a different human chromosome 5 allele, were karyotyped and each found to contain a normal appearing chromosome 5.

Hybrids HHW 1155 and 1159

The lymphoblastoid cell line L3214 was derived from a patient with FAP. On pulse field gel electrophoresis a normal band and an altered smaller band was detected in *Bgl* II digests of these cells by the probe L 5.79 which is closely linked to the *APC* gene. However, the shifted band was found to come from the unaffected parent and the affected parent found to have a normal size band. Densitometric analysis of southern blots of L3214 probed with polymorphic markers in the *A P C* region was interpreted as showing hemizygosity in this region and it was concluded that a deletion was present (R.L. White, personal communication).

Somatic cell hybrids were made from L3214 by J.J. Wasmuth (University of California, Irvine) using the temperature-sensitive system described above. HHW1155 and HHW 1159 are two hybrid clones that each retain one of the human chromosome 5s from L3214 .

Applications of the somatic cell hybrids

Mapping Panels

The construction of the hybrids MD/TS-1, PD/TS-1 and PN/TS-1 allowed the formation of a panel to map DNA probes on chromosome 5 and to determine which probes mapped into the *APC* gene region.

The parental and hybrid DNAs were digested with *Hinf* I, separated on an agarose gel, Southern blotted and probed with MS8. The parental cell lines Del 5, PW and L3214 were heterozygous and the segregation of one parental allele into each hybrid demonstrated (Figure 12). DNA from JT when digested with *Msp* I and probed with YN 5.48 was heterozygous and the segregation of one parental allele into each hybrid also demonstrated (Figure 13).

DNA from the human parental cells, the hybrids and from the hamster parent tsH1 was digested with *Eco* RI and hybridised with clones potentially on chromosome 5. A hamster signal would be seen in all four lanes. A probe on human chromosome 5 would give a human band in the PN/TS-1 lane but would not give a human signal in MD/TS-1 and PD/TS-1 lane if it mapped into both of the deletions.

C11p11, the first probe with which linkage was shown to FAP, did not map into either of the deletions on chromosome 5, a human signal being detected in the PN/TS-1, MD/TS-1 and PD/TS-1 lanes. Thus although C11p11 was sufficiently close to give linkage in family studies it is not in the immediate vicinity of the *APC* gene. This result was not unexpected as the maximum LOD score between C11P11 and FAP is at a recombination fraction of approximately 5%.

Dr Lilly Varesco (Molecular Analysis of Mammalian Mutation Laboratory, ICRF; presently at I.S.T., Genova, Italy) used the panel to map probes from an end clone library into the deletions. This library had been constructed from H64 a human: hamster somatic cell hybrid which retained human chromosomes 4 and 5

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HHW 1159

HHW 1155

L 3214

PN/TS-1

PD/TS-1

Patient PW

Hybrid 991

MD/TS-1

Patient M

Hamster A23
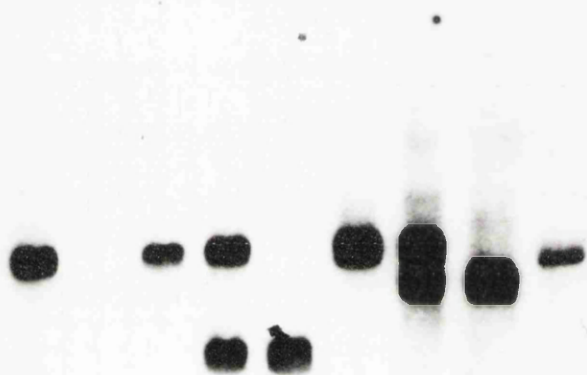
Figure 12

Autoradiograph of a panel of parental cells and human-hamster somatic cell hybrids, which retain human chromosome 5, digested with H inf I and probed with MS8. One of the two parental MS8 alleles is present in each hybrid.

94
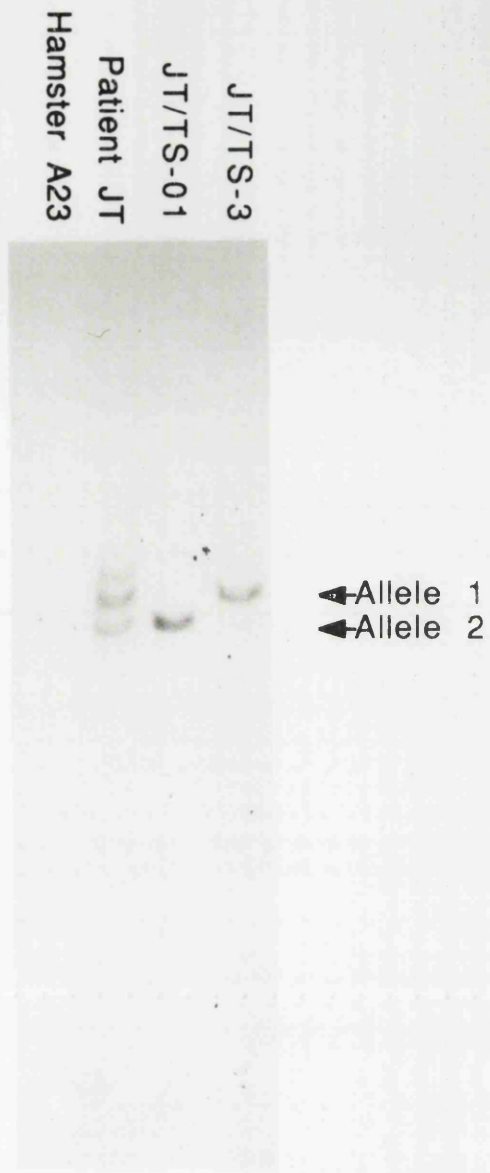
Figure 13

Autoradiograph of the two parental cells and two human-hamster somatic cell hybrids digested with Msp I and probed with YN 5. 48. One of the parental YN 5.48 alleles is present in each hybrid.

95
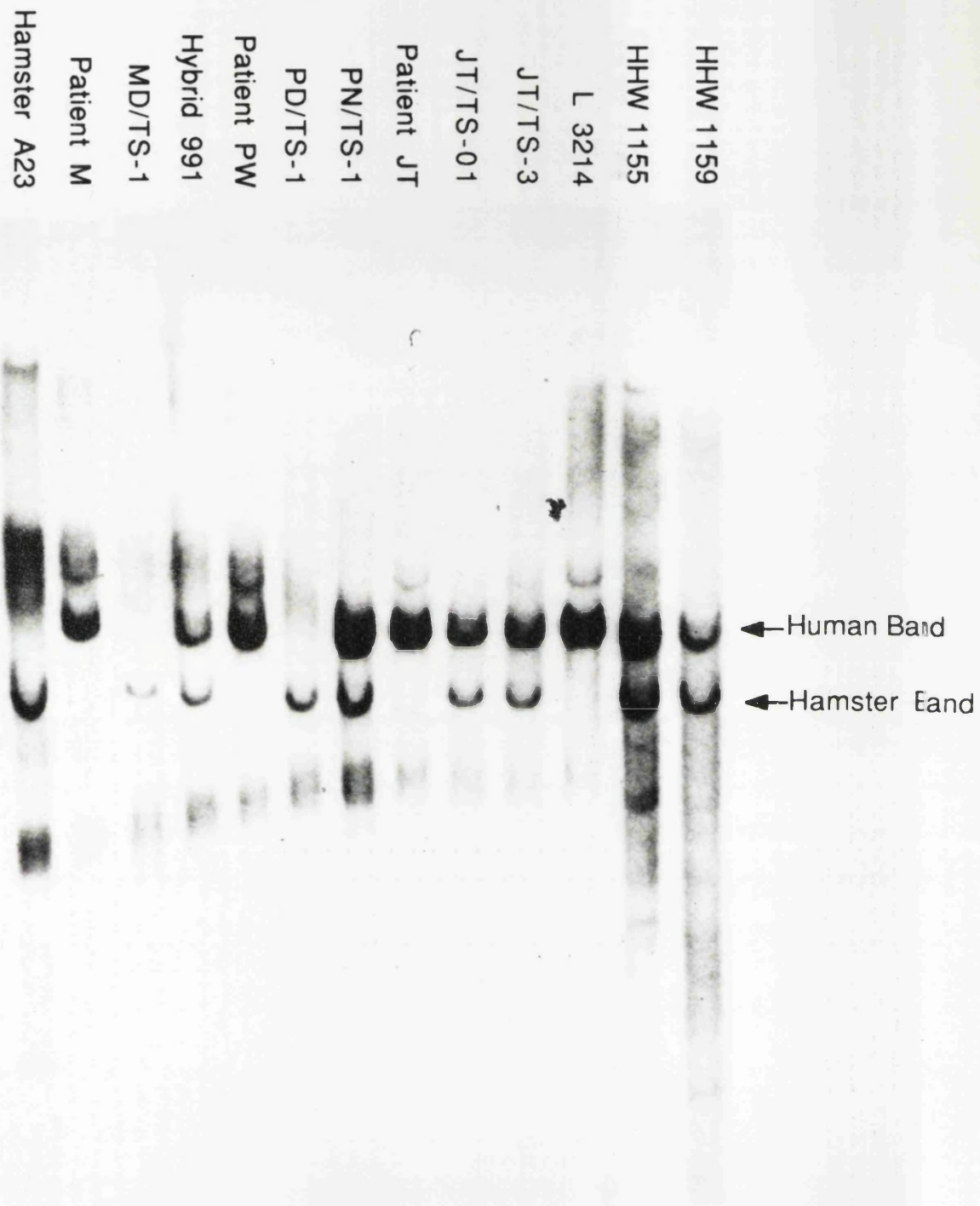
Figure 14

Autoradiograph of a panel of parental cells and human-hamster somatic cell hybrids, which retain human chromosome 5, digested with Eco RI and probed with ECB 27. No human signal is present in hybrids MD/TS-1 and PD/TS-1.

(MacDonald et al., 1987; Varesco et al., 1989). Initially three clones were identified that mapped onto chromosome 5 and into one or both of the deletions. ECB 27 was deleted in MD/TS-1 and PD/TS-1 (Figure 14), as was ECB 220. ECB 134 was only deleted in MD/TS-1. As yet no probes have detected deletions in the hybrids derived from L3214 or JT. These results indicate that the two deletions are overlapping but that the deletion of MD/TS-1 is more extensive in at least one direction than that of PD/TS-1.

The mapping panel has provided a way to rapidly screen which probes from a library map into the *APC* gene region without the necessity to find polymorphisms and perform family linkage studies.

Source of DNA for Cloning

Another use for the hybrid PN/TS-1 is as a source of probes on human chromosome 5. There are two possible approaches to isolating human chromosome 5 clones from PN/TS-1, either DNA libraries constructed from it may be screened for human clones or alternatively the human chromosome 5 may be flow sorted from the hamster chromosomes and the human chromosome 5 enriched material used to make DNA libraries.

Radiation Hybrids

The construction of a panel of human chromosome 5 radiation hybrids from PN/TS-1 is described in the next chapter.

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CHAPTER FOUR

RADIATION HYBRIDS

Introduction

The aim of the experiments described in this chapter was to isolate a small fragment of human chromosome 5, including the *APC* gene, in a somatic cell hybrid. Such a hybrid could then be used as a source of DNA for cloning the *APC* gene and could also be used for mapping.

There is no dominant selectable marker in the *APC* gene region of chromosome 5 and therefore the construction of hybrids using chromosome-mediated gene transfer is not feasible. Cox et al. (1989) and Benham et al. (1989) have recently described a modification of the technique of Goss and Harris (1975) for the production of radiation hybrids. The technique involves the irradiation of a somatic cell hybrid containing a single human chromosome with X rays. This results in double-stranded breaks in the DNA, the fragmentation of the hamster and human chromosomes and the death of the cells. The irradiated cells are rescued by fusion to a recipient hamster cell line which is deficient of either TK or HPRT and the products incubated in HAT selective medium. The innovatory feature of the technique is that the selection is for a hamster gene product and the human chromosomal fragments in the hybrid cells are present as a result of random co-transfection. Thus, the technique does not require a positive selectable marker and results in the retention of unselected human chromosomal fragments.

The size of the chromosomal fragments produced is dependant on the dosage of irradiation used. A high radiation dosage would be expected to lead to more frequent chromosomal breaks and to result in smaller chromosomal fragments. We used a very high radiation dosage of approximately 50,000rad with the aim of producing hybrids retaining small chromosomal fragments.

We planned to screen rapidly the colonies picked from the fusion for the retention of DNA in the *APC* region by amplification using oligonucleotide primers from probes closely linked to *APC*.

5×10^6 PN/TS-1 cells, a somatic cell hybrid whose only human genetic material is chromosome 5 (see previous chapter), were irradiated with 50,000 rads. The cells were then added to 5×10^6 A23 TK$^-$ cells, washed in serum-free medium and spun to produce a cell pellet. The supernatant was aspirated and a polyethylene glycol fusion performed. The products were plated on 20 x 90mm petri dishes in HAT medium and incubated at 37^0C (see Materials and Methods).

Colonies were visible after 10 days at which time 172 colonies were picked and expanded. Cells from each clone were grown to confluence in two 25cm^2 flasks and harvested. The cells from one flask were frozen in liquid nitrogen and the cells from the second flask were expanded to a 75cm^2 flask while PCR analysis was performed on a small cell pellet.

PCR of Cell Lysates

DNA from hybrid cell lysates may be used as a substrate for amplification by oligonucleotide primers, as was described in the characterisation of the temperature-sensitive hybrids in the previous chapter.

Pellets of approximately 10^6 cells were washed in PBS and stored at -40^0C. The cells were resuspended and incubated in 500μl with proteinase K for 45 minutes followed by heat inactivation of the proteinase K at 95^0C. Amplification using Alu sequence oligonucleotides was successful on control DNA and aliquots of PN/TS-1 cell lysate but unsuccessful on the radiation hybrid lysates.

Amplification using the ECB 27 oligonucleotides (Varesco, personal communication) gave a product with PN/TS-1 DNA but also gave a faint band with hamster DNA. This made these oligonucleotides

unsuitable to distinguish between clones that had and had not retained human DNA homologous to ECB 27.

Inhibitor of Amplification

The PCR was repeated and again there was no amplification product in the cell lysates of the radiation hybrids. The presence of an inhibitor was demonstrated by the failure of a sample containing a mixture of PN/TS-1 DNA and a cell lysate to amplify despite the normal amplification of the PN/TS-1 DNA alone. This inhibition could be overcome by increasing the amount of PN/TS-1 DNA in the reaction mix but not by increasing the concentration of *Taq* polymerase used in the reaction.

Alternative methods of lysing the cells were attempted to see if the inhibition could be avoided. The cells were lysed by boiling in distilled water, by proteinase K digestion followed by one phenol: chloroform extraction and by boiling in 1% SDS followed by one phenol: chloroform extraction. All of these failed to allow amplification of the cellular DNA and inhibited the amplification of low concentrations of PN/TS-1 DNA.

The growth of PN/TS-1 cells in HAT medium did not result in inhibition of amplification by the cell lysate nor did the addition of HAT to the amplification reaction. To test for digestion of the amplification product the reaction mix of a PN/TS-1 DNA amplification and a radiation hybrid lysate amplification were incubated at 37^0C for 30 minutes separately and as a mixture but no evidence of digestion was detected.

DNA Extraction

The failure of amplification using cell lysates of the radiation hybrids made the early analysis of which hybrids retained fragments in the *APC* region impossible by PCR. As the hybrids were still being expanded while these experiments were taking place it was decided to grow each clone to confluence on a 600cm^2 plate to allow the extraction of DNA for southern or PCR analysis.

150 of the colonies originally picked were expanded to confluence on a 600cm² plate and DNA extracted.

PCR of Radiation Hybrid DNA

Amplification of DNA from 20 radiation hybrid clones using human-specific Alu sequence oligonucleotides (Nelson et al., 1989) demonstrated the presence of amplification products in all of the clones. This indicated that all the radiation hybrid clones tested retained human chromosomal fragments.

Southern Hybridisation Analysis of Radiation Hybrids

DNA from each of the radiation hybrid was digested with *Eco* RI, size separated on an agarose gel and blotted onto a nylon membrane. PN/TS-1 DNA was included as a control on each filter.

The filters were initially hybridised with ECB 27 as this probe mapped into the deletions of both MD/TS-1 and PD/TS-1 and also showed close linkage to FAP in pedigree studies. 14 out of 138 hybrids retained human DNA homologous to ECB 27. The filters were then stripped and rehybridised with YN 548, a flanking marker which also shows close linkage to FAP (Nakamura et al., 1988; Tops et al., 1989). 12 out of 142 hybrids retained a human DNA homologous to YN 548 and five hybrids were positive for both markers.

The five hybrids retaining both markers may either have retained a single fragment of human chromosome 5 spanning the region between them or they may have retained two separate fragments each of which retains one marker. The radiation hybrids retaining ECB 27 and YN 548 were used as a source of DNA for the production recombinant DNA libraries (see following chapter). Further mapping of the radiation hybrids with additional DNA markers is described in the Radiation Hybrid Mapping Chapter.

Chromosomal Analysis of Radiation Hybrids

Five different radiation hybrid clones (12, 13, 25, 78 and 158) were regrown from the frozen stock and metaphase spreads prepared. Further stocks were also frozen. *In situ* hybridisation of the metaphase spreads with human genomic DNA was used to demonstrate the presence of retained human chromosomal fragments (Figure 15).

Human chromosomal fragments were present in most metaphases. The human material was usually present as relatively small insertions within hamster chromosomes and no long human chromosomal fragments were seen (for example, the retention of a chromosome arm was not seen).

Several different metaphases from each radiation hybrid were analysed to see if the same marker chromosomes were retained in all the cells. There was considerable heterogeneity. In radiation hybrid 12, for example, three marker chromosomes were retained in 93%, 67% and 47% of the cells and eight other markers were seen in between 7% and 27% of cells (S. Williams, personal communication). These results suggest that there is segregation of human genetic material within individual radiation hybrids.

Single Cell Cloning of Radiation Hybrids

To analyse the segregation of markers within individual radiation hybrids single cells were picked from each of four radiation hybrids and plated into 24 well plates. The following day the dishes were inspected to determine which wells contained only one hybrid cell and these were expanded in 25cm^2 flasks. The cells were harvested and DNA extracted.

The human DNA content of the single cell clones was analysed by amplification using a human-specific Alu sequence oligonucleotide (Nelson et al., 1989). The reaction product was run out on a 1% agarose gel which was then stained with ethidium bromide and photographed on an ultra-violet light transilluminator. The number

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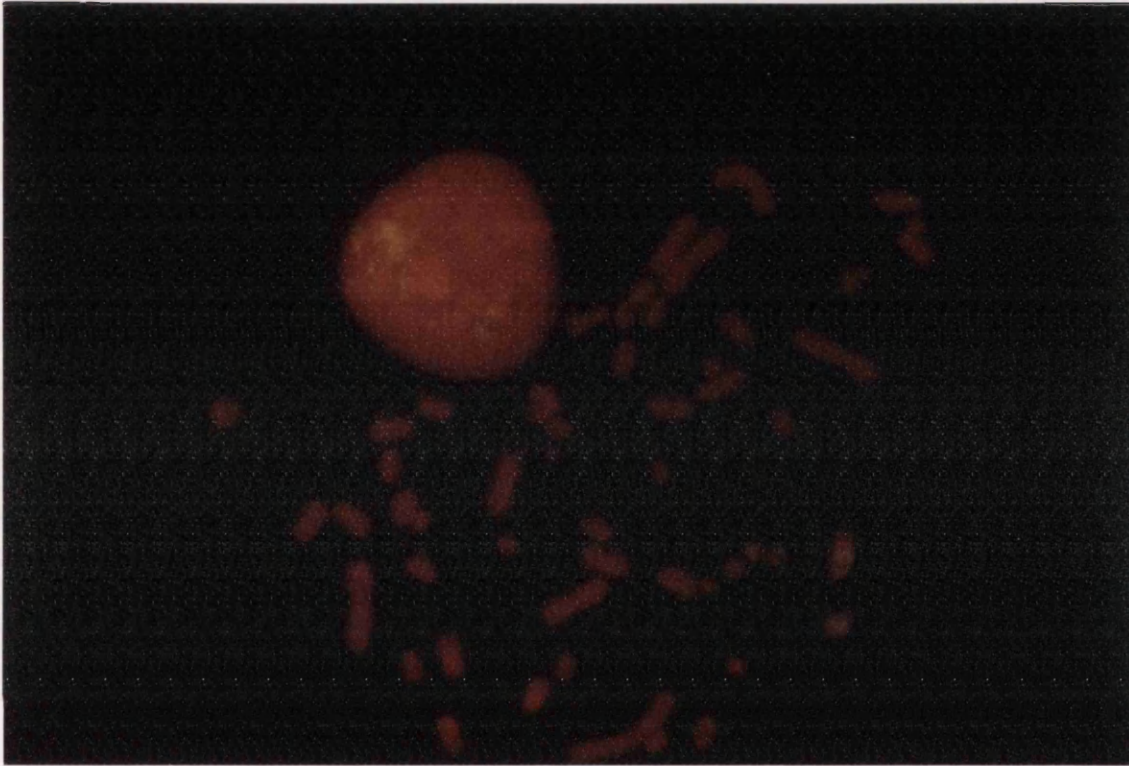
Figure 15

In situ hybridisation of a metaphase spread of Radiation Hybrid 12 with fluoroscein-labelled human genomic DNA. Two human chromosomal fragments, which are integrated into a hamster chromosome, are seen to fluoresce.
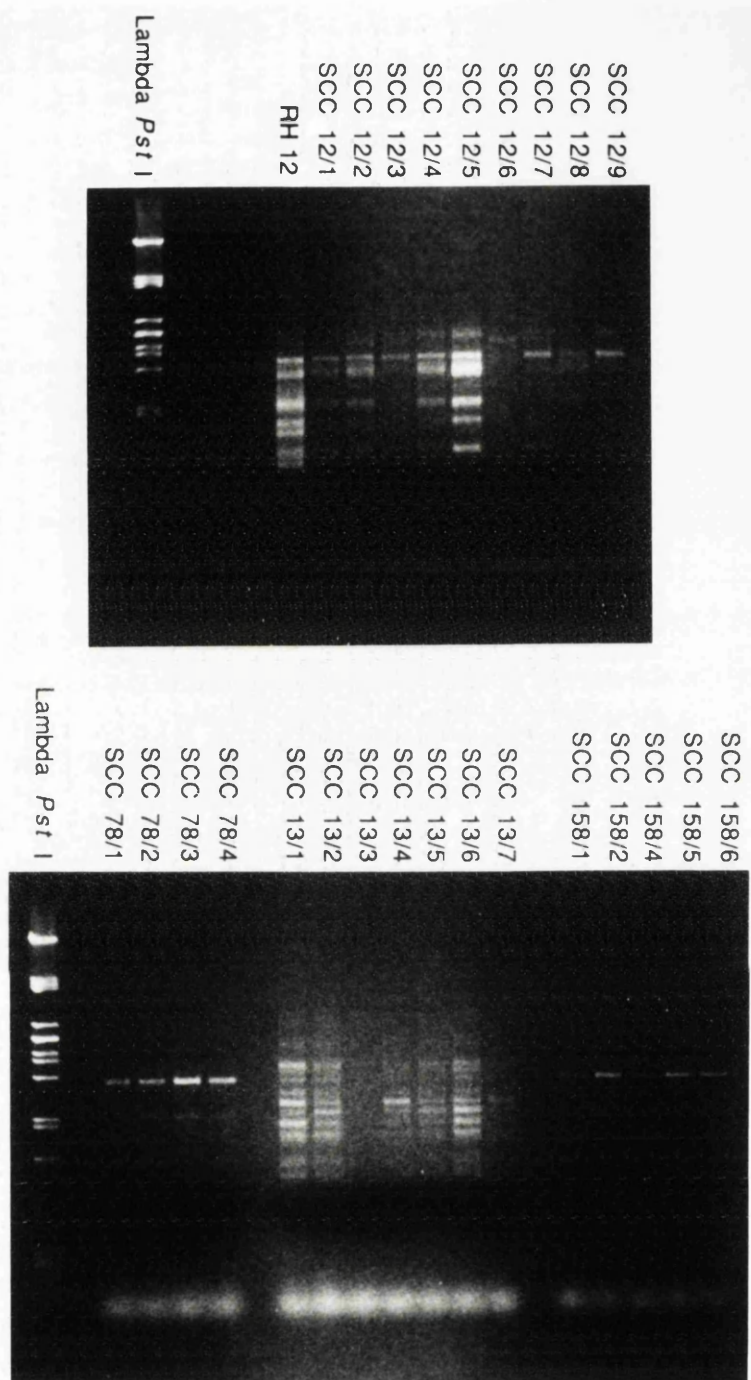
<u>Figure 16</u>
Photograph of an ethidium-stained agarose gel in which the Alu-Alu amplification products of radiation hybrids 12, 13, 78 and 158 and of single cell clones derived from them. Some of the single cell clones from RH 12 have fewer amplification products than the parental cells.

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and size of the bands in the amplification product was compared between different single cell clones from the same radiation hybrid (Figure 16).

The segregation of human DNA was demonstrated between individual single cell clones of radiation hybrid 12 in which Alu-Alu amplification products which were present in some clones were absent in others (Figure 16). A smaller number of single cell clones from the other three radiation hybrids analysed did not demonstrate segregation of the Alu amplification products.

Discussion

The radiation hybrids retained human DNA despite selection for only a hamster gene product in the donor hybrid. Fragments of human DNA were demonstrated to be integrated into hamster chromosomes on *in situ* hybridisation of metaphase spreads with fluoroscein-labelled human DNA. The single cell cloning experiment demonstrates that individual radiation hybrid clones are heterogeneous and have segregated different human DNA fragments. For example, the Alu amplification products of radiation hybrid 12 present in different single cell clones varied.

CHAPTER FIVE

LAMBDA LIBRARY OF RADIATION HYBRID 158

Introduction

The aim of the construction of a DNA library was to isolate further probes from the *APC* gene region. It was proposed that the selection of a radiation hybrid that retained DNA from this region would enhance the proportion of probes in the library from the region. Radiation hybrid 158 was chosen as it retains human DNA homologous to the probe YN548 This probe shows close linkage to FAP in pedigree studies and is deleted in the hybrids MD/TS-1 and PD/TS-1.

It was felt that a bacteriophage λ library would be simpler to construct and faster to screen than a cosmid library. The vector λEMBL3 was chosen. This vector has a polylinker sequence that allows the stuffer fragment to be restricted with a second enzyme preventing re-ligation of wild-type vector. The presence of *red* and *gam* genes in the stuffer fragment allows Spi (Sensitive to P_2 interference) selection to be used. With Spi selection only recombinant bacteriophage will undergo growth on a selective P_2 lysogen host such as NM646. An additional advantage of using λEMBL3 was that there was extensive experience of its use in the laboratory.

Library construction

10μg of vector DNA were digested with *Bam*HI. An aliquot was separated on a gel and demonstrated complete digestion. The vector was then digested with *Eco*RI. This removes the *Bam*HI site from the stuffer fragment and prevent ligation of the stuffer fragment and vector arms to reform wild-type vector.

Insert DNA

Analytical digestion of the insert DNA was performed. A digestion time of between five and ten minutes produced DNA fragments of a size suitable for cloning. The reaction was scaled up to 10μg of

insert DNA. The DNA was restricted, EDTA added and the enzyme heat-inactivated. An aliquot was retained for control ligation. The remaining DNA was then treated with alkaline phosphatase which was then inactivated with nitrilotriacetic acid. The DNA was then re-extracted with phenol: chloroform and ether.

Dephosphorylation Control Ligation

A ligation reaction was performed to test the completeness of the dephosphorylation of the insert DNA. Dephosphorylated DNA is unable to ligate to other dephosphorylated DNA. In contrast, the aliquot of phosphorylated insert DNA would be expected to ligate to form high molecular weight DNA which would run as a single band on an agarose gel.

The initial test ligation of the insert DNA was unsuccessful and resulted in the degradation of the DNA. This was thought to be due to incomplete extraction of the *Mbo*I after the preparative digestion resulting in the remaining *Mbo*I restricting the insert DNA during the ligation reaction.

A further preparative digestion was performed. The control ligation reaction performed on the insert DNA was unsuccessful with failure of the phosphorylated DNA to ligate. Failure to ligate may result from contamination of the DNA with either phenol or or EDTA. The DNA was therefore extracted once again with phenol: chloroform and then with ether. The yield of DNA following the re-extraction of the DNA was greatly reduced. This reduction in the yield may have been due to the use of ether that was not water-saturated.

The preparative digestion was repeated with careful phenol: chloroform and water-saturated ether extraction. The control ligation of phosphorylated insert DNA was successful and resulted in the formation of a higher molecular band of DNA. The dephosphorylated DNA failed to ligate.

Ligation

A ligation reaction was performed between the dephosphorylated *Mbo*I-digested insert DNA and the λEMBL3 vector that had been restricted with *Bam*HI and *Eco*RI. Digestion with the enzymes *Mbo*I and *Bam*HI results in the formation of compatible DNA ends for ligation.

Packaging and Test Plating

1 μg of recombinant phage DNA was packaged and aliquots of diluted packaged ligation mixture plated on three different strains of *Escherichia coli*. The three strains of plating cells were used to distinguish between recombinant and wild-type (non-recombinant) vector.

The non-selective strain NM621 allows the growth of both recombinant and non-recombinant vector. The selective strain NM 646 will only allow the replication of recombinant vector λEMBL3. Growth of wild-type bacteriophage is restricted by P_2 lysogen which expresses the cI repressor protein. This repressor inhibits the transcription in wild-type but not recombinant bacteriophage.

The strain ED8767*rec*A⁻ will only allow the replication of wild-type vector. The replication of bacteriophage λ DNA requires a recombination event to allow the formation of concatamers. The recombinant vector is unable to perform this recombination event itself, having lost the *red* and *gam* genes with the stuffer fragment. This function can be also be performed by a *rec*A⁺ host cell, however, if the host cell is *rec*A⁻ only non-recombinant vector will grow.

The plating efficiency of the packaged ligation mixture was calculated as 9×10^5 plaques/μg recombinant DNA on the selective strain NM646. There was also a high proportion of uncut wild-type vector on the non-selective and *rec*A⁻ strains of plating cells. This plating efficiency was considerably less than that of several other λEMBL3 libraries constructed by Dr A-M. Frischauf (Molecular

Analysis of Mammalian Mutation Laboratory, ICRF)from radiation hybrid DNA which had plating efficiencies of over 10^7 plaques/μg DNA. The low plating efficiency was thought to be due to incomplete digestion of the vector DNA and the insert DNA being too large. It was decided to use the libraries with higher plating efficiencies in subsequent experiments.

Plating of Library

100μl of 10^{-2} diluted packaged ligation mix was plated to give approximately 10^5 plaques on a 23 x 23cm plate. Two lifts were taken with nylon membranes and probed with total human DNA after incubation with competitor hamster DNA. 100 human plaques were picked into phage dilution buffer and incubated at 4^0C overnight. Twenty-five bacteriophage lysates from the primary screen were replated at various dilutions. Lifts were taken from the secondary plates and probed with total human DNA as above.

Preparation of Bacteriophage λ DNA

25 human plaques were picked from the secondary screen plates and grown in 3mls of L-broth. The bacterial cells were lysed and the bacteriophage DNA extracted.

Mapping of λ Clones

10μl of each DNA sample was labelled with ^{32}P and incubated with sheared human competitor DNA before being hybridised to a mapping panel. The mapping filters each had four DNA samples, Hamster, PN/TS-1, MD/TS-1 and PD/TS-1, digested with EcoRI. Twenty recombinant bacteriophage λ clones from the library of radiation hybrid 158 were mapped. Sixteen gave a clear human band on PN/TS-1, which retains a normal human chromosome 5, but none of these mapped into the deletion of either MD/TS-1 or PD/TS-1.

Two further bacteriophage λ libraries constructed by Dr A-M. Frischauf were also screened in the same way to map clones into the deletions of MD/TS-1 and PD/TS-1. Hybrid 58 retained human DNA homologous to ECB27 and hybrid 115 retained human DNA homologous to YN 548.

One recombinant clone from library 58 mapped into the MD/TS-1 deletion but not into the PD/TS-1 deletion, the remaining 24 did not map into either deletion. None of 18 clones screened from library 115 mapped into either deletion.

Discussion

The proportion of clones in these three radiation hybrid libraries mapping into either of the deletions was disappointing. Further libraries constructed by Dr A-M. Frischauf from the radiation hybrids were screened by colleagues at The ICRF working on the familial adenomatous polyposis project. The mapping results of all the libraries screened are presented below in Table 11.

Table 11 Mapping of radiation hybrid lambda clones

Hybrid	Positive Markers		Probes		Screened By
	ECB27	YN5.48	Deleted	Total	
4	+		0	8	G. Hampton
12	+	+	3	30	S. Cottrell
13	+	+	0	17	S. Searle
14	+		5	22	S. Cottrell
32	+	+	0	18	S. Searle
58	+		1(MD)	25	H. Thomas
115		+	0	18	H. Thomas
137		+	0	30	A. McKie
158		+	0	16	H. Thomas

In total eight new probes in both deletions were isolated from 184 probes which were mapped. The failure of these radiation hybrids to greatly enhance the proportion of probes mapping into the region of the *APC* gene and the mapping data from the radiation hybrid panel are discussed together in the next chapter.

CHAPTER SIX

RADIATION HYBRID MAPPING

Introduction

The aim of the experiments described in this chapter was to determine the pattern of retention of human DNA markers in the radiation hybrids and to see whether this provided information on the order of the probes on chromosome 5.

The radiation hybrid panel was initially probed with ECB27 and YN5.48, two flanking markers of the APC gene (Tops et al., 1989; Varesco et al., 1989). Hybrids that retained either or both of these markers were used as a source of DNA for the construction of the lambda libraries as described in the previous chapter.

Two panels of radiation hybrids were screened with further human DNA probes on chromosome 5. A selected panel, consisting of those hybrids which had retained either or both of the flanking markers ECB27 and YN5.48, was initially probed with any new markers. If the hybridisation of the probe was satisfactory and the pattern of retention of interest the complete radiation hybrid panel was also screened with the probe.

Selected Panel

This panel of radiation hybrids was used to test the hybridisation conditions required by new probes and to give an indication as to their retention pattern. The radiation hybrid DNA was digested with EcoRI, separated on an agarose gel and blotted onto a nylon membrane.

Four groups of probes were screened over the panel:

1. Probes which showed linkage to FAP in family studies.

2. Probes deleted in either MD/TS-1 or PD/TS-1 or in both.

3. Probes which showed linkage to Spinal Muscular Atrophy.

4. A probe near the telomere of the long arm of chromosome 5.

Table 12   Probes mapped on radiation panel

Probe	Locus Symbol	Linkage to	Deleted in	Reference/ Source
M4	D5S6	SMA		Dietzsch et al., 1986
C11p11	D5S71	FAP		Bodmer et al., 1987
YN5.48	D5S81	FAP	MD+PD	Nakamura et al., 1988
YN5.64	D5S82	FAP	MD+PD	Nakamura et al., 1988
ECB27	D5S98	FAP	MD+PD	Varesco et al., 1989
MS8	D5S43			Wong et al., 1987
pi227	D5S37	FAP		Stewart et al., 1987
p105/153	D5S39	SMA		Gilliam, et al., 1989
JK53		SMA		T.C. Gilliam
LGH5.4			MD+PD	G. Hampton
12:3			MD+PD	S. Cottrell
12:46			MD+PD	S. Cottrell
12:75			MD+PD	S. Cottrell
14:16			MD+PD	S. Cottrell
14:17			MD+PD	S. Cottrell
14:20			MD+PD	S. Cottrell
14:54			MD+PD	S. Cottrell
14:61			MD	S. Cottrell

Results of selected Panel

[32]P-labelled DNA probes were hybridised to the nylon filter. The approximate location of the probes used in the radiation mapping is given in Figure 17. An example of the signal given on an autoradiograph following hybridisation is illustrated in Figure 18. The retention of human DNA homologous to the probes in the selected radiation hybrids is presented in Table 13.
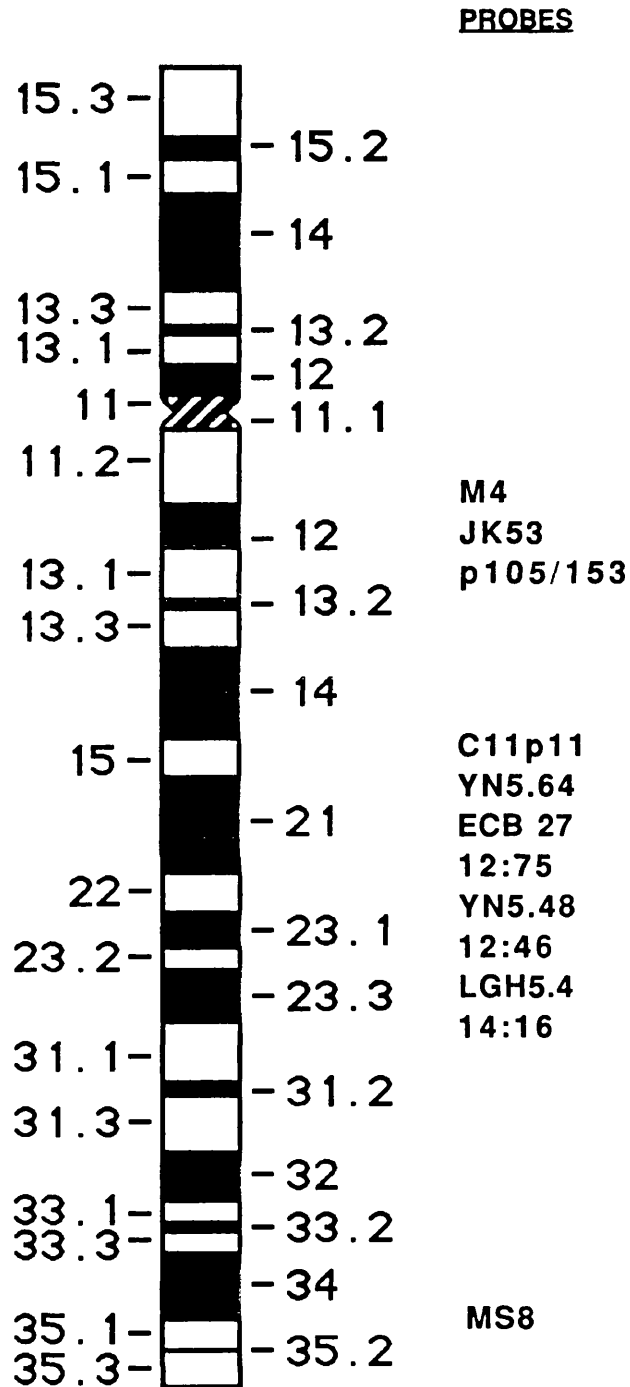
Figure17

Ideogram of human chromosome 5 showing the approximate locations of the probes used in the radiation hybrid mapping.

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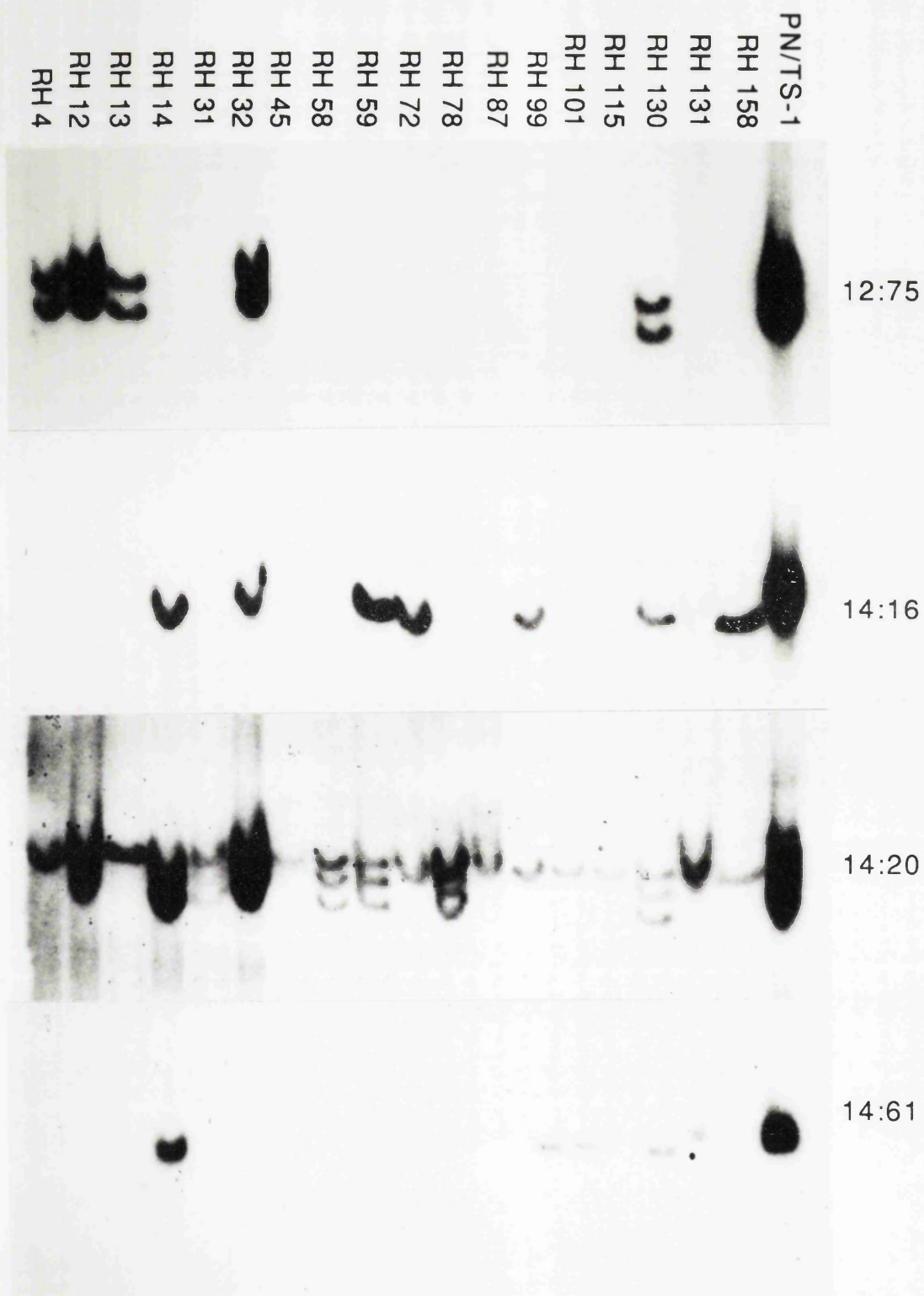
Figure18

Autoradiograph of the selected panel of radiation hybrids digested with *Eco* RI and probed with 12:75, 14:16, 14:20 and 14:61.

Hybrid	M4	153	p227	C11P11	YN564	14;20	12;3	ECB27	12;75	YN548	12;46	LGH5.4	14;16	14;17	14;61	MS8
4	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1
12	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0
13	0	0	0	0	1	0	0	1	1	1	0	0	0	0	0	0
14	0	1	0	0	1	1	1	1	0	0	0	0	1	1	1	0
31	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0
32	0	0	1	1	1	1	1	0	1	1	0	1	1	1	0	1
45	0	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0
58	1	0	0	0	0	1	1	1	0	0	1	1	0	0	0	0
59	1	0	0	0	0	1	1	1	0	0	0	0	1	0	0	0
72	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1
78	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0
87	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1
99	1	1	1	0	0	0	0	0	0	1	1	1	1	1	1	0
110	0	0	0		0	0	0	0	0	1	0	0	0	0	1	0
115	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
130	0	0	0		0	1	1	1	1	1	0	1	1	1	1	1
131	0	1	1		0	0	0	0	0	1	0	0	1	1	1	0
137	0	0	0		0			1	1	1		1	1			0
158	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0

Table13

Retention of human DNA probes in the selected radiation hybrid panel. 1=present, 0=absent.

Discussion of Selected Panel Results

Two groups of markers show a very similar retention pattern in the selected panel. The first group is ECB27, 12:3 and 14:20, and the second group is 14:16, 14:17, and 14:61. This suggests that the probes within each set are close to each other on chromosome 5. Therefore, only one probe from each of these groups was hybridised to the complete radiation panel.

The bacteriophage lambda library of radiation hybrid 14 contained a high proportion of probes which mapped into the deletions of MD/TS-1 and PD/TS-1 (5/22). On the selected radiation panel the probes 14:16, 14:17, and 14:61 have a similar retention pattern and this suggests that they are derived from a small region of chromosome 5. 14:20, on the other hand, shows similarity in its retention pattern to ECB27 and 12:3, which are also retained in hybrid 14.

These results suggest that hybrid 14 has retained at least two small fragments of human chromosome 5 within the deletions of MD/TS-1 and PD/TS-1 and that these represent approximately a fifth of the human material retained in the hybrid. The fragment retaining 14:16, 14:17, and 14:61 includes one margin of the PD/TS-1 deletion as 14:16 and 14:17 are deleted in both MD/TS-1 and PD/TS-1 whereas 14:61 is only deleted in MD/TS-1.

Statistical analysis of the selected panel was felt to be inappropriate in view of the bias inherent in the selection.

Complete Radiation Hybrid Panel

*Eco*RI digests of the 153 radiation hybrids were included in this panel. The filters were hybridised to ^{32}P-labelled DNA probes. The retention of human DNA homologous to the probes in the radiation hybrids is presented in Table 14.

Hybrid	M4	JK53	105/153	C11P11	YN564	ECB27	12;75	YN548	12;46	LGH5.4	14 16	MS8
1	0	0	0	0	0		0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	1
3	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	1	1	0	0	0	0	1
5	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0
7						0						
8	1	1	0	0	0	0	0	0	1	0	0	0
9												
10	0	0	0	0	0	1	0	0	0	0	1	0
11												
12	1	1	1	1	1	1	1	1	1	0	0	0
13	0	1	0	0	1	1	1	1	0	0	0	0
14	0	0	0	0	1	1	0	0	0	0	1	0
15	0	0	0	1	1	0	0	0	0	0	0	0
16	0	0	0		0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0	0	0	0
18	1	0	1	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0		0	0	0			0
20	0	0	0	0	0	0	0	0	0	0	0	0
21	0	1	1		1	0	0	0	0	1	1	0
22	0	0	0	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0	0	0	0
24												
25	0	0	0		0	0	0	0	0	1	0	0
26	0	1	1	0	0	0	0	0	0	0	0	0
27	0	0	0	0	0		0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0	0	0	0	0
30	1	0	0		0	0	0	0	0	0	0	0
31	0	0	0	0	0	0	0	0	0	0	0	0
32	0	0	0	1	1	1	1	1	0	1	1	1
33	0	0	0	0	0	0	0	0	0	0	0	0
34	0	0	0		0		0	0	0	0	0	1
35	0	0	0		0	0	0	0	0	0	0	0
36												
37	0	0	0	0	0	0	0	0	0	0	0	0
38	0	0	0	0	0	0	0	0	0	0	0	1
39	1	0	0	0	0	0	0		0	0	1	0
40	0	0	0		0	0	0	0	0	0	0	
41	0	0	0	0	0		0	0	0	0	0	0
42	1	0	0		0	0	0	0	0	0	0	0
43	0	0	0	1	0	0	0	0	0	0	0	0
44	0	0	0		1	0	0	0	0	0	0	0
45	0	0	0	1	0	0	0	1	0	0	0	0
46	0	0	0	0	0	0	0	0	0	0	0	0
47	1	0	0	0	0	0	0	0	0	1	0	0
48	0	0	0	0	0	0	0	0	0	0	0	0
49	0	0	0	1	0	0	0	0	1	1	0	0
50	0	0	0	0	0	0	0	0	0	0	0	0
51	0	0	0	0	0	0	0	0	0	0	0	0
52	0	1	0	0	0	0	0	0	0	0	0	0
53	0	1	1		1	0	0	0	0	0	0	0
54	0	0	0	0	0	0	0	0	0	0	0	1
55	0	0	0	0	0	0	0	0	0	0	0	0
56	0	0	1	0	0	0	0	0	0	0	0	0
57	0	0	0	0	0	0	0	0	0	0	0	0
58	1	1	0	0	0	1	0	0	0	1	0	0
59	1	0	0	0	0	1	0	0	0	0	1	0
60	0	0	0	0	0	0	0	0	0	0	0	1
61	0	0	0	0	0	0	0		0	0	0	0

Table14
Retention of human DNA probes in the Complete radiation hybrid panel.

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Hybrid	M4	JK53	105/153	C11P11	YN564	ECB27	12;75	YN548	12;46	LGH5.4	14 16	MS8
62					0							
63	0	0	0	0	0	0	0		0	0	0	0
64	0	0	0		0	0	0	0	0	0	0	0
65	0	0	0		0	0	0	0	0	0	0	0
66	1	0	1		0	0	0	0	0	0	0	1
67	0	0	0	0	0	0	0		0	0	0	0
68	1	0	0	0	0	0	0	0	0	0	0	0
69	0	0	0	0	0	0	0	0	0	0	0	
70												
71	0	0	0	0	0	0	0	.	0	0	0	1
72	0	1	0	0	0	0	0	1	0	1	1	1
73	0	0	0	0	0	0	0		0	0	0	0
74	0	0	0	0	0		0		0	0	0	0
75	0	0	0	0	0	0	0	0	0	0	0	0
76	1	0	0		0	0	0	0	0	0	0	1
77	0	0	0	0	0	0	0	0	0	0	0	0
78	0	0	0	0	1	1	0	0	0	0	0	0
79												
80												
81	0	0	0	0	0	0	0	0	0	0	0	0
82	0	0	0	0	0	0	0		0	0	0	0
83	0	0	0	0	0	0	0		1	0	0	0
84	0	0	0		0	0	0	0	0	0	0	0
85	0	1	1	0	0	0	0		0	0	0	0
86	0	0	0		0	0	0	0	0	0	1	0
87	0	0	0	0	0	1	1	0	0	0	0	1
88	0	1	0	0	0	0	0	0	1	0	0	
89	1	0	0	1	0	0	1		0	0	0	0
90	0	0	1	0	0	0	0	0	0	1	0	0
91												
92	0	0	0	0	0		0	0	0	0	0	0
93	0	0	0		0		0	0	0	0	0	0
94	0	0	0		0	0	0	0	0	0	0	0
95	0	0	0	0	0	0	0	0	0	0	0	0
96	0	0	0		0		0	0	0	0	0	0
97	0	0	0		0	0	0	0	0	0	0	0
98	0	0		0	0	0	0	0	0	0	0	0
99	1	1	1	0	0	0	0	1	1	1	1	0
100		0	0	0	0	0	0	0	0	0	0	0
101	0	0	0	0	0	0	0	0	0	0	0	0
102												
103												
104	0	1	1	1	0	0	0	0	0	0	0	0
105	0	0	0		0	0	0	0	0	0	0	0
#### 106	0	0	0		1	0	0	0	0	0	0	0
107	0	0	0		0	0	0	0	0	0	0	0
108												
109	0	0	0		0		0	0	0	0	0	0
110	0	0	0		0	0	0	1	0	0	0	0
111	0	0	1	0	0	0	0	0	0	0	0	0
112	0	0	0	0	0	0	0	0	0	0	0	0
113	0	0	1	0	0	0	0	0	0	0	0	0
114	0	1	0	0	0	0	0	0	0	0	1	0
115	0	0	0	0	0	0	0	1	0	0	0	0
116												
117	0	0	0		0		0	0	0	0	0	0
118	0	0	0		0	0	0	0	0	0	0	1
119												
120	0	0	0	0	0	0	0	0	0	0	0	0
121	0	0	1	0	0	0	0	0	0	0	0	0
122	0	0	0	0	0	0	0	0	0	0	0	0

Table14 (continued)

Retention of human DNA probes in the radiation hybrid panel.

Hybrid	M4	JK53	105/153	C11P11	YN564	ECB27	12;75	YN548	12;46	LGH5.4	14 16	MS8
123	0	0	0	1	0	0	0	0	0	0	0	0
124	0	0	0		1	0	0	0	0	0	0	1
125	0	0	0		1	1	1	0	0	1	0	1
126	0	1	1	0	0	0	0	0	0	0	0	0
127	0	0	0	0	0	0	0	0	0	0	0	0
128	1	0	0		0	0	0	0	0	0	0	0
129	0	0	1		0	0	0	0	0	0	0	0
130	0	1	0		0	1	1	1	0	1	1	1
131	0	0	1		0	0	0	1	0	0	1	0
132	1	1	0	0	1	0	0	0	0	0	0	0
133	0	0	0	0	0	0	0	0	0	0	0	0
134	0	0	0	1	0	0	0	0	0	0	0	0
135	0	1	1	1	0	0	0	0	0	0	0	0
136	0	0	0	0	0	0	0	0	0	0	1	0
137	0	0	0		0	1	0	1	1	1	1	0
138												
139	0	0	0	0	0	0	0	0	0	0	0	0
140	0	0	0	0	0	0	0	0	0	0	0	0
141	0	0	0	0	0	0	0	0	0	0	0	0
142												
143	0	0	0	0	0	0	0	0	0	0	0	0
144	0	0	0	0	0	0	0	0	0	0	0	0
145	0	0	0	0	0		0	0	0	0	0	0
146	0	0	0	0	0	0	1	0	0	1	1	1
147												
148												
149	0	0	0	0	0	0	0	0	0	0	0	1
150	0	0	0		0	0	0	0	0	0	0	0
151	0	0	0	0	0	0	0	0	0	0	0	0
152	0	0	0	0	0	0	0	0	0	0	0	0
153	0	0	0	0	0	0	0	0	0	0	0	0
154	0	0	0	0	0	0	0	0	0	0	0	0
155	0	0	0	0	0	0	0	0	0	0	0	0
156	0	0	0		0		0	0	0	0	0	0
157	0	0	0	0	0	0	0	0	0	0	0	
158	0	0	0	0	0	0	0	1	0	0	1	0
159	1	0	0	0	0	0	0	0	0	1	0	
160	0	0	0	0	0	0	0	0	0	0	0	0
161												
162	0	1	1		0	1	0	0	0	0	0	0
163	0	0	0		0		0	0	0	1	1	0
164	0	0	0	0	0	0	0	0	0	0	0	0
165	0	0	0	0	0	0	0	0	0	0	0	0
166	0	0	0	0	0	0	0	0	0	0	0	1
167	1	0	0	0	0	0	0	0	0	0	0	0
168	0	0	0	0	0	0	0	0	0	0	0	0
169												
170												
171												
172	0	0	0	0	0	0	1	0	0	0	0	0
173												
Y	0	0	0		0		0	0	0	0	0	0
Z	0	0	0	1	1	0	0	0	0	0	1	1
Hybrid	M4	JK53	105/153	C11P11	YN564	ECB27	12;75	YN548	12;46	LGH5.4	14 16	MS8

Table14   (continued)

Retention of human DNA probes in the radiation hybrid panel.

Statistical Analysis of Radiation Hybrid Data

Statistical analysis of the radiation hybrid mapping data was kindly performed by Dr D.T. Bishop (ICRF, Leeds) according to the protocol described below.

Retention of DNA Markers

The proportional retention of each DNA marker was calculated as was the mean proportional retention of all the markers. The proportional retention of each of the different DNA markers was compared and a χ^2 test performed to see whether the observed frequencies were consistent with random variation or whether some probes were incorporated significantly more, or less, frequently.

Expected Frequencies of Marker Pairs

If the retention of all the DNA markers in the radiation panel was consistent with random variation the mean proportion retained (p) was used to calculate the expected proportion of hybrids to retain both, one or neither marker under the assumption of independent retention. The formula used is the same as that for genotype proportions in a population as defined by the Hardy-Weinberg Equilibrium. The proportions of hybrids with both markers, either marker or neither marker were calculated as below:

Both Markers	One Marker	Neither Marker
(++)	(+-/-+)	(--)
p^2	$2p(1-p)$	$(1-p)^2$

If the retention of DNA markers in the hybrids had not been random the expected proportions would have been calculated from the proportional retention of each DNA marker in the pair.

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Segregation of Pairs of DNA markers.

The segregation of pairs of DNA markers within the radiation hybrid panel was analysed by the χ^2 test to see if any two markers segregated together more frequently than predicted by comparing the observed patterns of cosegregation with those predicted under independent segregation. The significance of any association between two markers was calculated from the χ^2 tables for two degrees of freedom.

Two Point Linkage Analysis

The breakage frequency (θ) between paired markers in the radiation panel was calculated by likelihood methods using the following equations (D.T.Bishop, personal communication):

Proportion both markers retained:

$$P[++] = p(1-\theta) + p^2\theta$$

Proportion one marker retained:

$$P[+-] = \theta p(1- p)$$

Proportion one marker retained:

$$P[-+] = \theta p(1- p)$$

Proportion neither marker retained:

$$P[--] = (1- p)(1- \theta) + (1- p)^2\theta$$

These equations were solved using the maximum likelihood method to estimate the breakage frequency (θ) between pairs of probes in the radiation panel.

The likelihood (probability) for the breakage frequency (θ) given the observed data was then calculated L(θ). The likelihood of no linkage given the observed data was also calculated.

The odds ratio was calculated by dividing the likelihood of the estimated breakage frequency (θ) by the likelihood of the breakage frequency for no linkage ($\theta=1$). The decimal logarithm of this odds

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ratio was used to express the log of odds (LOD) for linkage at breakage frequency θ.

$$\text{LOD} = \log_{10} \frac{\text{Probability at } \theta}{\text{Probability at } \theta = 1}$$

Probe Order on Chromosome 5

A computer programme was written by Dr D.T. Bishop to attempt to place the DNA markers in order. The programme calculated the minimum number of obligatory breaks in the retained human chromosomal fragments of the radiation hybrids for every possible probe order. The results were tabulated as the probe order for the lowest number of breaks and the orders compatible with successive additional breaks.

Retention of DNA Markers

The mean retention of DNA markers in the radiation panel was calculated and the proportional retention of each of the different DNA markers was compared and a χ^2 test performed (see table 15).

Table 15 Retention of human DNA markers

Probe	Positive hybrids	Total hybrids	Proportion Positive
C11p11	11	113	0.10
YN564	14	153	0.09
ECB27	14	138	0.10
12;75	10	153	0.07
12;46	7	153	0.05
YN548	12	142	0.08
14 16	18	152	0.12
LGH5.4	15	152	0.10

$\chi^2 = 7.39154$ (7 degrees of freedom)
Significance level = 0.39
Mean frequency = 0.09

The χ^2 was 7.39 which for 7 degrees of freedom gives a significance level of only 0.39. Thus, the observed variation in the retention frequency was consistent with random variation.

Segregation of pairs of DNA markers and two point linkage analysis

The retention of pairs of markers within the panel was compared to see if markers co-segregated more frequently than expected. The data was analysed by the χ^2 test. The breakage frequency (θ) between paired markers in the panel was also calculated and the LOD score calculated for the most likely estimate of breakage frequency (Table 16).

Seventeen of the twenty-eight marker pairs analysed showed a significant ($p<0.05$) association in their retention in the radiation hybrid panel. This high proportion of markers showing co-segregation is not unexpected as only markers from the APC region were analysed. The breakage frequency of the marker pairs varied between 0.46 and 0.93 and in only a few pairs was the LOD score greater than 3.

The most significant association was shown by ECB27 and 12:75 which were both retained together in 7/17 (41%) hybrids. This is a considerably higher proportion than expected by chance given the mean retention frequency of probes of 0.09. The χ^2 was calculated as 40.4 which, for two degrees of freedom gives a significance of 0.00. (highly significant) The breakage frequency between ECB27 and 12:75 was calculated as 0.46 with a LOD of 5.03.

The variation in breakage frequency between pairs of markers of between 0.46 and 0.93 and the low values of most of the LOD scores does not provide sufficient information to determine the order of the probes.

Probe Order on Chromosome 5.

To try to order the DNA markers in the APC gene region the number of breaks associated with every possible order of probes

Probe 1	Probe 2	++	+-	-+	--	Total	Both Included	Chi Sq. 2df	Sig	Theta	90% CI		LOD
YN564	C11P11	4	4	7	98	113	0.27	14.3	0.00	0.62	0.34	0.92	2.02
ECB27	C11P11	2	8	9	87	106	0.11	1.8	0.41	0.90	0.62	1.00	0.20
ECB27	YN564	6	8	8	116	138	0.27	24.9	0.00	0.65	0.41	0.89	2.68
12;75	C11P11	3	5	8	97	113	0.19	6.8	0.03	0.74	0.44	1.00	1.05
12;75	YN564	4	6	10	133	153	0.20	10.0	0.01	0.70	0.44	0.95	1.62
12;75	ECB27	7	3	7	121	138	0.41	40.4	0.00	0.46	0.22	0.74	5.03
12;46	C11P11	2	4	9	98	113	0.13	3.1	0.22	0.81	0.47	1.00	0.53
12;46	YN564	1	6	13	133	153	0.05	1.5	0.48	0.95	0.65	1.00	0.04
12;46	ECB27	2	5	12	119	138	0.11	2.1	0.34	0.86	0.56	1.00	0.36
12;46	12;75	1	6	9	137	153	0.06	4.4	0.11	0.87	0.52	1.00	0.20
YN548	C11P11	3	5	7	87	102	0.20	7.5	0.02	0.74	0.42	1.00	1.07
YN548	YN564	3	9	11	119	142	0.13	3.7	0.16	0.85	0.58	1.00	0.55
YN548	ECB27	5	7	9	107	128	0.24	17.5	0.00	0.70	0.44	0.94	1.92
YN548	12;75	4	8	5	125	142	0.24	12.3	0.00	0.64	0.36	0.92	2.03
YN548	12;46	3	9	3	127	142	0.20	9.0	0.01	0.65	0.35	0.95	1.58
14 16	C11P11	2	10	9	91	112	0.10	1.7	0.44	0.93	0.65	1.00	0.14
14 16	YN564	4	14	10	124	152	0.14	7.0	0.03	0.86	0.62	1.00	0.71
14 16	ECB27	6	11	8	113	138	0.24	23.7	0.00	0.72	0.47	0.94	2.15
14 16	12;75	3	15	7	127	152	0.12	3.1	0.21	0.87	0.62	1.00	0.47
14 16	12;46	2	16	5	129	152	0.09	1.1	0.58	0.91	0.64	1.00	0.20
14 16	YN548	7	10	5	119	141	0.32	35.1	0.00	0.60	0.35	0.85	3.61
LGH5.4	C11P11	2	7	9	94	112	0.11	1.7	0.42	0.88	0.57	1.00	0.28
LGH5.4	YN564	3	12	11	126	152	0.12	3.0	0.23	0.88	0.63	1.00	0.41
LGH5.4	ECB27	5	9	9	115	138	0.22	15.5	0.00	0.74	0.47	0.95	1.73
LGH5.4	12;75	4	11	6	131	152	0.19	9.3	0.01	0.73	0.46	0.97	1.46
LGH5.4	12;46	3	12	4	133	152	0.16	6.0	0.05	0.75	0.47	1.00	1.04
LGH5.4	YN548	5	10	7	119	141	0.23	15.7	0.00	0.71	0.45	0.94	1.91
LGH5.4	14 16	8	7	10	127	152	0.32	42.5	0.00	0.61	0.38	0.83	4.02

Table 16

Pairwise analysis of the retention of DNA in the radiation panel.

Number
of Breaks   Probe order

Number of Breaks								
97	C11P11	YN564	ECB27	12;75	YN548	12;46	LGH5.4	14 16
98	C11P11	YN564	ECB27	12;75	12;46	YN548	14 16	LGH5.4
99	C11P11	YN564	12;75	ECB27	YN548	12;46	LGH5.4	14 16
99	C11P11	YN564	ECB27	12;75	12;46	YN548	LGH5.4	14 16
99	C11P11	YN564	ECB27	12;75	LGH5.4	12;46	YN548	14 16
100	14 16	LGH5.4	12;46	C11P11	YN548	12;75	ECB27	YN564
100	C11P11	YN564	12;75	ECB27	12;46	YN548	14 16	LGH5.4
100	C11P11	YN564	ECB27	12;75	12;46	LGH5.4	14 16	YN548
100	C11P11	YN564	ECB27	12;75	LGH5.4	14 16	YN548	12;46
100	C11P11	YN564	ECB27	12;75	YN548	14 16	LGH5.4	12;46
101	C11P11	YN564	12;75	ECB27	12;46	YN548	LGH5.4	14 16
101	C11P11	YN564	12;75	ECB27	LGH5.4	12;46	YN548	14 16
101	C11P11	YN564	ECB27	12;75	12;46	LGH5.4	YN548	14 16
101	LGH5.4	14 16	YN548	12;46	C11P11	12;75	ECB27	YN564
102	14 16	LGH5.4	12;46	YN548	C11P11	12;75	ECB27	YN564
102	14 16	LGH5.4	12;46	YN548	ECB27	12;75	C11P11	YN564
102	14 16	LGH5.4	YN548	12;46	C11P11	12;75	ECB27	YN564
102	C11P11	12;46	LGH5.4	14 16	YN548	12;75	ECB27	YN564
102	C11P11	12;46	YN548	14 16	LGH5.4	12;75	ECB27	YN564
102	C11P11	YN564	12;75	ECB27	12;46	LGH5.4	14 16	YN548
102	C11P11	YN564	12;75	ECB27	14 16	LGH5.4	12;46	YN548
102	C11P11	YN564	12;75	ECB27	14 16	YN548	12;46	LGH5.4
102	C11P11	YN564	12;75	ECB27	LGH5.4	14 16	YN548	12;46
102	C11P11	YN564	12;75	ECB27	YN548	14 16	LGH5.4	12;46
102	C11P11	YN564	ECB27	12;75	YN548	12;46	14 16	LGH5.4
103	14 16	LGH5.4	12;46	C11P11	YN564	ECB27	12;75	YN548
103	14 16	YN548	12;46	C11P11	YN564	ECB27	12;75	LGH5.4
103	C11P11	LGH5.4	14 16	YN548	12;46	12;75	ECB27	YN564
103	C11P11	YN548	12;75	ECB27	YN564	12;46	LGH5.4	14 16
103	C11P11	YN548	14 16	LGH5.4	12;46	12;75	ECB27	YN564
103	C11P11	YN564	12;46	LGH5.4	14 16	YN548	12;75	ECB27
103	C11P11	YN564	12;46	YN548	12;75	ECB27	LGH5.4	14 16
103	C11P11	YN564	12;46	YN548	14 16	LGH5.4	12;75	ECB27
103	C11P11	YN564	12;46	YN548	ECB27	12;75	LGH5.4	14 16
103	C11P11	YN564	12;75	ECB27	12;46	LGH5.4	YN548	14 16
103	LGH5.4	12;46	C11P11	YN564	ECB27	12;75	YN548	14 16
103	LGH5.4	14 16	YN548	12;46	C11P11	YN564	12;75	ECB27
103	LGH5.4	14 16	YN548	12;46	C11P11	YN564	ECB27	12;75
103	LGH5.4	14 16	YN548	12;46	ECB27	12;75	C11P11	YN564
103	LGH5.4	14 16	YN548	C11P11	12;46	12;75	ECB27	YN564
103	YN548	12;46	C11P11	YN564	ECB27	12;75	LGH5.4	14 16
103	YN548	14 16	LGH5.4	12;46	C11P11	12;75	ECB27	YN564

Table 17

Probe orders on chromosome 5.

was calculated. The possible orders were ranked from that requiring the least number of breaks progressively upwards (see Table 17).

The probe order with the lowest number of breaks is consistent with recombination data from family linkage studies and from physical mapping data (Nakamura et al., 1988; Varesco et al., 1989; Tops et al., 1989; Ward, personal communication). However, five additional breaks (an increase of 5%) would result in a further 24 different possible probe orders.

Discussion

The high radiation dose of 50,000 Rads used to fragment the donor chromosomes in the construction of these radiation hybrids appears to have resulted in the production of small chromosomal fragments as anticipated.

The mean retention of DNA markers in 9% of hybrids suggests that each hybrid retains on average approximately 16 Mb of human chromosome 5 DNA (9% of 180Mb). Pulsed field mapping using DNA from the radiation hybrids has suggested that the average fragment size is less than 1Mb. DNA blocks of the parental hybrid PN/TS-1 and five radiation hybrids were digested with either *Eag* I or *Bss*H1, separated by pulsed-field electrophoresis, blotted and probed with YN5.48. In PN/TS-1 a band of approximately 1Mb was detected in digests with both enzymes, whereas in all the radiation hybrids there was alteration of the size of the band detected (Searle, personal communication). From these figures it can be calculated that, on average, each hybrid retains 20 fragments of human chromosome 5.

The retention of multiple small fragments of chromosome 5 in the majority of the radiation hybrids helps to explains the results of mapping probes from the radiation hybrid lambda libraries. The libraries were constructed from the DNA of radiation hybrids retaining a marker in the *APC* gene region. However, the presence of up to 20 other fragments of human chromosome 5, not

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necessarily in the *APC* gene region, would result in a low proportion of probes in a library mapping into the MD/TS-1 and PD/TS-1 deletions.

The observed retention of human DNA markers in the panel was consistent with random variation. These results are in contrast to those of Cox et al. (1990) who found a wide variation in retention of markers in their radiation panel. This panel was constructed using a lower radiation dose (6,000 Rads) and hence contained larger fragments of human DNA. It appears in their panel that markers near the centromere are retained more frequently. This may be due to the centromere itself being selectively retained along with other DNA markers in continuity on the chromosomal fragment (Bishop, personal communication).

There is insufficient information available in the panel for two point linkage analysis to give breakage frequencies with significant LOD scores. It is therefore not possible to construct a "linkage" map of the probes in the panel.

The order of probes produced by minimising the number of breaks in the hybrid panel is consistent with family linkage studies and physical mapping. However, this is not a powerful way to determine the order of DNA markers as one or two additional breaks are consistent with multiple further probe orders.

CHAPTER SEVEN

CHROMOSOME 5 ALLELE LOSS IN COLORECTAL NEOPLASIA

Introduction

The experiments described in this chapter were performed to see how frequent genetic alterations on chromosome 5 were in common colorectal neoplasia. Knudson (1985) proposed that the same genes that predispose to inherited tumours in an autosomal dominant manner may also be involved in the aetiology of sporadic (non-familial) cancer. DeMars (1969) had previously proposed that autosomal dominant predispositions to cancer may result from the inheritance of an autosomal recessive allele and that a mutation of the normal allele led to tumour development.

The presence of tumour suppressor genes may be demonstrated by the loss of genetic material when pairs of normal and tumour tissue are compared. Originally this could only be demonstated by looking for cytogenetic abnormalities in tumours and by the loss of polymorphic protein alleles (Benedict et al., 1983; Sparkes et al., 1983). More recently it has been possible to look for the loss of restriction length fragment polymorphism alleles in tumour DNA when compared to the DNA from the patient's normal tissue (Cavenee et al., 1983).

The experiments described are an extension of those of Solomon et al. (1987) in which they compared normal and tumour DNA from patients with common colorectal cancer and found loss of heterozygosity for markers on chromosome 5 in between 20% and 40% of colorectal carcinomas. These results suggested that alterations of the *APC* gene are involved in the aetiology of common colorectal cancer. However, they found no evidence of allele loss in FAP adenomas.

The majority of colorectal carcinomas are believed to develop from pre-existing colorectal adenomas (Muto et al., 1975). To determine whether alterations on chromosome 5 are an early event in colorectal neoplasia thirty common adenomas were also investigated for loss of heterozygosity on chromosome 5.

Chromosome 5 allele loss in common colorectal carcinomas

The panel of colorectal carcinomas used by Solomon et al. (1987) was expanded to a 100 tumour: normal pairs. The paired tumour and normal DNA samples were digested with enzymes appropriate for additional polymorphic DNA markers on chromosome 5. The digested DNA was size-separated by agarose gel electrophoresis and then transferred to a nylon filter. The filter was hybridised to ^{32}P-labelled probes.

Table 18 Polymorphic probes

Probe	Enzyme	Reference
C11p11	*Taq* I	Bodmer et al., 1987
π 227	*Pst* I/ *Bcl* I	Stewart et al., 1987
L1.4	*Eco* RI	Pearson, 1985
MS8	*Hinf* I	Wong et al., 1987

The signal from the normal DNA of each pair was examined to see if the patient was heterozygous for the DNA probe. If the normal tissue was heterozygous the ratio of the intensity of the two alleles from the normal DNA was compared to the ratio of the tumour alleles. The loss of an allele in the tumour, which was present in the normal DNA, was noted. Contamination of the tumour with normal tissue may lead to apparent incomplete loss of heterozygosity. Any tumour that showed an altered ratio of allele intensity from the normal tissue was scanned by a laser densitometer. The ratio of the two tumour alleles divided by the ratio of the two normal allele $(T_1/T_2: N_1/N_2)$ was calculated.

Solomon et al. (1987) had previously shown a bimodal distribution in the ratio $T_1/T_2: N_1/N_2$. A ratio of less than 0.6 was associated with allele loss in the tumour and contamination with normal tissue and a ratio of between 0.6 and 1 was taken as showing no evidence

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Table 19 Chromosome 5 allele loss in common colorectal carcinomas

Patient No	L1.4		π227		C11p11		MS8	
	N	T	N	T	N	T	N	T
10							2	1
12	2	1	2	2				
14							2	1
17	2	1	2	1			2	1
21	2	2					2	1
22							2	1
26			2	1				
27			2	2	2	1	2	1
29			2	1			2	1
30	2	1	2	1			2	1
32	2	2					2	1
39			2	2			2	1
40	2	1			2	2	2	2
41			2	1				
48	2	1	2	1			2	1
50					2	1	2	1
51			2	1			2	1
53	2	2					2	1
58			2	1				
62	2	1	2	1			2	1
69			2	1	2	1	2	1
70			2	2			2	1
72	2	2					2	1
75	2	2	2	1				
76	2	2					2	1
82							2	1
84			2	1	2	1	2	2
90			2	1				
91			2	1			2	1
93			2	1				
94							2	1
98	2	2			2	1		

Loss of Individual Markers

15.3–
15.1–
14–
13–
11–
11.2–
12–
13–
14–
15–
21–
22–
23–
31–
32–
33–
34–
35–

APC

L1.4   19%

π 227   33%
C11p11   28%
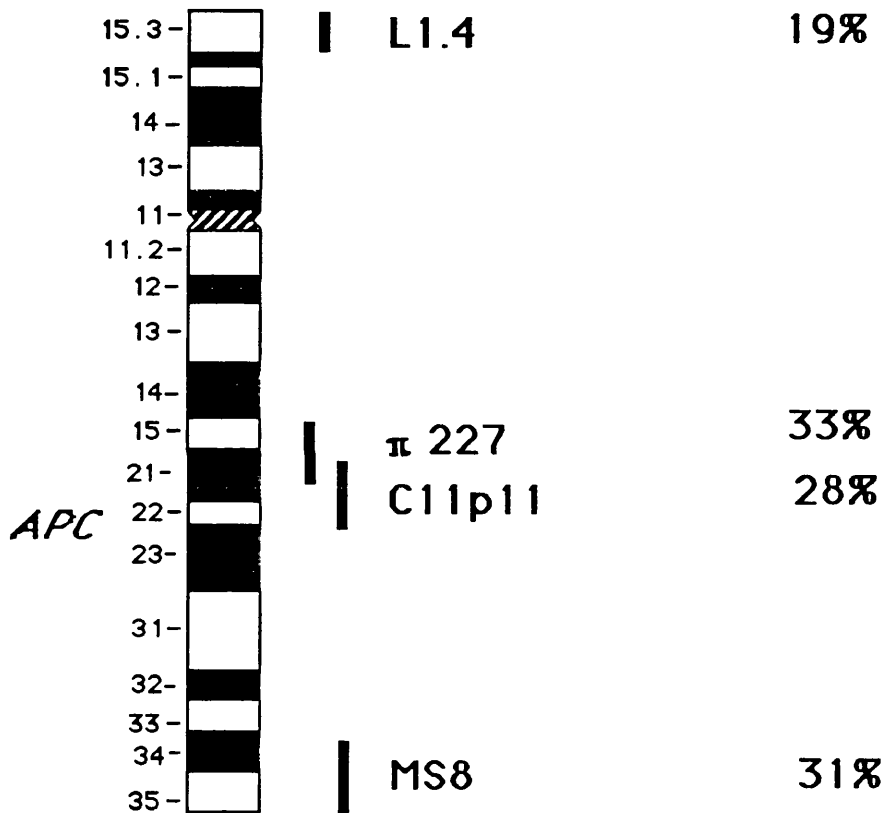
MS8   31%

<u>Figure 19</u>

Ideogram of chromosome 5 with the position of the probes and the percentage loss of constitutional heterozygosity in common colorectal cancer.

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of allele loss. In the present study ratios of less than 0.6 were also taken to indicate allele loss. The specificity of these changes to chromosome 5 was demonstrated by Solomon et al (1987) by showing that allele loss was rarely detected in colorectal carcinomas by polymorphic DNA markers on six other chromosomes. However, allele loss on chromosome 17 was not detected in these controls.

Results

Table 19 includes the colorectal carcinomas from the panel in which loss of constitutional heterozygosity was detected. The percentage loss of heterozygosity of individual polymorphic markers on chromosome 5 is presented in Figure 19.

Discussion

Allele loss of one or more polymorphic DNA marker on chromosome 5 was detected in 32/91 (35%) of common colorectal carcinomas.

The results of individual tumours in Table 19 demonstrate the various genetic mechanisms involved in allele loss, namely, whole chromosome loss, mitotic recombination, large chromosomal deletion and interstitial deletions. Whole chromosome loss was demonstrated in tumour 17 in which loss of heterozygosity was seen in markers both on the long arm and on the short arm of chromosome 5. Either a large deletion or a mitotic recombination would be consistent with the loss of heterozygosity seen in tumour 27 in which the two DNA markers distal to the *APC* gene showed loss of heterozygosity but no loss of heterozygosity was seen in a marker proximal to *APC* on the long arm of chromosome 5. Finally, in tumour 84 the results are consistent with an interstitial deletion, distal markers on the long arm of chromosome 5 remain heterozygous but a proximal long arm marker shows loss of heterozygosity.

Two tumours (12 and 40) show allele loss with L 1.4 , a marker on the short arm of chromosome 5, but no loss of heterozygosity of markers proximal to *APC* on the long arm of chromosome 5. These

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results are inconsistent with a simple chromosomal mechanism leading to loss of the *APC* gene and probably represents the background level of genetic loss in colorectal carcinomas on all chromosomes of approximately 10% (Vogelstein et al., 1989).

Allele loss was detected in a higher proportion of tumours with polymorphic markers mapped to the long arm of chromosome 5 (30%) than with a polymorphic marker on the short arm of chromosome 5 (20%). This supports the mapping of the *APC* locus to the long arm of chromosome 5.

There was little variation in percentage of allele loss seen with MS8, a probe mapped to the telomere of chromosome 5, and the two markers, π227 and C11p11, which show linkage to the *APC* gene. The proportion of tumours showing allele loss might have been expected to increase with probes closer to the *APC* gene as interstitial deletions around the *APC* gene would also be detected. The failure to detect any increase in allele loss in the markers linked to *APC* may be due to the smaller numbers of informative tumours.

Chromosome 5 allele loss in Sporadic adenomas

Thirty non-FAP adenomas were investigated for allele loss on chromosome 5. The adenoma samples were small and thus limited amounts of DNA were available. The DNA was digested with *Hinf* I and *Bcl* I and hybridised to the polymorphic DNA probes MS8 and π227. The results are presented in Figures 20 and 21 and Table 20.

Bcl I Digests
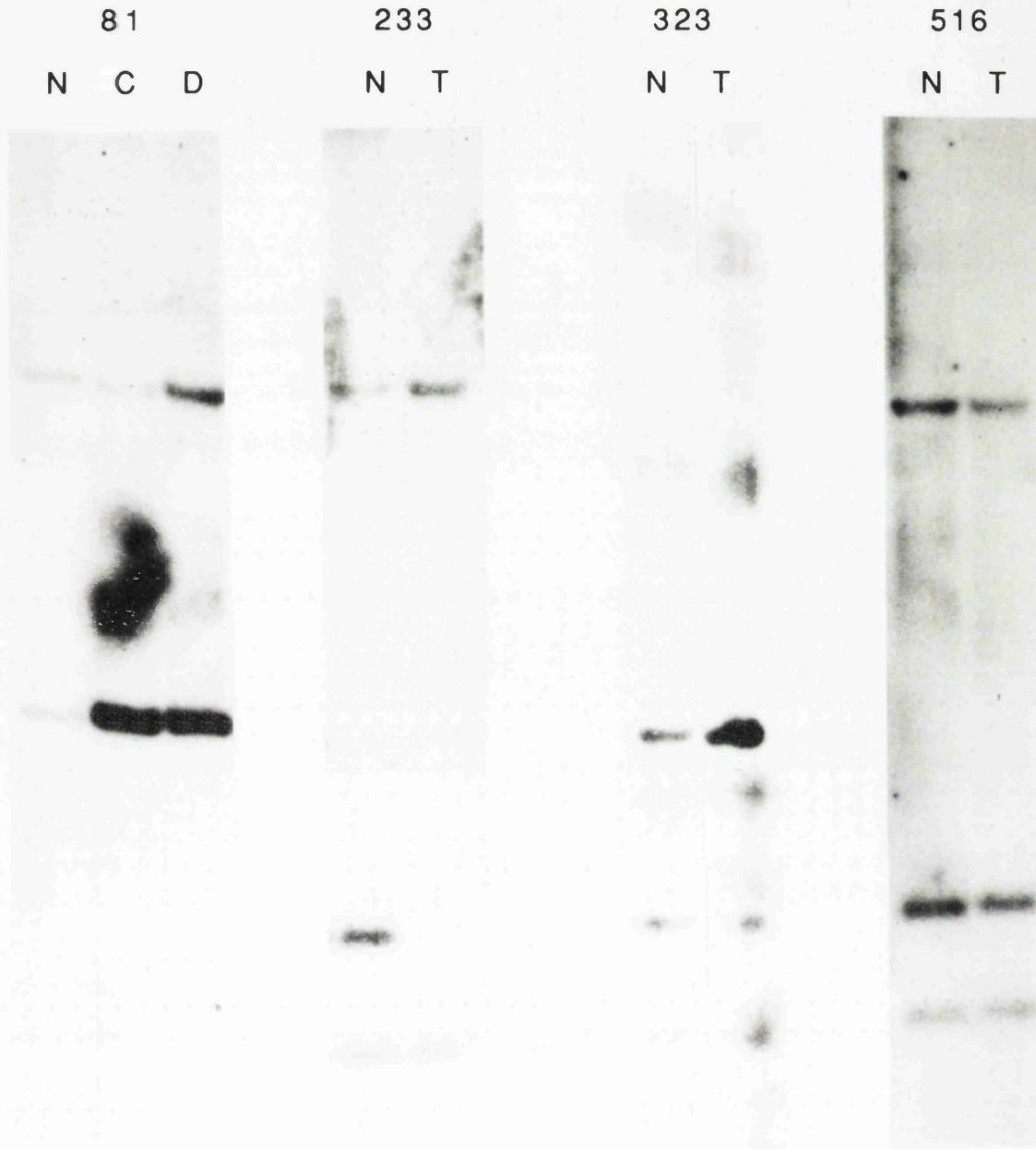
Figure 20
Autoradiograph of normal and adenoma DNA digested with *Bcl* I
and probed with π 227. Loss of alleles is demonstrated in the
adenomas. Unequal DNA loading gives the appearance of the
amplification of some adenoma alleles.

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N = normal
T, C and D = adenoma

Hinf I Digests

233 562
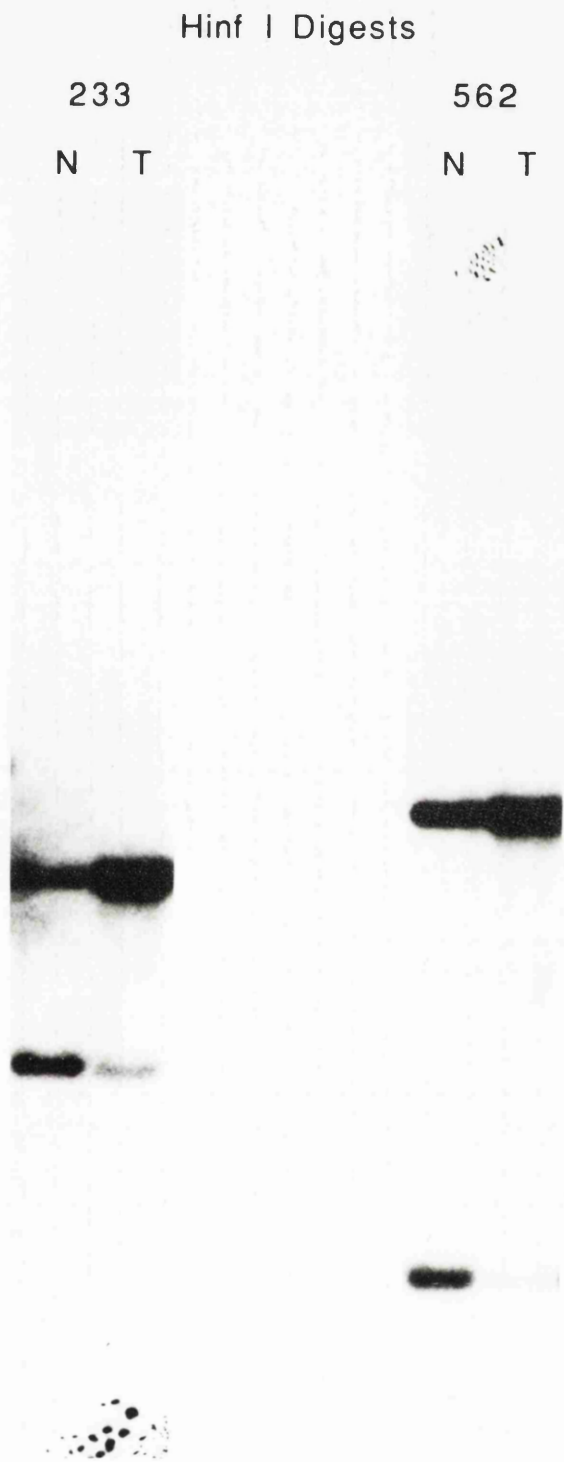
N T   N T

Figure 21
Autoradiograph of normal and adenoma DNA digested with *Hinf* I
and probed with MS8. Loss of alleles is demonstrated in the
adenomas.

Table 20 Chromosome 5 allele loss in common colorectal adenomas

Patient No		PROBE π227 N	T	MS8 N	T
1	1			2	2
2	17C	2	2		
3	17E	2	2		
4	24	2	2	2	2
5	27	2	2		
6	81C	2	1		
7	81D	2	1		
8	128			2	2
9	214	2	2	2	2
10	225	2	2	2	2
11	226	2	2	2	2
12	233	2	1	2	1
13	292	2	2	2	2
14	323	2	1		
15	334	2	2		
16	344	2	2		
17	516	2	1		
18	525	2	2		
19	540	2	2		
20	550/1	2	2		
21	550/2	2	2		
22	550/3	2	2		
23	562AD			2	1
24	562P			2	2

Results

Allele loss of either one or both probes was seen in six of twenty-four adenomas (25%). As a control MS 31, a minisatellite probe on chromosome 7 (Wong et al., 1987), was also hybridised to the *Hinf* I filters. Allele loss was only seen in one of nine informative adenomas (11%).

Discussion

Genetic alterations on chromosome 5 were demonstrated in 25% of common colorectal adenomas and appear to be a relatively specific change and not due to random loss as in a small control series allele loss on chromosome 7 was only seen in 11% of adenomas. It was not possible to perform further controls as no further DNA was available from the adenomas.

Loss of heterozygosity on chromosome 5 appears to be a relatively common early event in colorectal neoplasia. This suggests that mutations of the *APC* gene are involved in the development of non-FAP adenomas. In contrast chromosome 5 allele loss has only rarely been detected in adenomas from patients with FAP (Solomon et al., 1987; Vogelstein et al., 1988; Rees et al., 1989).

In a recessive model of tumour suppressor gene function (see Introduction Chapter Figure 2) loss of heterozygosity represents the second 'hit', the first 'hit' usually being a localised mutation inactivating one allele. This model has been shown to be too simplistic in the case of p53 in which heterozygous mutations have been shown to alter tumour growth, possibly by a 'dominant negative' mechanism (see Discussion Chapter). Thus, mutations of one *APC* allele may be sufficient to lead to a hyperproliferative colonic mucosal epithelium.

There are two possible explanations as to why chromosome 5 allele loss is seen in sporadic adenomas but not in FAP adenomas. In general FAP adenomas are extremely small whereas sporadic

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polyps are usually only detected when relatively large. Allele loss on chromosome 5 has been shown to become more frequent with increasing size of sporadic adenomas (Vogelstein et al., 1988). Alternatively, in FAP patients the constitutive presence of the FAP mutation in all colonic tissue may abrogate the need for chromosome 5 allele loss in adenomas (Paraskevas and Williams, 1990).

CHAPTER EIGHT

DISCUSSION

The construction of a panel of somatic cell hybrids retaining human chromosome 5 proved a successful method of rapidly mapping probes into the *APC* region. The publication of linkage between FAP and probes on chromosome 5 and the case report of an individual with FAP and a constitutional interstitial deletion of chromosome 5 led to the description of several further FAP patients with interstitial chromosome 5 deletions. Cell lines from these patients were used in the construction of somatic cell hybrids.

A panel of hybrids in which one hybrid retained a normal human chromosome 5 with no other detectable human material (PN/TS-1) and two hybrids retained human chromosome 5s with deletions in the *APC* region in the absence of the normal homologue of chromosome 5 (PD/TS-1 and MD/TS-1) allowed the mapping of DNA probes to the *APC* region of chromosome 5. DNA markers isolated by Dr Lilly Varesco (Molecular Analysis of Mammalian Mutation Laboratory, ICRF; presently at I.S.T., Genova, Italy) from an end clone library demonstrated that the PD and MD deletions were overlapping. ECB27 and ECB220 were mapped to both deletions whereas ECB 134 was only present in the MD deletion indicating that this deletion was, in one direction at least, larger than the PD deletion.

Further somatic cell hybrids were constructed from an individual with FAP and intellectual handicap but no visible karyotypic abnormality. The phenotype suggests that a sub-microscopic deletion may be present which would be of great value for mapping. At the time of writing somatic cell hybrids are being made from an individual (PS) with FAP and intellectual handicap and a more distal deletion on chromosome 5.

In 'reverse genetic' strategies, which aim to clone a gene based on its chromosomal location rather than its function, the importance of chromosomal rearrangements which involve the gene, such as interstitial deletions and reciprocal translocations, is emphasized by

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the recent cloning of the Wilms tumour and von Recklinghausen neurofibromatosis (NF1) genes.

In Wilms tumour a panel of somatic cell hybrids retaining human chromosome 11s with deletions in the Wilms region and a tumour with a homozygous deletion in the region greatly aided the identification of the gene (Rose et al., 1990; Call et al., 1990; Gessler et al., 1990). In NF 1 somatic cell hybrids from two patients with translocations of chromosome 17 allowed probes to be mapped to the region between them and led in the cloning of the NF 1 gene (Wallace et al., 1990; Viskochil et al., 1990; Cawthon et al., 1990).

Radiation Hybrids

Radiation hybrids were constructed with the intention of isolating random fragments of human chromosome 5 in a panel of human-rodent hybrids. The panel was screened by southern hybridisation with probes from the *APC* region to detect which radiation hybrids retained human DNA from the region.

The presence of DNA homologous to the markers in the radiation hybrid panel was analysed and shown to be consistent with random retention. Analysis of the segregation of pairs of DNA probes in the radiation panel showed that 17/28 pairs from the *APC* region showed a significant association in their retention. However, in linkage analysis only three pairs gave a significant LOD score (i.e. greater than three). Thus insufficient information is present to construct a linkage map of the the region from the radiation hybrid data. The probe order derived from minimising the number of chromosomal breaks in the panel was consistent with pedigree linkage analysis and physical mapping data. However, this method was not a powerful way of determining order as a few extra breaks were consistent with multiple further probe orders.

The radiation hybrids clones proved a disappointing source of DNA for isolating probes from the *APC* region. The dosage of radiation used (50,000 rad) was chosen to produce small chromosomal

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fragments. However, it would appear to have resulted in the retention of multiple small chromosomal fragments. Hence the presence of DNA from the *APC* region within a hybrid clone resulted in little enhancement of the overall proportion of human DNA within the hybrid from the region. This interpretation is consistent with the findings of Goodfellow et al (1990) who also used a high radiation dosage to construct hybrids.

Single cell cloning of four radiation hybrids which retained human DNA in the *APC* region demonstrated the segregation of human genetic material in the single cell clones. There was loss of Alu-Alu amplification products in some of the single cell clones that had been present in the original radiation hybrid. In some single cell clones there might therefore be an enhanced proportion of human DNA from the *APC* region.

Cox et al. (1990) constructed a panel of radiation hybrids retaining fragments of human chromosome 21 and used it to produce a high resolution map of chromosome 21. The donor human-hamster hybrid, which only retained human chromosome 21, was irradiated with 8000 rad. After the fusion 102 hybrid colonies were picked and screened by southern hybridisation with 14 DNA probes spanning a region of 20Mb on chromosome 21. The DNA markers were retained in between 41% and 59% of hybrids, a higher proportion than our radiation hybrids, and did not appear to be randomly retained, centromeric markers being retained more frequently than telomeric markers.

Two point linkage analysis was performed between pairs of DNA markers and the breakage frequency and LOD score calculated. Many pairs of markers gave a significant LOD score and from the breakage frequency it was possible to construct a linkage map of the region. Pulsed-field mapping of the same markers was entirely consistent with the radiation hybrid mapping data.

Radiation hybrids constructed using a lower radiation dosages (6,000rad-8000rad) have a higher retention frequency of human DNA markers and appear to be considerably more informative

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when used for mapping probes than those made using higher radiation dosages (Cox et al., 1990; Burmeister et al., 1991). Radiation hybrids produced using a lower radiation dosage retain fewer human chromosomal fragments and enhance the isolation of DNA probes from the region of interest (Cox et al., 1989; Pritchard et al., 1989).

Chromosome 5 allele loss in colorectal neoplasia

Chromosome 5 allele loss was detected in 25% of common colorectal adenomas and in 35% of common colorectal carcinomas. These results suggest that somatic mutations of the *APC* gene are an early event in colorectal neoplasia. Allele loss has been found in up to 60% of colorectal carcinomas when polymorphic DNA markers more closely linked to the *APC* locus are used (Ashton-Rickardt et al., 1989).

In contrast, studies of FAP adenomas have rarely shown chromosome 5 allele loss (Solomon et al. 1987; Vogelstein et al., 1988; Rees et al., 1989; Sasaki et al, 1989). A recessive model of action for the *APC* gene product would predict allele loss in FAP adenomas. In this model clonal expansion would occur when a localised mutation inactivates the first allele and the second allele is lost by whole chromosome loss, mitotic recombination or a more localised mutation. The generalised abnormalities of cellular proliferation in the FAP colonic epithelium and the absence of allele loss in FAP adenomas suggest that hemizygosity at the *APC* locus may be sufficient to cause these changes. It has been proposed that in FAP patients the constitutive presence of hemizygosity at the *APC* locus may permit colonic epithelial proliferation in the absence of inactivation of the remaining normal *APC* allele (Paraskevas and Williams, 1990).

Loss of heterozygosity on chromosome 5 is found in the same proportion of FAP carcinomas as non-FAP carcinomas (Sasaki et al., 1989; Okamoto et al., 1990; Miyaki et al., 1990). Okamoto et al. (1990) have demonstrated that in FAP carcinomas and desmoid tumours the chromosome 5 from the normal parent is lost and the

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chromosome 5 from the FAP parent retained in the tumour. This is consistent with a recessive model of *APC* gene function in the formation of carcinomas even if in the formation of FAP adenomas hemizygosity of the *APC* gene may be sufficient. It also indicates that the same genetic events are involved in the development of FAP and non-FAP tumours.

Tanaka et al. (1991) demonstrated that the introduction of a normal chromosome 5 into a human colorectal carcinoma cell line suppressed tumourigenicity. Following the transfer of a normal chromosome 5 into a colorectal cell line, in which loss of heterozygosity on chromosome 5 had previously been demonstrated, the cells no longer formed tumours in nude mice and displayed an altered phenotype on cell culture. They were unable to back select for the elimination of the normal chromosome 5 to demonstrate a reversion to malignancy. This experiment suggests that replacement of a normal *APC* gene is sufficient to suppresses tumourigenicity.

Other somatic mutations in colorectal neoplasia

In addition to the changes on chromosome 5 many other genetic events have been described in association with colorectal neoplasia. These include the activation of dominantly acting oncogenes and the loss of genetic material associated with tumour suppressor genes.

K-ras is a cytoplasmic proto-oncogene that encodes a membrane-bound signal transduction protein with intrinsic GTPase activity. Single base pair mutations of $p21^{k\text{-}ras}$ have been shown to lead to its activation and ability to transform cultured cells in vitro. Somatic mutations of codon 12 of $p21^{k\text{-}ras}$ occur in up to 60% of sporadic colorectal adenomas and 50% of carcinomas but are present in only 7% of FAP adenomas (Bos et al, 1987; Forrester et al, 1987; Vogelstein et al, 1988; Farr et al, 1988).

C-myc expression and tyrosine kinase activity are both increased in colorectal tumours. Increased c-myc expression appears to be more

common in left-sided (distal) colorectal cancers and tyrosine kinase activity to be associated with pp60^{c-src} a known oncogene (Rothberg, 1985; Bolen et al, 1987; Cartwright et al, 1990). No specific mutations have been shown to cause these changes and at present it is unclear whether they are the result or the cause of neoplastic transformation.

The DNA from colorectal tumours is hypomethylated when compared to DNA from surrounding normal tissue (Goelz et al, 1985; Feinberg et al, 1988). Hypomethylation is associated with gene activation and also has been shown to inhibit DNA condensation during mitosis. It has been proposed that decreased condensation of DNA may lead to an increased incidence of chromosomal translocations during mitosis and to further genetic alterations in the neoplastic cell (Schmid et al, 1984).

Karyotypic analysis of colorectal carcinomas has shown the frequent occurrence of cytogenetic abnormalities particularly of chromosomes 17 and 18 (Reichmann et al., 1981; Muleris et al., 1985). These chromosome alterations have been investigated using RFLPs and loss of heterozygosity on chromosomes 17 and 18 shown to be extremely common (Fearon et al., 1987; Monpezat et al., 1988).

The majority of colorectal carcinomas are thought to arise from pre-existing adenomatous polyps. Vogelstein et al. (1988) looked at which genetic events had occurred at various stages of colorectal tumourigenesis. Chromosome 5 allele loss was commonly detected in non-FAP adenomas and *ras* mutations in larger adenomas. Chromosome 17 and 18 allele loss were commonly found in large adenomas and carcinomas. Thus the progression from normal mucosa to adenoma to carcinoma is associated with the accumulation of somatic mutations. Particular mutations occur more commonly at certain stages of tumourigenesis but there does not appear to be a rigid sequence in which these changes occur.

Baker et al (1989) used polymorphic markers to localise the region of allele loss on chromosome 17 to 17p12-p13.3. This region was

noted to include the gene for the oncoprotein p53. No large rearrangements of the p53 gene were detected on Southern and Northern analysis of colorectal tumours. However, in two colorectal cancers showing chromosome 17 allele loss the remaining p53 alleles were sequenced and both were found to contain point mutations in a highly conserved region of the gene.

P53 mutations have since been demonstrated in most other adult solid tumours known to show chromosome 17 allele loss, including breast, lung, brain and mesenchyme and point mutations demonstrated in one p53 allele in two tumours without chromosome 17 allele loss (Nigro et al, 1989). Rodrigues et al (1990) have used monoclonal antibodies specific to p53 to demonstrate increased levels of p53 in 50% of colorectal cancers on immunohistological staining. In six out of ten colorectal cancer cell lines p53 was increased and this was shown to be associated with mutations in conserved sequences of the gene, however, in two cell lines similar mutations were present without increased p53 expression. They concluded that mutant p53 is often associated with increased levels of expression in colorectal cancer.

P53 was originally considered to be a dominantly-acting oncogene as mutant p53 together with activated ras transforms primary rat embryonal fibroblasts. However, wild-type p53 has recently been shown to act as a tumour suppressor; transfection of the wild type gene in addition to mutant p53 or E1A inhibits expression of the neoplastic phenotype in rat embryonal fibroblasts. A model has been proposed in the rodent in which mutant p53, which is frequently expressed at high levels in tumours, has a dominant negative effect on wild-type p53, possibly by forming oligomeric complexes with the wild-type gene product (Finlay et al, 1989). In human colorectal tumours a heterozygous p53 mutation has been proposed to cause tumour progression and the later loss of the remaining wild-type allele to lead to further loss of growth control (Nigro et al, 1989). The transfection of wild-type p53 into colorectal cancer cell lines has recently been shown to inhibit their proliferation, whereas mutant p53 does not, providing further

evidence for p53 acting as a growth suppressor in the human colon (Baker et al, 1990).

Germline mutations of the p53 gene have been described in Li-Fraumeni syndrome, an dominantly inherited predisposition to early onset breast cancer, sarcomas and other malignancies (Malkin et al., 1990; Srivastava et al., 1990). In this situation it would appear that the inherited mutant allele does not have a dominant negative effect.

Fearon et al. (1990) defined the common region of deletion on chromosome 18 in colorectal cancers in which a polymorphic DNA probe detected a homozygous deletion in one tumour and a new polymorphism in another. A 370 kilobase region around this probe was cloned and a comparison of human and rodent sequence made to look for potential exons, conserved sequences were then used to screen cDNA libraries. They identified a cDNA, which they named deleted in colorectal carcinomas (DCC), with significant sequence homology to neural cell adhesion molecules and other related cell-surface glycoproteins. DCC is expressed in most normal tissue, including colon, and its expression is greatly reduced in most colorectal carcinomas. Alterations in cell-surface interactions have previously been implicated in abnormal colonic differentiation by several studies (eg Pignatelli et al, 1988).

In addition to the somatic genetic alterations on chromosomes 5, 17 and 18 loss of heterozygosity has also been detected on chromosomes 1q, 4p, 6p, 6q, 8p, 9q and 22q in between 25 and 50% of colorectal carcinomas (Vogelstein et al, 1989). In individual colorectal carcinomas a median of four to five chromosome arms show allele loss. These other sites of allele loss may either represent random events associated with other genetic changes or represent the sites of other tumour suppressor genes in the genome. Allele loss on these chromosomes is also seen in a number of unrelated tumours (see Table 1). Chromosome 5 allele loss has only been detected in colorectal and hepatocellular carcinomas and appears to be a genetic event confined to gastro-intestinal tumours. The prognosis of patients with tumours in which there was a greater

than the median number of chromosomes with allele loss was considerably worse than those with four to five chromosomes affected (Kern et al., 1989).

The number of chromosomes with allele loss has been found to be higher in distal (left-sided) tumours than proximal (right-sided) tumours although the incidence of *ras* mutations is the same in both (Dellatre et al., 1989; Kern et al., 1989). This increased incidence of allele loss mirrors the increased incidence of colorectal carcinomas from proximal to distal in the colon. The chromosomal mechanisms responsible for revealing recessive mutations, such as non-disjunction and recombination, may occur more frequently in the distal colon and be partly responsible for the excess of tumours.

The multiple somatic mutations described in colorectal tumourigenesis accord well with the multistep model of tumourigenesis predicted by studies of tumour kinetics. Each chromosome with allele loss represents two genetic events and thus the median loss of four to five chromosome arms in colorectal cancers and the activation of dominantly acting oncogenes gives a total greater than that predicted by kinetic studies. However, the original kinetic studies assumed a constant mutation rate throughout life whereas each mutation that results in clonal expansion would be expected to increase the rate. In these circumstances a higher number of mutations would be associated with tumourigenesis.

There is considerable evidence for the familial clustering of colorectal cancer and pedigree studies that suggest a partially penetrant autosomal dominant predisposition to colorectal adenomas in cases of apparently sporadic colorectal cancer. Leppert et al. (1990) have reported linkage to markers in the *APC* region of chromosome 5 in an atypical non-FAP kindred with a high incidence of early onset colorectal cancer and a highly variable number of colonic adenomas in affected members. This raises the possibility that different mutant alleles of the *APC* gene may be responsible for other inherited predispositions to colorectal cancer.

Further DNA markers in the *APC* region are still required. A microdissection library of chromosome 5q21-q22 has provided a large number of new probes which map into the PD/TS-1 and MD/TS-1 deletions. These deleted probes will also be screened on the new chromosome 5 hybrids derived from PS, a patient who has FAP and a more distal chromosome 5 deletion (del 5 (q22.1-23.2), which will shortly be available. This should allow the region of the *APC* gene to be more clearly defined.

These new probes are being used to screen southern blots of 150 pairs of normal tissue and common colorectal cancer DNA and pulsed-field filters of 100 colorectal cancer cell lines and cell lines from FAP patients. It is hoped that probes close to the *APC* gene will detect homozygous deletions or band shifts in some of the tumours or FAP patients. The microdissection probes are being used to construct a pulsed-field map of the region and to screen a flow-sorted chromosome 5 cosmid libraries. The cosmids can then be used to look for RFLPs and for the presence of conserved sequences.

Probes from the *APC* region which detect sequences that are conserved in other species will be used to screen cDNA libraries and to look for gene expression on northern blots. Mutations will need to be demonstrated in a candidate gene which affect its expression or function in FAP patients with new mutations, but not in the parents, or in common colorectal carcinomas to confirm that it is indeed the *APC* gene.

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