EXPRESSION OF ONCOGENES IN

HUMAN COLORECTAL NEOPLASMS

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ABSTRACT

Oncogenes are now recognised to be fundamentally important in regulating cellular proliferation and differentiation, and alterations in their expression have been described in spontaneous human neoplasms. This study has sought to identify changes in ras and myc oncogene expression during the process of human colorectal carcinogenesis. The introduction presents a review of current knowledge of oncogenes and their involvement in neoplasia, and relates this information to the context of colorectal carcinogenesis. The original work of the thesis examines techniques of demonstrating oncogene expression in tissue sections. Immunohistochemical methods using specific antibodies to oncogene proteins were compared with in situ hybridisation techniques using radioactively labelled DNA probes containing oncogene sequences. The latter methods were found to have many problems and disadvantages compared with immunohistochemistry. Provided that the specificity of the antibodies was confirmed, that fixation conditions were optimised, and that a sensitive detection system was used, specific localisation of ras and myc oncogene proteins could be consistently demonstrated in human colorectal tissues by immunohistochemistry.

The monoclonal antibody Y13-259 was used to study ras gene expression. Its specificity was confirmed by immunoblotting, and by its use in immunohistochemistry

with a positive control cell line (FHO5T1) which was genetically manipulated to express Ha-ras at artificially high levels. In a series of human colorectal tissues studied by frozen section immunohistochemistry, it was found that ras gene expression was increased in the early (adenoma) stage of carcinogenesis, but that this increase was not consistently sustained in carcinomas. These results were confirmed in a larger series of tissues, fixed by the specialised fixative PLPD, which offered improved morphology with retention of optimal immunoreactivity.

c-myc expression was investigated using the monoclonal antibody Myc1-9E10 to the myc protein product. Tissue fixation conditions were found to have an important effect on staining distribution. 50 colorectal carcinoma resection specimens were specially handled to optimise fixation. Positive staining was observed in all epithelial tissues, but a significant difference occurred in subcellular distribution of stain. Nuclear staining predominated in non-neoplastic mucosa whilst carcinomas consistently showed cytoplasmic staining. The findings suggest that an alteration occurs in the cellular processing of the c-myc oncogene protein during the process of colorectal carcinogenesis.

It is suggested that cooperation of ras and myc oncogenes may occur at an early stage in colorectal carcinogenesis, and play an important role in the generation of the

malignant phenotype. This suggestion is discussed in the context of existing knowledge of oncogene invovement in colorectal carcinoma.

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DECLARATION OF ORIGINALITY

I declare that the contents of this thesis were composed entirely by myself. In addition, all the work described herein was performed by myself, with the following exceptions:

1. All cell lines were maintained in culture by R. Morris and staff of the Cell Culture Laboratory.

2. All sections were cut by staff of the Research Histology Laboratory.

3. Monoclonal antibodies were prepared from culture supernatants by R. Morris.

4. The RAP-5 immunoblots shown in this thesis were performed by A. Robinson.

5. Immunoprecipitation experiments were performed by A. Robinson and A.H. Wyllie.

6. Animal inoculations and post-mortems were performed by A.H. Wyllie.

7. Final stage incubations of ^{125}I-protein-A 'Western' blots were performed by D. Watson.

8. Independent assessment of immunohistochemical staining was performed by J. Piris.

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CHAPTER 1

INTRODUCTION

INTRODUCTION

1.1 ONCOGENES - AN OVERVIEW

1.1.1 <u>Retroviruses</u> <u>and</u> <u>oncogenes</u>

It is a central belief of modern oncology that genetic changes are fundamental to carcinogenesis. Evidence for this belief comes from a wide variety of different sources - from the study of cancer in populations to the analysis of DNA of individual cells. We are now at a stage in the study of cancer where the techniques and resources exist to analyse these genetic changes at the molecular level.

Recently oncological research has witnessed a remarkable convergence of two apparently unrelated avenues of investigation, which has led to important advances in the understanding of genetic events of carcinogenesis. The first of these concerns the work of tumour virologists.

Viruses have for long been suspected of playing a critical role in the development of at least some human cancers. It was many years before the full significance was realised of the early observations of Rous, who showed that a cell-free filtrate from tumours of fowl could induce similar tumours in recipient birds. It was subsequently shown that the agent responsible is a virus of the retrovirus family.

Retroviruses are unusual in several respects. Their life cycle involves integration of their genome into the host cellular chromosome by the unique process of reverse transcription, in which the viral enzyme reverse transcriptase allows the synthesis of a DNA copy of the viral genome, which is then integrated into the host cell's DNA. A subgroup of the retroviruses - the oncogenic retroviruses - is able to cause transformation of appropriate recipient animal cells in culture (Duesberg, 1983). Transformation in this context refers to a heritable change whereby affected animal cells lose growth restraints of normal cells while adopting some of the characteristics of malignant cells, including in some cases the ability to induce tumours when injected into syngeneic animals. Dissection of the genome of the oncogenic retroviruses led to the identification of a group of genes which were necessary for the initiation and maintenance of transformation. This group is termed the oncogenes, onc genes or transforming genes, and in the retroviruses is quite separate from the viral genes controlling integration and replication.

The first of these genes to be characterised was the src gene of the Rous sarcoma virus (Stehelin et al, 1976), which is the gene enabling the virus to transform chicken fibroblasts in culture, and to induce sarcomas in vivo. These workers made the tantalising discovery that src is

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not a bona fide viral gene at all, but is a close copy of
the coding sequence of a gene found in all normal chicken
cells.

The full significance of these observations to the
context of human carcinogenesis was initially obscure.
Formal proof was still lacking that any virus could cause
a human malignant neoplasm, and indeed the mode of action
of the acutely-transforming oncogenic retroviruses
causing tumours in experimental animals seemed
incompatible with the natural history of the majority of
human malignant tumours. The retroviruses seemed to
transform cells to the malignant phenotype in a single
step, whereas many of the spontaneously-occurring human
cancers appeared to occur by the accumulation of several
changes, occurring step-wise, leading to a progressive
loss of growth control, apparently without the assistance
of viruses.

1.1.2 Oncogenes identified by transfection
A new and different approach to the investigation of the
cancer-causing genes of transformed cells was provided by
experiments involving the transfer of functional genes
between different cell types. There are a number of
different methods of performing this procedure which is
known as transfection. In the most commonly-used
technique, a non-transformed embryonic rodent fibroblast
cell line was exposed to a calcium phosphate precipitate

of high molecular weight DNA derived from transformed cell lines (Cooper, 1982). The recipient cell line most frequently used was the cell line NIH/3T3. When DNA from control untransformed cells is used, there is no observable effect, but with DNA from transformed cells, the NIH/3T3 recipients sometimes undergo transformation. It was possible by the techniques of molecular cloning to identify the genes responsible for the change. Some workers anticipated that these would emerge as an unfamiliar, previously uncharacterised group of genes, but they were at least partly mistaken. Examination of nucleotide sequences defined very close homologies between the transforming genes identified by transfection and the already familiar oncogenes of the oncogenic retroviruses (Bishop, 1983). The earliest reports of such work include those of Parada et al (1982) and Santos et al (1982), who showed the transforming genes of the EJ and T24 human bladder carcinoma cell lines to have very close homology with the oncogene carried by the Harvey strain of the murine sarcoma virus. Subsequently, many further similar types of homology were demonstrated between transforming genes of cell lines and the oncogenes of retroviruses.

1.1.3 Cellular oncogenes

Clues to the evolutionary origin of the retroviral oncogenes were initially supplied by the work of Stehelin et al (1976), and Spector et al (1978), who demonstrated

the DNA of avian and mammalian cells to contain sequences of very close homology to the src gene of the Rous sarcoma virus. Analysis of cellular RNA also showed transcripts of this gene to be present in uninfected normal cells. Further work along similar lines showed that each retroviral oncogene possesses a close homologue in normal cells. Significantly, the oncogene sequence found in normal cells possesses no adjacent viral sequences such as the gag, pol or env genes, nor does it contain the viral long terminal repeat sequences (LTR's) whose presence would have been expected if the oncogene sequence was of viral origin. In addition, the cellular gene has the characteristic structure of alternating exons (sequences coding for the final protein product) and introns (intervening sequences not represented in the protein product) which distinguishes eukaryotic genes from viral genes which do not possess introns. These are compelling pieces of evidence that the cellular oncogenes are the progenitors of the viral oncogenes, and were presumably picked up by the viruses during the processes of integration and replication. Whilst such transduction is a rare event, the oncogene confers the property of inducing increased cellular proliferation, and hence presumably gives the carrier virus a selective advantage. The cellular oncogene sequence is termed a cellular oncogene (c-onc) or proto-oncogene, to distinguish it from the viral oncogene v-onc.

Molecular cloning techniques have allowed characterisation of c-oncs from many species, widely separated in evolutionary terms, and a most remarkable degree of inter-species conservation of gene structure is observed. Creatures as different as man and the Drosophila fruit fly share c-oncs of striking homology. Indeed it has now been shown that baker's yeast (Saccharomyces cervisiae) possesses genes whose structure and protein products are remarkably similar to corresponding genes in mammals (Papageorge et al, 1984).

Such fidelity of conservation across aeons of evolutionary time is truly extraordinary, and argues strongly for some crucial function basic to cellular homeostasis. Furthermore, transcription from c-oncs occurs in a variety of tissues, and in every vertebrate species that has so far been examined, albeit often at very low levels (1-10 copies per cell). However, expression does not occur coordinately, and each oncogene may have its own specific function in different tissues and at different stages in development (Bishop, 1983).

The speculation follows quite naturally that oncogenes are central to the regulation of cellular proliferation and differentiation, functions which are manifestly deranged in tumours. The cellular progenitors of the retroviral oncogenes are clearly likely to be direct instruments in the process of carcinogenesis.

1.1.4 Oncogenes and control of cellular proliferation

All proliferating cells progress through a highly ordered sequence of events referred to as the cell cycle, during which protein and DNA synthesis occur followed by mitosis. Evidently controls are exerted at certain critical points in the cell cycle. In normal cells, such controllers are sensitive to environmental stimuli, and complex transmembrane and intracellular signalling systems exist to transfer information from the extracellular environment to the cell nucleus in the induction of cell division. Accumulating evidence indicates that oncogene products may have important functions as links in these systems.

Growth stimulation of quiescent non-transformed cells in culture leads to elevated expression of c-myc only in the first cell cycle following stimulation, whereas c-Ki-ras shows cell-cycle dependent levels of expression, the relative abundance of ras mRNA increasing in mid-to-late G_0/G_1. Conversely, a teratocarcinoma cell line (F9), induced to undergo terminal differentiation to non-proliferating endoderm, shows markedly diminished c-myc expression, but unchanged levels of ras (Campisi et al, 1984). The c-fos oncogene shows elevated transcription following growth stimulation of 3T3 cells in culture (Greenberg & Ziff, 1985).

In vivo, there is some evidence that oncogene expression shows regulated variation at different phases of embryonic development, and in adult tissues in varying physiological states. In the mouse embryo, there are 2-5 fold increases in mRNA transcripts of c-myc, c-erb-A and c-src during late gestation, whilst c-Ha-ras appears to be transcribed constitutively (Muller et al, 1982; Slamon et al, 1984). In human placenta, there is evidence of significant increases in c-myc mRNA levels in cytotrophoblast during early gestation (Pfeifer-Ohlsson et al, 1984). c-Ha-ras transcription has been observed to increase 3-fold during hepatic regeneration induced by partial hepatectomy in rats (Goyette et al, 1984).

Elucidation of the biochemical functions of the protein products of oncogenes initially resisted straightforward definition, partly because of the difficulty in isolating the proteins in any quantity. With the advent of techniques allowing efficient expression of cDNA in bacteria and yeasts, it has become evident that a variety of different functions are subserved, the products of the different oncogenes segregating into a number of major groups.

The first to be identified was the protein encoded by the well-studied retroviral transforming gene v-src, a 60 kDa protein termed pp60-v-src (Brugge and Erikson, 1977). Soon, the uncommon enzymatic function of protein-tyrosine

kinase activity was demonstrated for this product (Collett and Erikson, 1978). Whilst phosphorylation had already been recognised as an important regulator of protein function, it had only infrequently been observed to involve tyrosine in more than a small proportion of total residues. Soon, the same tyrosine kinase activity had been shown for the proteins of a number of retroviral oncogenes as well as for their corresponding c-oncs (Hunter and Cooper, 1985). All were found to share a recognisably similar domain of around 30 kDa in which resides the specific catalytic activity. This group of proteins is generally associated with the plasma membrane of cells, some spanning the membrane, others being cytoplasmic proteins, closely apposed to the inner face.

The strategic location of this group of proteins at the plasma membrane of the cell is suggestive of a role as receptors in signalling systems analogous to those shown by endocrine cells, in which cellular proliferation is triggered by the action of an extracellular stimulus on a membrane receptor. The epidermal growth factor (EGF) receptor of normal cells was shown to have tyrosine kinase activity (Buhrow et al, 1983), and later it was shown by Downward et al (1984) that the protein product of the v-erb-B oncogene shows very close amino acid sequence homology with the sequence of the intracellular part of the EGF receptor. The v-erb-B product appears to be a truncated version of the receptor, the retained

portion being the transmembrane and intracytoplasmic part of the molecule. The intracytoplasmic domain is the part likely to be responsible for the delivery of the signal to the cell to proliferate. In its truncated form, the receptor protein is no longer under the influence of extracellular EGF, and presumably delivers a continuous unregulated signal to the cell to proliferate.

A family of oncogene products exists without known membrane receptor function, but which also show tyrosine-kinase activity (abl, fes and fps). In addition to their tyrosine kinase activity (the exact significance of which remains to be determined), the protein products of the src and ros oncogenes have been shown to have inositol lipid kinase activity, having the ability to convert phosphatidyl inositol to phosphatidyl inositol biphosphate (PtdIns(4,5)P2). The latter has emerged as playing a central part in an important intracellular signal transduction system (Berridge and Irvine, 1984). In this system, binding of an agonist to a receptor on the cell membrane leads to the diversion of PtdIns(4,5)P2 from a 'futile cycle' of phosphorylation and dephosphorylation by kinases and esterases in the cell membrane, towards a phosphodiesterase which splits it into inositol triphosphate (IP3) and diacyl glycerol (DAG). Both of these molecules function as intracellular second messengers, mediating stimulation of cellular proliferation by mobilisation of sequestered

intracellular calcium, and enhancement of protein kinase C (PKC) activity respectively.

The downstream target of the ras proteins has recently been identified (Trahey & McCormick, 1987) (see section 1.2.4), and it appears to couple a membrane receptor for growth factors to the phosphodiesterase phospholipase C which cleaves PtdIns(4,5)P2 to IP3 and DAG (Berridge, 1986).

Further links have been demonstrated between oncogene products and previously described pathways controlling cellular proliferation. Sequence analysis of the 28kDa protein product of the v-sis oncogene showed close homology to one of the subunits of the well-studied growth factor, platelet derived growth factor (PDGF) (Waterfield et al, 1983). The attractive implication of this finding is that cells transformed by this oncogene escape the influence of normal growth restraints by autocrine synthesis of growth factor.

The protein products of the oncogenes described above appear to exert their action either at the cell membrane or within the cytoplasm of the cell. There is however a functionally separate group whose action appears to be exerted in the nucleus. Some members of this group, whose members include myc, myb, fos and erbA, show some structural homology with each other, and their functions

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are also similar. As a group, the most conspicuous of their actions is that of immortalisation of cells in culture, whilst they are much more limited in their ability to induce anchorage-independent growth (Weinberg, 1985). This is in contrast to the larger group of oncogenes whose action is exerted in the cytoplasm.

The mechanisms of action of these 'nuclear oncogenes', and their roles in normal cellular homeostasis, are largely unknown. It is apparent that many of their products are reversibly bound to nuclear structures, and transcription of myc increases in growing cells shortly before the S-phase of the cell cycle. Cells transfected with myc appear to acquire an enhanced ability to express other cellular genes, including artificially introduced genes (Kingston et al, 1984). It is speculated that myc may exert effects on transcription itself, or on the apparatus responsible for it regulation or specificity.

1.1.5 Activated oncogenes in carcinogenesis
Cellular oncogenes, which after all are normal cellular genes, do not in their native form cause neoplastic transformation of cells. How then do they become activated? Their structural and functional heterogeneity becomes further manifest in the variety of ways in which activation takes place. As a generalisation, oncogene activation involves alteration from the normal

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physiological state either by enhanced transcription or a structural change in the gene itself.

The first of the mechanisms to be described involved the experimental ligation of strong transcriptional promoting sequences to oncogene DNA sequences (Blair et al, 1981). Oncogenes which were previously non-transforming acquired activity when transcribed at artificially high levels.

This experimentally contrived mechanism was soon found to have several close parallels in vivo. A rather perplexing problem was posed to the tumour virologists by a group of retroviruses, of which the avian leukosis virus (ALV) is an example, which repeatedly induce specific neoplasms in animals, yet do not carry a v-onc. This puzzle was elegantly resolved when it emerged that the viral DNA in the tumours caused by ALV is specifically integrated in close proximity to the c-myc oncogene, whose expression is greatly increased as a consequence of the action of viral promoter or enhancer sequences in the long terminal repeat (LTR) (Payne et al, 1982). A similar mechanism has been shown to exist for other oncogenes in animal tumours, including erb-B, but there is little evidence that this mechanism is of major importance in human carcinogenesis.

A second mechanism, with some points of similarity to the first, emerged from study of oncogenes in the process of

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chromosomal translocations. Translocations are common in tumour cells, and certain types of neoplasms repeatedly show the same ones, the most familiar example being the distinctive Philadelphia chromosome of chronic myeloid leukaemia. Human Burkitt's lymphoma cell lines in the majority of cases show a translocation in which the distal portion of chromosome 8 is transferred to a chromosome bearing an immunoglobulin gene which is active in the initial tumour (usually chromosome 14). The c-myc oncogene has been mapped to a position near to the break point on the translocated portion of chromosome 8, and it seems to come under the influence of neighbouring powerful gene regulator sequences involved in immunoglobulin production (Dalla Favera et al, 1982). Again, inappropriately high expression leads to cellular transformation.

Gene amplification is yet another means whereby oncogene expression may be sufficiently boosted to cause transformation. Distinctive chromosomal abnormalities have been described which signify DNA amplification, namely double minute chromosomes, and homogeneously staining regions (HSR) (Alitalo, 1984). Amplification of c-myc has been mapped to HSR of chromosomes in the colon carcinoma cell line COLO320 (Alitalo et al, 1983) and c-Ki-ras amplification is described in double minute chromosomes and HSR in a mouse adrenocortical tumour cell line (Schwab et al, 1983). Amplification of the

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oncogene N-myc has been reported in unmanipulated human tumours (principally of neuro-endocrine histogenesis - small cell undifferentiated carcinoma of lung, and childhood neuroblastomas). The level of amplification of N-myc broadly reflects the clinical aggressiveness of the tumour (Schwab, 1985). The Ki-ras oncogene has been reported to be amplified in ovarian carcinoma (Filmus & Buick, 1984).

A very different mechanism of activation is shown by a number of oncogenes, most notably the members of the ras gene family. In this group, an alteration in gene structure by mutation leads to important conformational and functional changes in the protein product. This was first discovered by analysis of DNA from EJ and T24 bladder carcinoma cell lines, and accumulating evidence indicated activation of ras oncogenes to occur by mutations at the specific points 12 or 61 in their coding sequences (Varmus, 1984). Tumours induced by the action of chemical carcinogens on rodent fibroblasts consistently show point mutations at these specific sites, the distribution of mutations suggesting interaction of the carcinogen with the ras gene directly (Balmain, 1985; Barbacid, 1987).

The epidemiology and natural history of most human cancers indicates that the process of carcinogenesis occurs in a stepwise fashion involving sequential

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acquisition of ever-greater deviations from the normal controls of cells' growth, differentiation and behaviour. It is a complex dynamic sequence which occurs over many cycles of cell replication affecting successive generations of cells. Moulding the processes of evolution in the cell population is the natural selection of those cells best fitted for autonomous replication. This is clearly a very different situation from experimental models in which cells acquire the transformed phenotype in a single step, either by infection with an acutely transforming retrovirus or by transfection with a single oncogene.

Activated oncogenes are often detectable in unmanipulated human cancers, but several observations indicate that they are rarely sufficient on their own to induce tumour formation, and probably represent only one component in a complex chain of alterations. There is good evidence that single oncogenes have limited powers. When early passage cells are used as recipients of transfection, single oncogenes do not on their own effect neoplastic transformation. They may, however, confer isolated components of the transformed phenotype - Land et al (1983a) observed changes in cells transfected with the ras gene indicating acquisition of cellular functions such as reduced anchorage dependence. Recipients of myc in contrast showed immortalisation in culture but were not transformed. Under experimental conditions in which

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cellular maturation could be studied, Kahn et al (1986) showed that myc alone could maintain self-renewal and block terminal differentiation. When the ras gene was introduced together with the active v-myc oncogene, the cotransfected cells underwent vigorous morphological transformation (Land et al, 1983a). Similar results were obtained when ras was cotransfected with the large T gene of the oncogenic DNA polyoma virus. This is one of three true viral genes which evolved in the polyoma virus genome, and which cooperate to induce the transformed phenotype in infected cells. The large T gene on its own confers decreased serum dependence and increased life span, but when it is cotransfected with ras, complete conversion to the transformed phenotype results (Land et al, 1983b).

Further evidence for the limited powers of a single oncogene come from experiments using transgenic mice. In this system, a gene is injected into a fertilised ovum, and typically within a few cell divisions, integrates into a host chromosome. Usually all tissues acquire the newly-inserted gene, although expression is often tissue-specific, directed by regulatory elements of the gene. Transgenic mice produced bearing the c-myc oncogene develop neoplasms in only a minority of cells expressing myc, indicating that further events are required for tumourigenesis (Adams et al, 1985).

The first cooperating pair of oncogenes recognised were Ha-ras and c-myc, but several others are described. The broad concept of cooperation was refined by Weinberg (1985) who introduced the idea of 'complementation groups' of oncogenes. Replacement of ras or myc in the cotransfection experiments with other oncogenes allowed the definition of two functionally different classes of oncogene, based on the nuclear or cytoplasmic (including plasma membrane) localisation of the gene product. He pointed out that whilst there are few examples of transformation of early passage cells by either a single oncogene or two oncogenes of the same functional type, there are many examples of nuclear and cytoplasmic oncogenes cooperating to produce transformation. In some of the few examples of apparent transformation by a single oncogene, a variety of chromosomal aberrations are observed in transformants (Spandidos and Wilkie, 1984), which could conceivably lead to activation of additional genes. Indeed, there is recent evidence indicating that insertion of an extraneous oncogene can lead to increased expression of endogenous oncogenes (Wyllie et al, 1987).

It seems likely that the transformed phenotype results from a complex interaction of different oncogenes, different growth signals and different cell receptors. A system such as this with overlap and interplay between the various components may allow proliferation to be very finely controlled, and this may also explain why multiple

genetic changes are needed for tumorigenesis (Land, 1986). The individual features which characterise the transformed phenotype, such as growth factor independence, reduced adhesiveness, immortalisation and metastatic potential, are often independent of each other, and it is likely that their acquisition by different routes and in different chronological orders may not affect the ultimate arrival of the cell at the end point of the fully transformed phenotype (Balmain, 1985).

1.2. THE RAS FAMILY OF ONCOGENES

1.2.1 <u>ras genes identified by transfection</u>

The NIH/3T3 cell line is a continuously passaged line of murine fibroblasts which has been widely used in transfection experiments in the identification and characterisation of transforming genes (Cooper, 1982). DNA from a number of sources including cultured cell lines, chemically induced animal tumours and unmanipulated primary human tumours, can elicit transformation of the recipient NIH/3T3 cells. DNA from corresponding normal tissues of the same tumour-bearing hosts has no such effect, suggesting that a qualitative change has taken place in the DNA of the tumour.

Collective experience of the NIH/3T3 assay system has provided two striking observations. Firstly, transforming activity is detected in only 10-20% of human tumours of widely varying histogenesis, and secondly, regardless of the tumour type, the activated <u>c-ras</u> oncogene is by far the most frequently detected transforming gene in this assay (Santos et al, 1985).

The NIH/3T3 cell line is considered to be an abnormal cell line, already immortalised or 'rescued from senescence', and which appears to be particularly sensitive to transformation by activated <u>c-ras</u> oncogenes. Neoplastic transformation of the recipient cells in the assay appears to depend on the acquisition from the

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tumour DNA of a mutant allele acting in a dominant manner. The NIH/3T3 cell is insensitive, however, to many other activated oncogenes, whose presence is almost certainly concealed in the 85% or so of human tumours which are negative in the assay. An important flaw in the transfection assay is therefore in its insensitivity - success of transfection requires a dominant effector; some active oncogenes may show limitations of species specificity; and the earliest steps in the acquisition of the transformed phenotype are unlikely to be detected in this system.

Despite these shortcomings, the transfection assay has provided valuable information. The availability of retroviral DNA probes allowed investigators to seek - and find - homologies between the previously known viral oncogenes and the cellular transforming genes of the NIH/3T3 transformant clones. The human EJ and T24 bladder carcinoma cell lines were first found to have an activated form of the ras oncogene carried by the Harvey strain of the murine sarcoma virus v-Ha-ras (Parada et al, 1982; Santos et al, 1982) and soon other human cell lines were found to possess the corresponding v-ras gene of the Kirsten strain (v-Ki-ras) (Der et al, 1982). A third member of the ras family - N-ras - was also detected as a functionally transforming gene in the NIH/3T3 assay, but this has no retroviral homologue (Shimizu et al, 1983).

1.2.2 Molecular structure of ras oncogenes

In the human genome, Ha-ras and Ki-ras are each represented by two loci, one with introns and one without (Ellis et al, 1981; DeFeo et al, 1981; Chang et al, 1982b). The genes with introns show a higher degree of inter-species conservation and are thought to represent the functional genes. For historical reasons, they are referred to as c-Ha-ras-1 and c-Ki-ras-2, and are located on the short arms of chromosomes 11 and 12 respectively. N-ras has been assigned to the short arm of chromosome 1.

Precise nucleotide sequence data are available for the three human ras oncogenes (Capon et al, 1983a; McGrath et al, 1983; Taparowski et al, 1983). There are wide divergences in the size and complexity of the non-transcribed intron sequences, but all three have four exons of the same size with closely homologous nucleotide sequences, and precisely corresponding splice points. (Ki-ras has an additional diverged fourth exon, and may code for two alternative products). Due to variations in intron length, the three genes have very different sizes - approximately 40 kb, 6.6 kb and 14 kb for c-Ki-ras-2, c-Ha-ras-1 and N-ras respectively. Each gene codes for proteins of 188 or 189 amino acids, with a molecular weight of 21,000.

There is evidence that the ras genes are members of a larger evolutionarily-conserved 'super-family' of related

genes which have been termed rho (ras-homologous) (Madaule & Axel, 1985). Significant homologies occur between the amino-terminal sequence of the ras proteins and a group of mammalian proteins termed G proteins, whose role is transduction of signals from extracellular stimuli into a variety of cellular responses (Hurley et al, 1984; Gilman, 1984). A chance finding during the screening of a cDNA genomic library prepared from the abdominal ganglion of the mollusc Aplysia was that of an open reading frame of 192 amino acids encoding a protein of predicted molecular weight of 21,000 (Madaule & Axel, 1985). Computer-assisted search of the NIH protein database allowed identification of several regions of close homology between the deduced protein and the three members of the ras family. Analysis of many different species subsequently confirmed rho genes to be universally present in all eukaryotes examined. There is a striking conservation in size of the protein products of ras and rho, all having a molecular weight of 21,000. (The implications of this are discussed in section 1.2.4).

1.2.3 Activation of ras oncogenes

ras genes are clearly present in an activated form in the genome of tumours which are positive in the NIH3T3 transfection assay, yet transfection in their native form from normal tissues fails to induce neoplastic transformation (Cooper et al, 1982; Chang et al 1982a).

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What changes in the structure or expression of the gene constitutes such activation? Comparison of the structures of normal and activated ras genes initially indicated subtle genetic changes of small physical proportions which were not detected by restriction mapping. Accumulating nucleotide sequence data from a number of different laboratories demonstrated that the transforming activity of the gene is the consequence of a point mutation occurring at one or other of two critical locations - codon 12, located in the first exon, or codon 61 of the second exon (Reddy et al, 1982; Tabin et al, 1982; Taparowsky et al, 1982 and 1983; Capon et al, 1983; Shimizu et al, 1983; Yuasa et al, 1983). Mutations at one or other of these locations have been demonstrated for all three ras genes from a wide range of cell lines and spontaneous human tumours. Each mutation is a single base change. By analysing experimentally mutated ras genes, it has been demonstrated that substitutions at positions 12, 13, 59, 61 and 63 can lead to activation (Levinson, 1986). By constructing Ha-ras genes bearing all possible mutations at codon 12, Seeburg et al (1984) showed that only one amino acid (proline) other than the glycine which is normally present, fails to lead to activation. Proline and glycine when they occur in alpha-helical polypeptide chains both tend to act as hinge points, so their substitution in the activated gene product may lead to an important conformational change in the polypeptide. This is supported by the fact that the p21 products of

the mutated ras gene show altered electrophoretic mobility (Seeburg et al, 1984).

Most reports have indicated that ras is activated by mutation, but this may reflect the inherent insensitivity of the NIH3T3 transfection assay. It is possible that activation from gene amplification may not be detectable in this assay. Amplification of non-mutated ras genes has been reported in a few spontaneous tumours, but it seems that this is a relatively inefficient way of inducing malignant change. Driven by its own cellular promoters, which are relatively weak, the normal ras gene is unable to induce transformation of transfected fibroblast cell lines. However, by placing the normal Ha-ras oncogene under the powerful enhancing influence of viral long terminal repeat sequences, Chang et al (1982a) were able to effect transformation in the NIH3T3 system, but not in primary embryo fibroblast cultures. Spandidos and Wilkie (1984) demonstrated single step transformation of early passage embryonic fibroblasts by the mutated T24 human Ha-ras oncogene ligated to strong viral promoting sequences. The normal non-mutated ras gene under the same promoting influence induced immortalisation but not transformation.

Over-expression of normal ras, whilst clearly demonstrated in several experimental systems, has less commonly been reported in authentic human tumours.

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However, elevated expression of ras has been detected at the RNA level in a number of spontaneous human malignancies of different types (Spandidos & Kerr, 1984; Spandidos & Agnantis, 1984; Spandidos, 1985).

1.2.4 ras p21 structure and function

The product of the ras gene family is a protein of molecular weight 21,000 daltons termed p21 (Shih et al, 1979). It has been the subject of intensive investigation since ras was first identified as the transforming gene of the murine sarcoma virus. The foundations of this work involved the use of specific antisera against the viral transforming protein. Rodents inoculated with syngeneic virally transformed cells produce antibodies to the product of the viral oncogene (Shih et al, 1979), and antisera produced in this way allowed partial purification and biochemical characterisation of p21.

Each ras gene encodes a product of 188 or 189 amino acids, and the three members show greatest divergence from each other between amino acids 121-140 and 165-180. In contrast, the amino terminal domain of the first 80 amino acids shows virtual identity of sequence between the three proteins, and the middle domain of 80 amino acids shows approximately 85% homology between any pair. The sites at which activating mutations occur (positions 12 and 61) are within domains of homology between the three proteins.

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The primary translation product is present in the cytosol of the cell where it undergoes post-translational acylation (Sefton et al, 1982), and is subsequently to be found on the inner aspect of the plasma membrane (Willingham et al, 1980) where it is bound with lipid (Capon et al, 1983b; Yuasa et al, 1983). Amino acids located at or near the carboxy terminal end of the p21 molecule are required for cellular transformation, membrane association and lipid binding (Willumsen et al, 1984).

The ability to efficiently express cDNA's encoding normal and activated p21 polypeptides in Escherichia coli has permitted more detailed biochemical and functional analysis of the ras products (Lautenberger et al, 1983; Feramisco et al, 1984; McGrath et al, 1984). Scolnick et al (1979) had previously demonstrated GTP-binding activity of p21, but the larger quantities of purified protein yielded by the bacterial expression system permitted the significant observation that in addition to guanine nucleotide binding ability, the normal p21 proteins have GTP'ase enzymatic activity, and that this is selectively impaired by 4 - 10 fold by the critical mutation at position 12 which activates the oncogenic potential of the gene (Gibbs et al, 1984; McGrath et al, 1984; Sweet et al, 1984). This was the first indication of a functional difference between the normal and

oncogenic forms of the protein.

The GTP-binding and GTP'ase activities of p21 prompted speculative comparisons with previously-described proteins with these properties, notably the mammalian G proteins (Gilman, 1984) and the bacterial elongation factor protein EF-Tu. Fascinating parallels emerged between these proteins of widely differing origins. Like p21, the G proteins are membrane-associated and catalyse GTP hydrolysis, a function performed in response to a variety of extracellular stimuli as diverse as photons and hormones. X ray crystallographic data are not available for p21 or G proteins as crystallisation has not been achieved, but such data is available for EF-Tu, which has 42% homology with p21 (McCormick et al, 1985). These data were used to generate a model of the tertiary structure of the p21 molecule. A striking finding was that the four loops of maximum homology between the ras gene products and those of the related homologous proteins constitute the proposed guanine nucleotide binding site. Significantly, amino acids 12 and 61 are located in this phosphoryl-binding loop. This is supported by the finding that an antibody raised against a synthetic peptide of amino acids 5-18 (ie spanning position 12) abolishes GTP binding by p21 (Clark et al, 1985).

It seems clear that the structural similarity between

GTP-binding domains of ras p21 and the G proteins indicates a similar function, namely that of a membrane-associated switch. Mutations at positions 12 or 61 do not affect GTP binding, but decrease hydrolysis by up to 10 fold. The GTP-bound form of p21 represents the active conformation of the protein with respect to signal delivery, and it appears that the oncogenic form of p21 when GTP-bound provides the cell with an unregulated proliferation signal.

Whilst GTP hydrolysis is the function of the constant domains of p21, the variable domains are likely to serve to link the protein to several different receptor or effector proteins. A flexible system of this kind may allow a single cell to respond identically to different stimuli, or alternatively allow two different cells to repond differently to identical ligands (Madaule & Axel, 1985). The striking conservation in size of the ras and rho gene products (21,000 kDa) may indicate that they are interchangeable units in an interacting system of proteins subject to strict constraints of size of individual units.

Very recently, a protein has been identified by Trahey & McCormick (1987) which is a strong candidate to be the target for action of the p21 protein in mammalian cells. This cytosolic protein termed GTP'ase activating protein (GAP) interacts with the normal p21 protein to stimulate

its GTP'ase activity by 100 fold. Significantly, GAP has no apparent effect on the GTP'ase activity of the mutationally activated p21 protein. The biologically active GTP-bound form of p21 is hence permitted to persist (Sigal, 1988).

In a different approach to the functional analysis of p21, Stacey & Kung (1984) micro-injected purified recombinant p21 proteins into various cell types. Micro-injection into NIH/3T3 cells led to morphological transformation. When quiescent NIH/3T3 cells were injected with non-mutated p21 they started to synthesise DNA; when injected with mutationally activated p21, such cells underwent rapid proliferation. Conversely, micro-injection of transformed cells with a monoclonal antibody to p21 can lead to temporary reversal of the transformed phenotype (Mulcahy et al 1985). Furthermore, such antibodies also block the transforming ability of a group of oncogenes with membrane-associated protein products (src, fes and fms), an observation in keeping with the proposal that p21 proteins act as transducers of receptor-mediated signals (Smith et al, 1986).

Thus, whilst the details of the physiological function of p21 remain unclear, a framework is beginning to emerge. It is speculated that the p21 proteins mediate signals from peptide growth factors such as EGF and PDGF. In support of this is evidence that p21 interacts with the

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EGF receptor (Kamata & Feramisco, 1984). This leads to binding of GTP by p21 resulting in a conformational change, initiating interaction with an effector protein. Whilst the nature of the effector protein is also unclear, an obvious candidate is phospholipase C, a phosphodiesterase playing a central role in the generation of IP_3 and DAG from PtdIns(4,5)P2 (see section 1.1.4). IP_3 and DAG act as 'second messengers' in the control of proliferation. Some evidence for the ras proteins playing a role in this relay system comes from observations of enhanced turnover of phosphoinositides in cell lines transformed by v-ras (Fleischman et al, 1986). Termination of activation of the normal p21 follows the hydrolysis of GTP, but mutants defective in this activity undergo a prolonged interaction with the effector protein, resulting in a continuous signal to the cell to proliferate.

The p21 protein product of ras has also been shown to possess important properties influencing cell differentiation. The cell line PC12 is derived from a rat phaeochromocytoma, and under the influence of nerve growth factor, its cells undergo morphological differentiation towards neuronal cells. Transfection of the mutationally activated ras gene into PC12 recipients reproduces this morphological differentiation, as does micro-injection of the oncogenic p21 protein. The non-mutated ras gene does not induce the change unless its

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expression is artificially enhanced, and micro-injection of the normal p21 protein is followed by no observable effect (Bar-Sagi & Feramisco, 1985; Satoh et al, 1987). Observations such as these suggest that, in addition to their role in the control of proliferation, the p21 proteins have a part to play in the induction of differentiation in certain cell types. Immunohistochemical observations of p21 expression in different tissues support this suggestion. Furth et al (1987) observed abundant p21 expression in certain fully differentiated cell types, notably neurones of brain. It was suggested that in these tissues, p21 proteins subserve specialised membrane functions unrelated to proliferation.

1.3 THE MYC ONCOGENE

The cellular oncogene c-myc is the eukaryotic genomic homologue of the transforming sequence v-myc first identified in the MC29 avian retrovirus, an acutely transforming retrovirus which causes a variety of neoplasms in birds. It is expressed in many different mammalian tissues, and abnormalities of its expression have been described in a variety of mammalian and avian tumours. The protein product of v-myc was one of the first transforming proteins found to be localised to the cell nucleus, and it has been proposed to function as a regulator of gene expression, and to control the entry and progression of cells through the cell cycle.

In the human, the single locus encoding c-myc has been mapped to chromosome 8q. It contains a single copy of the gene, consisting of three exons, only two of which (exons 2 and 3) encode a protein product. Exon 1, although non-coding, shows a high degree of evolutionary conservation, and it is therefore believed that regulatory elements are situated in this part of the gene. Exons 2 and 3 encode a polypeptide product of 62-64 kDa (Hann & Eisenmann, 1984), which has been localised to the nucleus in immunofluorescent and biochemical studies. The protein is labile, with a half-life of approximately 15 minutes, and in vitro it shows DNA-binding properties (Beimling et al, 1985), a property which is impaired in the proteins of

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non-transforming mutant forms of v-myc (Donner et al, 1983). These features of the p62 protein product of myc are suggestive of a regulatory role in gene expression. An early model proposed that p62 exerted a repressor function by binding to exon 1, but more recent evidence indicates that the situation is more complex, as the translation efficiency of c-myc has been found to be independent of the presence or absence of exon 1 (Cole, 1985; Butnick et al, 1985).

Expression of c-myc has been investigated in untransformed cells and their transformed counterparts by studying the effects of growth stimulation of mouse fibroblasts (Kelly et al, 1983; Campisi et al, 1983; Greenberg & Ziff, 1984; Muller et al, 1984). Within 1 hour of growth factor stimulation, levels of c-myc mRNA showed a 20-fold increase, returning to basal levels by 18 hours. In this situation, it appears that the concentration of c-myc mRNA is enhanced more by post-transcriptional mechanisms (ie enhanced messenger stability) than by transcriptional activation, although the relative contributions are still uncertain.

While cells participate in the cell cycle, levels of c-myc mRNA transcription appear to be constant, whether the cell is of normal or transformed phenotype (Kelly et al, 1983). However, when growth is arrested by depletion of growth factors, levels of c-myc transcript fall

dramatically in normal cells within 2 hours, but remain high in chemically transformed counterparts (Campisi et al, 1984). The levels of c-myc transcript also decrease during terminal differentiation, for example HL60 cells induced by dimethyl sulphoxide or the tumour promoter TPA to undergo differentiation towards the macrophage or neutrophil (Westin et al, 1982). The p62 protein product appears to be qualitatively normal in most transformed cells.

Deregulation of the normal pattern of c-myc expression in a number of transformed cell lines and authentic tumours is associated with structural changes involving the gene. Mechanisms so far recognised in which there is loss of regulatory control are chromosomal translocations, retroviral insertions and gene amplification. In chromosomal translocations and retroviral insertions, the c-myc gene appears to come under the influence of constitutively active gene enhancing sequences which subvert the normal controls of expression. The avian leukosis virus, for example, integrates close to c-myc and lymphomas develop in infected birds as a result of this mechanism (Payne et al, 1982).

Chromosomal rearrangements of a characteristic type are often associated with distinctive tumours, and consistent translocations are seen in Burkitt's lymphoma cell lines. The commonest involves translocation of a terminal

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fragment of chromosome 8 to the distal part of the long arm of chromosome 14. Molecular analysis has shown that this characteristic rearrangement leads to the juxtaposition of c-myc to close proximity with sequences of the immunoglobulin heavy chain locus. The translocation may transfer c-myc intact, and it is proposed that in this case, the constitutive activity of the immunoglobulin locus leads to uncontrolled expression of myc. In different tumours, the c-myc-carrying chromosome may break within the first exon or the first intron, but never within exon 2 or 3. It has been suggested that exon 1 of c-myc may come under the influence of the mechanism (as yet largely not understood) which generates somatic mutations in the variable region of the immunoglobulin gene in the process of generation of antibody diversity (Taub et al, 1984). Such mutations would lead to disruption of control sequences, but retention intact of exons 2 and 3. The implication is that an intact c-myc protein is required for transformation, but that the presumed regulatory function of exon 1 (and perhaps other upstream sequences) is lost in tumour cells.

Gene amplification as a mechanism of oncogene activation has been described in section 1.1.5. Many cellular oncogenes are capable of undergoing amplification, but so far, myc genes account for more than 90% of amplifications described (Schwab, 1986). In most cases of

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amplification, despite relocation of the oncogene-containing sequences to diverse positions on other chromosomes, structural alterations of the gene itself are relatively infrequent. However, it was during the analysis by low-stringency probing of double-minute (DM) chromosomes and homogeneously staining regions (HSR) for the presence of amplified oncogenes that two previously undescribed structural variants of the myc gene were discovered. N-myc was found amplified in neuroblastoma cells (Schwab, 1985), and L-myc in small cell lung cancer (Nau et al, 1985). The complete structure of these genes awaits elucidation, but they show a similar 3-exon structure to c-myc with only exons 2 and 3 translated, and there are regions of close sequence homology with c-myc. In human neuroblastomas, there is a correlation between the presence of amplified N-myc and advanced tumour stage and aggressiveness (Seeger et al, 1985). Again there is evidence that terminal differentiation (in this case to ganglion cells) is associated with decreased expression of N-myc (Schwab, 1985). Amplification of myc seems to be one of the mechanisms leading to the emergence of clones of cells with increasingly malignant properties, and is unlikely to be the initiating event in carcinogenesis.

The reason is unknown for selective amplification in tumours of c-myc as opposed to other cellular oncogenes. A selective advantage appears to be conferred, as myc-

rich double-minute chromosomes which lack centromeres would be expected to disappear in the absence of selection pressure. It is possible that amplification of cellular oncogenes other than c-myc confers decreased survival ability to most cell types.

1.4 PATHOGENESIS OF COLORECTAL CANCER

There are striking differences in the incidence of colorectal cancer in different parts of the world. In Northwest Europe, North America and Australia, the large bowel is amongst the three commonest sites of carcinogenesis, whereas in Africa, Asia and South America it is uncommon. Within high risk populations, distinct variations in incidence occur which are largely unexplained. Migrant studies indicate an environmental factor or factors to be of importance, and with the exception of a few uncommon conditions discussed below, hereditary factors appear to play a lesser role. Broadly speaking, populations at high risk enjoy an affluent lifestyle (Japan is an exception), and the speculation follows that diet has an influence on the development of the disease.

There is a small number of conditions which are known to predispose to the development of large bowel cancer, most notably long-standing ulcerative colitis. There is reason to believe, however, that cancer occurring in this situation differs in several important respects from the much commoner sporadic variety; such tumours are usually poorly differentiated histologically and exhibit aggressive, diffusely infiltrative behaviour. They appear to take origin from flat, dysplastic mucosa, which is in marked contrast to sporadic cancers which are believed to

arise by malignant transformation of a pre-existing adenoma, and are frequently well or moderately well differentiated histologically.

1.4.1 Bile, bacteria and bowel carcinogens

Much effort has been spent attempting to identify potential dietary carcinogens of the colorectum, and it is clear that a complex interaction occurs between the components of the diet and the resident bacterial flora of the large intestine. It has for long been suspected that metabolites of bile acids are potential carcinogens, and it is postulated that bacterial flora of the large intestine are responsible for converting benign substrates into carcinogens. The composition of the diet has an influence both on the concentration of the bile acids in the intestinal lumen, and on the composition and activity of the bacterial flora. High levels of meat (particularly beef) consumption in a population correlate with the incidence of colorectal cancer, and it has been proposed that carcinogens are formed in situ by microbial modification of dietary neutral sterols, or of bile acids, increased by the presence of high levels of fat in the diet. In vitro mutagenicity assays have been used to investigate carcinogens in the faeces, and have identified a potentially important group of substances termed fecapentaenes, which are polyunsaturated derivatives of glycerol, probably of bacterial origin (Gupta et al, 1983). Whilst carcinogenic activity has not

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yet been formally demonstrated, the fecapentaenes are very potent mutagens. Animal studies have also indicated that bile acids may act as tumour promoters, and microbial mutagenicity testing has shown that, although they are not mutagenic by themselves, bile acids and their metabolites act as co-mutagens, significantly potentiating the mutagenic activity of other substances. Recently, a mechanism has been postulated which may explain the toxic effect of bile acids. This proposes that mucosal damage occurs as a consequence of the calcium-binding action of these substances, and that the increased cell turnover required to replace cells lost through this effect may result in an increased chance of alterations in DNA and mutant progeny leading to neoplasms (Newmark et al, 1984). Human studies have shown a correlation between the mean faecal bile acid concentration and the incidence of colorectal cancer in a population. Whilst there is no evidence that bile acids can initiate the carcinogenic process (by the formation of adenomas), it has been postulated that their role may be in the promotion of premalignant neoplasms into those with the potential for invasive growth (Hill, 1983).

1.4.2 The adenoma-carcinoma sequence

There is a considerable body of evidence which, although largely circumstantial, indicates that the great majority of colorectal carcinomas arise by malignant transformation of pre-existing adenomas. The evidence is

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principally derived from epidemiological and pathological observations (Day, 1984). The prevalence of adenomas in a population is not easy to study, and the most reliable information comes from careful prospective autopsy studies. (Radiological studies tend to underestimate the incidence of small lesions, and there is a bias in many of the surgical studies towards proximal lesions within endoscopic range). A correlation has been shown to exist between geographical areas of high carcinoma incidence and high adenoma prevalence. The correlation is far from absolute, however, and a difference of up to 70% in carcinoma incidence has been observed in different populations showing similar prevalence of adenomas (Vatn & Stalsberg, 1982). Adenomas tend to show a relatively even distribution throughout the colon and rectum, although in populations with high risk of cancer, adenomas tend to be larger in the sigmoid colon and rectum, the favoured sites of development of carcinomas in these groups. This suggests that different factors may be involved in the causation of adenomas (iniation) and their subsequent development into invasive carcinomas (progression). It is clear from migration studies that environmental influences are again important factors in the initiation of adenomas, but genetic factors are known to play a role in a proportion of cases. Excluding the familial polyposis syndromes, 'cancer families' have been identified in whom an increased susceptibility to colorectal cancer, not accounted for by shared

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environmental factors, is accompanied by a high incidence of adenomatous polyps in the large bowel, a trait which shows an autosomal dominant mode of inheritance (Burt et al, 1985). Inheritance of this trait may have a more significant role in the pathogenesis of adenomatous polyps and colorectal cancer than has hitherto been appreciated.

In populations at high risk for carcinoma, adenomas are very common, with autopsy incidence rates of up to 50%. Clearly therefore, only a small minority of adenomas undergo malignant transformation. Much effort has been spent on the important task of identifying selective markers of high risk, both within the individual lesion and in subsets of patients. Adenomas are histologically divisible according to the predominant pattern of glandular architecture, into tubular, villous and mixed tubulo-villous types, but the three represent variants of the same neoplastic process, and cytologically they are very similar. All have the potential for malignant transformation by undergoing the progressive changes of dysplasia, in which histological changes of increasing cellular atypia, architectural disorder and aberrant cellular differentiation occur. All adenomas can, by these criteria, be regarded as circumscribed foci of dysplastic epithelium (Jass, 1987), and by convention, histopathologists often attempt to assess the degree of dysplasia in order to predict the risk of malignant

transformation, The highly subjective nature of this assessment has been emphasised by Brown et al, (1985), but the fact remains that those lesions showing severe dysplasia have a higher risk of progression to malignancy. The fact that dysplasia can occur in flat mucosa, for example in long-standing ulcerative colitis, has led to the concept of the 'dysplasia-carcinoma' sequence, advocated by some to supersede that of the adenoma-carcinoma sequence, (Jass, 1987).

In addition to dysplasia, two other factors have been identified which are associated with malignancy in adenomas. Firstly, the size of an adenoma correlates closely with the risk of malignancy - lesions less than 1 cm in diameter show less than 2% risk, whilst those greater than 2 cm carry up to 50% risk. Secondly, the architectural pattern of villous diferentiation carries a risk of approximately 40% of malignancy compared with 5% for pure tubular adenomas, and an intermediate level of risk for mixed tubulo-villous lesions. Villous adenomas are in general larger than the tubular forms, but even when this influence of size is taken into account, their tendency for malignant change is significantly higher. In a large series of adenomas studied colonoscopically, adenomas of 5mm or less showed a relatively even distribution throughout the large bowel, whereas there was a significantly higher incidence of larger lesions in the sigmoid colon (Morson et al, 1983). Indeed, when

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size, growth pattern and degree of dysplasia are considered, a close correlation occurs between the distribution in the colon of 'high risk' adenomas and colorectal cancer (Matek et al, 1986).

Adenomas and carcinomas coexist not only within the same populations but frequently in individual patients. 30% of surgical resection specimens for carcinoma show the presence of one or more adenomas, and such patients are at higher risk of development of metachronous carcinomas (Bussey et al, 1967). When adenomas coexist with carcinoma, they show a higher degree of dysplasia than those seen in patients without carcinoma (Heald & Bussey, 1975).

Further strong histopathological evidence for the adenoma-carcinoma sequence is provided by the occurrence of residual benign elements within colorectal carcinomas. This feature is seen in approximately 15% of cases overall, but significantly more frequently in lesions at an early clinical stage, which have not penetrated the bowel wall (Dukes' stage A). Histologically, the residual adenomatous elements show approximately equal occurrence of tubular, villous or mixed tubulo-villous morphology. However, as the incidences of these respective patterns in adenomas not associated with carcinoma are 75%, 15% and 10%, this is a further indication of the apparently greater propensity of villous lesions to undergo

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malignant transformation (Morson, 1984).

If carcinoma of the colorectum were to arise de novo from normal colonic mucosa with any degree of frequency, one would expect there to be descriptions of incidentally discovered small carcinomas of less than 0.5 cm in diameter, both in surgical resection specimens and in autopsy studies. This is not the case, and in contrast, early invasive carcinoma is commonly seen occurring within a pre-existing adenoma. This is regarded as supportive evidence that the great majority of cancers arise by this mechanism.

Perhaps the strongest evidence for a direct transition from adenoma to carcinoma comes from study of the uncommon hereditary condition familial adenomatous polyposis (FAP). There are a number clinical variants of this condition, but all show autosomal dominant mode of transmission. Patients develop hundreds of adenomatous polyps throughout the gastro-intestinal tract, but most notably in the large intestine. Adenomas usually become clinically apparent in adolescence and early adulthood, and without treatment invariably carcinoma supervenes in the large bowel, usually by the age of 40 years. Whilst this condition is uncommon, it is a most valuable model for the study of colorectal cancer in general, because apart from their occurrence in large numbers, there are no discernible clinical or pathological differences

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between the lesions of FAP and their sporadic counterparts. The various histopathological types of adenomas are found in very similar proportions to those occurring sporadically. The evolution of lesions can be traced histologically from the earliest changes affecting a single crypt, through the formation of an adenoma with increasing degrees of dysplasia to the development of a typical invasive adenocarcinoma. Study of individual lesions in patients with this condition (as well as the rare cases of sporadic adenomas in which treatment is refused) has allowed an approximate estimate to be made of the time scale of the adenoma-carcinoma sequence. Most adenomas never develop malignant change, but in those which do, the transition seems to occur over a protracted time scale of at least 5 years.

1.4.3 Genetic mechanisms

Over the years, many attempts have been made to identify early changes in the colonic mucosa which may relate to the development of neoplasia. Examination of mucosa adjacent to carcinomas has revealed a range of alterations in antigen expression (Skinner and Whitehead, 1981), type of mucin secretion (Williams, 1985), mitotic activity (Romagnoli et al, 1984) and pattern of DNA synthesis (Lipkin, 1974), but the associations may be coincidental or consequential to neoplasia rather than of aetiological significance. Recently, technological advances in the field of molecular biology have been

applied to the study of the fundamental genetic mechanisms of colorectal carcinogenesis. Investigations have focussed on the genome at various levels, from the identification of cytogenetic abnormalities in tumour cells, to investigation of the role of oncogenes and the alterations which can occur in their regulatory mechanisms.

Analysis of ploidy in adenomas and carcinomas by flow cytometry of paraffin-processed tissues has shown that approximately 60% of colorectal carcinomas show DNA aneuploidy compared with around 10% or less of adenomas (Quirke et al, 1986). Cytogenetic analysis of individual tumours has identified non-random chromosomal abnormalities in carcinomas, in particular involving deletions of 5q (Herrera et al, 1986), and 17p (Muleris et al, 1986). In 1971, Knudson proposed that both dominantly-inherited cancer susceptibility of the FAP type and the sporadic form of carcinoma may be due to recessive mutations occurring at the same gene. For cancer to develop, both alleles must be lost - in sporadic cases both by somatic events, whilst in familial cases one allele already carries a mutation through the germ line, and only one somatic change is needed in the homologous allele. There is recent evidence to support this hypothesis. Bodmer et al (1987) have mapped the FAP gene to a locus on chromosome 5q, near bands 5q21-q22, and in an accompanying paper, Solomon et al (1987)

demonstrated partial or complete loss of an allele in the 5q31-q34 locus in 20% of sporadic colorectal cancers. These authors have proposed that becoming recessive for the FAP gene at this locus is a key event in the development of both the sporadic and familial forms of cancer.

A similar approach reported by Fearon et al (1987) yielded important results which are, however, to some extent discordant. Following several reports of non-random chromosomal deletions involving chromosome 17p in sporadic colorectal cancers, these workers discovered clonal loss in tumour cells only of one allele of 17p in 76% of a series of 34 such sporadic carcinomas. (Polymorphic gene probes for a number of other chromosomes were used, but chromosome 5 was not included; less frequent deletions were occasionally observed on other chromosomes). Interestingly, the 17p deletion was only found in 1 out of 30 benign adenomas and 1 out of 3 areas of residual adenoma from frank carcinomas. It was concluded that loss of a 17p allele occurs relatively late in the multi-stage process of colorectal carcinogenesis.

The same workers also reported in their paper results of experiments investigating the clonality of colorectal neoplasms. Using a Southern blotting technique with a methylation-dependent restriction endonuclease, they

studied restriction fragment length polymorphisms in DNA
from colonic tumours of female patients heterozygous for
a polymorphic locus on the X chromosome, and demonstrated
inactivation of the same X chromosome in all neoplastic
cells of the same individual tumours, including both
adenomas and carcinomas. This is good evidence for the
clonal nature of both adenomas and carcinomas.

There is evidence from a variety of sources which
indicates   that oncogene expression is abnormal   in
colorectal cancer. At present, much of the evidence is
fragmentary and its significance uncertain, but there
seems little doubt that both qualitative and quantitative
abnormalities of ras family oncogene expression occur.
Pulciani et al (1982) used the NIH3T3 transfection assay
to demonstrate the presence of Ki-ras transforming
sequences in DNA derived from 2 unmanipulated human
colonic carcinomas. Such transforming activity was absent
from the DNA from normal tissues of the same patients.
Whilst it was recognised that the NIH3T3 assay was an
insensitive technique, particularly for the detection of
oncogenes other than activated ras, other pieces of
evidence accumulated indicating a specific role for ras.
Spandidos and Kerr (1984) demonstrated a significant
elevation in the levels of ras mRNA transcripts in both
carcinomas and premalignant adenomas of colon compared
with normal colonic mucosa. Similar results were reported
by Slamon et al (1984) in a systematic survey of a large

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number of different human tumours including colorectal adenomas and carcinomas.

Increased <u>ras</u> transcript levels do not appear to be a consequence of gene amplification. Pulciani et al (1985) found no evidence of amplification of <u>ras</u> DNA sequences in 13 adenomatous polyps and 22 colonic cancers. However, there is evidence for increased expression at the protein level. Using immunoblotting techniques, Gallick et al (1985) detected elevated levels of the <u>ras</u> p21 protein product in extracts of colonic cancers compared with those from adjacent uninvolved mucosa. They also noted that levels of p21 appeared to be higher in carcinomas at an earlier clinical stage compared with advanced lesions which had metastasised.

A generalised decrease in the methylation state of DNA in both adenomas and carcinomas of colon has been reported (Goelz et al, 1985). DNA methylation occurs almost exclusively at cytosine-guanine dinucleotides. Its role is not fully understood, but it appears to be associated with down-regulation of gene expression, and conversely, a decrease in methylation is associated with gene activation. Methylation changes are a normal feature of cellular differentiation, and also occur in neoplasia, but it is uncertain whether they are an invariable property of tumours. Hypomethylation has been shown to inhibit chromosomal condensation, and may lead to mitotic

non-disjunction, resulting in a loss or gain of chromosomes. Clusters of hypomethylated sequences upstream of actively-transcribed genes (termed 'HTF islands') have been identified, and are believed to contain regulatory elements controlling gene transcription. Changes in the methylation state of these regions may have effects on levels of gene expression. It is perhaps significant in this regard that the Ha-ras oncogene shows a much higher transforming activity in the hypomethylated state (Borrello et al, 1987).

Mutational activation of ras has been sought by a variety of methods of varying sensitivity. Early studies relied on the NIH3T3 assay, and mutational activation detected relatively infrequently (reviewed by Barbacid, 1987). Alexander et al (1986) examined DNA derived from 9 primary colorectal carcinomas, and using a Southern blotting technique to detect restriction fragment length polymorphisms showed no evidence of mutations occurring at codon 12 of Ki-ras or N-ras. There was no evidence of rearrangement of either of these genes, but in one of their cases, deletion of a Ha-ras allele was reported. Heterozygosity of Ha-ras was also mentioned in a study by Yokota et al (1986) who reported apparent loss of one c-Ha-ras allele in a number of colonic cancers; it is not specifically stated in their report how commonly this abnormality was detected.

Recently, more sophisticated and sensitive methods have been applied to the detection of mutational activation of ras. Simultaneous reports from Bos et al and Forrester et al (1987) highlight a high incidence of activating mutations at codon 12 of the Ki-ras gene in colorectal cancers. Despite some remaining limitations to the sensitivity of both of the techniques applied, both studies reported a 30 - 40% incidence of the activating mutation. Furthermore, a similarly high occurrence of this mutation was present in dysplastic villous adenomas and residual adenomatous elements of the carcinomas studied. This suggests that the activating mutation occurs early in the development of malignancy.

Evidence for the primary involvement of other cellular oncogenes in colorectal cancer is less abundant, and although there are many indications of alterations in oncogene expression, some caution must be used in the interpretation of these data, particularly with regard to genes whose expression is cell cycle related. Increased expression may simply relate to increased growth fraction in tumours compared with normal mucosa (Torelli et al, 1987).

Several groups have reported alterations of c-myc expression - significantly elevated levels of c-myc mRNA transcript occur (Slamon et al, 1984; Erisman et al, 1985; Alexander et al, 1986; Sikora et al, 1987; Meltzer

et al, 1987), but only in a small minority of cases has gene amplification been identified, and gene rearrangement also seems to be uncommon. At the protein level, immunoblotting and immunohistochemical studies have described increased levels and altered distribution of the p62 c-myc protein product in colorectal carcinomas (Stewart et al, 1986; Sikora et al, 1987; Sundaresan et al, 1987). Bolen et al (1987) described increased tyrosine kinase activity of the protein product of the c-src oncogene, pp60-c-src, in colorectal carcinomas compared with normal colonic mucosa. It was suggested that this alteration may have a direct role in colonic carcinogenesis.

The current state of understanding of the processes of human colorectal carcinogenesis is far from comprehensive, but some parts of the puzzle are falling into place. The neoplastic process appears to commence with a clonal expansion from a single cell of the crypt, escaping from normal growth controls, and forming a small adenoma. Specific gene deletions on chromosome 5q and probably other loci may be important in initiating the process, and luminal carcinogens may play a potentiating role. By the stage of adenoma formation, a generalised diminution has occurred in the methylation state of DNA in the lesion, consequences of which may include an increased incidence of mitotic non-disjunction leading to gain or loss of chromosomes, and increased expression of

many genes, including oncogenes. During adenoma development, an infrequent but important event is the development of an activating mutation of the Ki-ras gene, probably a consequence of increased cell turnover and the further action of luminal carcinogens. Adenomas acquiring the activating ras mutation may increase in size and degree of dysplasia. Acquisition of the malignant phenotype may be associated with loss of tumour suppressor gene activity by deletion of chromosomes 5q or 17p.

This sequence of events is certain to be an oversimplification. For example, less than 50% of carcinomas have been shown to possess the activating mutation of Ki-ras, and only a similar proportion show the 5q deletion. It is likely that a considerable degree of heterogeneity exists between individual lesions at the detailed genetic level, but the techniques are now in place to examine the molecular pathology in experimental systems. The implications for diagnosis and treatment are likely to be far-reaching.

1.5 AIMS OF THE STUDY

Colorectal neoplasia is common in the UK and its high incidence poses problems in population screening and patient management. The detailed events of colorectal carcinogenesis are unknown, but the adenoma-carcinoma sequence is generally accepted in the large intestine, and the availability of appropriate surgical resection specimens affords the opportunity to study lesions at various stages in the evolution from normal mucosa to invasive adenocarcinoma. Increasing evidence of the important roles of oncogenes in carcinogenesis has accumulated, and activation of individual oncogenes at different stages may account for the multi-step process of colorectal carcinogenesis.

Several studies have indicated alterations in oncogene expression in neoplasms of the large intestine, but most have studied established malignant tumours, and have often involved investigation of DNA, RNA or proteins extracted from homogenates of solid tumours. The results of such studies represent an overall picture of expression in the total cell population in the tumour, but may be misleading. All tumours consist of highly heterogeneous populations of neoplastic cells and stromal cells, and it would be of great interest to determine any alterations in oncogene expression which might occur within individual cells of the tumour.

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An important aim of this study was to develop reliable methods of identifying oncogene expression at the individual cell level in tissue sections of colorectal neoplasms. It was of interest to compare two potentially useful methods, in situ hybridisation and immunohistochemistry. In situ hybridisation is a technique of great potential value in which messenger RNA transcripts of an expressed gene are detected in tissue sections by the hybridisation of a labelled complementary nucleic acid strand, making use of the highly specific nature of such interactions between the two strands. Appropriate DNA probes containing oncogene sequences were available. Immunohistochemistry is a longer established technique which utilises the specificity of binding of antibodies to particular epitopes in tissues, with subsequent detection usually by enzyme-linked methods. The availability of monoclonal antibodies with specificity to oncogene protein products allowed investigation of this method to be undertaken.

A flexible positive control system was also available in the form of rodent cell lines in culture which had been genetically manipulated to express certain oncogenes at artificially high levels.

Accumulating evidence for the importance of involvement of ras and myc oncogenes in the carcinogenic process in

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general, and in colorectal tumours in particular, prompted special attention being paid in this study to analysis of their patterns of expression. It was of particular interest to attempt to define alterations at different stages of the multistep process of carcinogenesis.

CHAPTER 2

IMMUNOHISTOCHEMICAL DETECTION OF RAS ONCOPROTEIN

SUMMARY

The monoclonal antibody Y13-259 was used to investigate
ras p21 distribution in human colorectal tissues by
immunohistochemistry. The specificity of Y13-259 was
confirmed by demonstration of its reactivity with a
control cell line (FHO5T1) which had been genetically
manipulated to express the mutationally activated human
Ha-ras oncogene at high levels. Reactivity was
significantly lower in the unmanipulated progenitor cell
strain (CHL). Immunoblotting experiments also confirmed
the specificity of Y13-259, demonstrating reactivity with
a single protein band of MW 21kDa in protein extracts of
FHO5T1 cells which was absent from extracts of CHL cells.
Formaldehyde fixation of tissues resulted in loss of Y13-
259 reactivity, so a series of acetone-fixed cryostat
sections of human colorectal tissues was investigated
using the ABC immunohistochemical technique. Colorectal
carcinomas showed a similar low intensity of staining to
normal mucosa, but adenomas showed consistently higher
staining intensity. A further series of similar tissues
was examined using periodate-lysine-paraformaldehyde-
dichromate (PLPD) fixation, which allowed use of paraffin
sections with improved morphology and retention of
immunoreactivity. Results of the frozen section series
were confirmed by titration experiments in which staining
was compared on serial sections at a range of
concentrations of Y13-259. The results suggest that

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elevated ras expression may be a significant early stage in the process of colorectal carcinogenesis, but that high levels of expression need not be sustained in the subsequent conversion to invasive carcinoma.

2.1 MATERIALS AND METHODS

2.1.1 Cell lines and tissues

Cell lines

A variety of cell lines were maintained in culture in which oncogene expression had been augmented by genetic manipulation. The cell lines were maintained in Dulbecco's modification of minimum Eagle's medium containing 10% newborn calf serum, and penicillin and streptomycin. The cell lines were originally produced by insertion of oncogenes in high expression vectors into non-transformed early or late passage rodent fibroblast cell lines by the calcium phosphate transfection technique (Spandidos & Wilkie, 1984). The non-transformed parental cell strains are contact-inhibited, non-tumourigenic and in the case of early passage cells, diploid or near-diploid, whilst the transfectants are transformed, aneuploid and give rise to rapidly-growing tumours in immune-deprived animals.

The ras-expressing transformed cell line FHO5T1 was derived by transfection of an early passage fibroblast cell strain from Chinese hamster lung, designated CHL, using the plasmid construct pHO5T1 (Spandidos and Wilkie, 1984). This plasmid has the mutationally-activated human T24 Ha-ras oncogene (originally derived from bladder carcinoma cells) flanked by transcriptional enhancing sequences derived from the SV40 virus, ensuring a high

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level of transcription of the oncogene. The resulting FHO5T1 cells are fully transformed, lack contact inhibition and are aneuploid. They induce rapidly-growing, infiltrative metastasising tumours when inoculated into immune-deprived mice. Histologically, these tumours have the appearances of undifferentiated spindle-cell tumours of fibrosarcoma type. Estimation of relative quantities of oncogene mRNA by dot-blot hybridisation has shown 20-50 fold increased Ha-$\underline{ras}$ transcript in FHO5T1 cells compared with the parental untransformed CHL cells (Spandidos and Wilkie, 1984; Wyllie et al, 1987).

Cytocentrifuge preparations

Monolayer cultures of cell lines were gently disrupted by treatment with 0.02% EDTA and 0.1% trypsin. The resulting cell suspension was washed in culture medium containing 10% newborn calf serum followed by a further wash in serum-free medium. The cells were counted, and their concentration adjusted to 1×10^5 cells per ml. 100ul of cell suspension was placed in each chamber of the cytocentrifuge. The suspension was spun onto gelatine-coated slides for 5 mins at 60 rpm. The slides were air-dried, then fixed in the appropriate fixative (see section 2.1.3). Slides were either used immediately or stored in sealed polythene bags at $-20\,^{o}C$.

Cell pellets for paraffin processing

Monolayer cultures were gently disrupted with 0.1% trypsin and 0.02% EDTA as above. Approximately 5×10^6 cells were used for each preparation. The cells were suspended in 5 ml of the appropriate fixative, and allowed to fix for 15 min at 4^oC. After washing in phosphate-buffered saline, the cells were resuspended in 100ul PBS and 100ul 4% low-temperature melting agarose at 40^oC. After the pellet had set (5 minutes at 4^oC) it was finally fixed for a further 15 mins in fixative, washed in PBS and processed to paraffin wax.

Animal tumours

FHO5T1 cells inoculated into immune-deprived mice engender rapidly-growing malignant tumours which are a valuable source of authentic tumour tissue which is known to express the T24 ras oncogene at high levels. The regime of immune deprivation and inoculation is described in detail by Wyllie et al (1987). Briefly, 6 week old mice were thymectomised, irradiated and treated with cytosine arabinoside. 0.1 ml of a cell suspension containing $1-2 \times 10^7$ FHO5T1 cells was inoculated subcutaneously. Animals were killed when tumours of 1 cm or greater were present (usually within 1-2 weeks). At autopsy, portions of tumour were taken and snap frozen in liquid nitrogen.

Human colorectal tissues

Human colorectal tissues were received on ice within minutes of resection in the operating theatre. Resection specimens were opened and examined immediately, and samples of tissue taken from tumour and normal mucosa. Samples were placed in plastic vials and 'snap' frozen in liquid nitrogen. Special attention was paid to the presence of polyps of the colonic mucosa. If present and of sufficient size, they were bisected, one fragment retained for diagnosis by paraffin section histology, the other frozen. In some cases, blocks were taken for fixation in special fixatives at this stage. The remainder of the resection specimen was pinned onto a cork board, allowed to fix in formalin, and blocks subsequently taken for paraffin section histology in the usual way. This allowed confirmation of the diagnosis, histological assessment of tumours, Dukes' staging , and was a source of paraffin sections for various techniques.

Frozen tissues aimed to include a block of the tumour-normal interface for frozen section immunohistochemistry. For protein extraction, samples were taken which aimed to consist entirely of viable tumour tissue. Frozen sections (6um) were cut, mounted on gelatine-coated slides, fixed in acetone for 15 minutes at room temperature and air dried. If necessary, sections were stored at this stage in sealed polythene bags at -20°C.

2.1.2 Immunoblotting for ras oncoproteins

Protein extraction

15-20 ml 0.02% EDTA was added to cell culture bottles containing cell monolayers and the cultures were gently agitated to detach the cells. After washing once in phosphate-buffered saline (PBS), the supernatant was decanted and 1 ml of lysis buffer was added (25mM TRIS pH 8.0, 50 mM NaCl, 0.5% NP40 (Nonidet p40), 0.1% SDS, 0.02% PMSF (phenylmethylsulphonylfluoride)) and refluxed vigorously through a 21 gauge needle. The lysate was then centrifuged at 10,000 rpm for 30 min at 4°C, and the supernatant collected. Although it was often necessary to store the lysate (200ul aliquots at -20 to -70°C), best results were obtained with freshly prepared specimens. It was noted that levels of immunoreactive p21 diminished very significantly in lysates stored for more than a few weeks.

Specimens of human colorectal tissues sampled as soon as possible after removal in theatre were 'snap' frozen in liquid nitrogen, and stored at -70°C for variable periods. A fragment of frozen tissue (approximately 1 gram) was rapidly chopped as finely as possible with a scalpel, and the fragments immediately transferred to a glass homogeniser with 1 ml of lysis buffer as above. After homogenisation, the lysate was centrifuged as above, and the supernatant collected.

Measurement of protein concentration

Protein concentration of lysates was measured using the Bio-Rad assay system. Dye reagent was freshly prepared and filtered according to the manufacturer's instructions. A range of known protein standards was prepared from stock bovine serum albumin solution (1mg/ml). To 100ul of standards and unknown samples was added 5ml of dye reagent, and the mixture vortexed. Samples were held at room temperature for 5 mins to 1 hr, and optical density measured at 595nm by spectrophotometry.

SDS-PAGE (SDS-Polyacrylamide gel electrophoresis)

A 15% acrylamide separating gel and 5% stacking gel were prepared as described in Appendix 2. Equal volumes of protein lysate and sample buffer (4% SDS, 40% glycerol, 125mM TRIS pH 7.0, 10mM EDTA, 10% B-mercaptoethanol, 0.04% bromophenol blue) were mixed, and the samples denatured by boiling for 5 mins. Variable amounts of protein up to 500ug were loaded onto each track of the gel. One or more tracks of the gel were reserved for a mixture of molecular weight standards, either as unconjugated proteins (Sigma MW markers kit) or as proteins conjugated to dyes of different colours ('Rainbow Markers', Amersham). Each electrode of the vertical Bio-Rad electrophoresis apparatus was immersed in electrode buffer (0.01% SDS, 0.05M TRIS pH 7.0, 0.4M glycine). Separation was allowed to occur at 10 mA

current until the sample fronts had entered the separating gel, after which it was increased to 30mA.

Transfer to nitrocellulose

After electrophoretic separation had occurred in the polyacrylamide gel (usually approximately 3 hours) the gel was removed from the electrophoresis apparatus and washed for 30 minutes with gentle agitation in transfer buffer (0.01M TRIS, 0.1M glycine, 20% methanol). A nitrocellulose sheet was cut to size (approximately 14x14 cm), and soaked in transfer buffer with two similar sized pieces of Whatman chromatography paper (thickness 0.92 mm). The nitrocellulose filter was applied to the gel, and air bubbles excluded by gentle rolling with a glass rod. The gel and nitrocellulose were sandwiched between the two sheets of chromatography paper, with a sponge pad on each outer side. This assembly was held in place in a perspex clip, and inserted into the transfer apparatus. 3 litres of transfer buffer were added, and electrophoretic transfer allowed to occur overnight at 50V, with constant stirring.

Staining of gel (total protein)

1. Kenacid blue method.

After electrophoretic transfer, the gel was stained for a minimum of 2 hours (or overnight) in kenacid blue solution (2g/l kenacid blue, 10% glacial acetic acid, 45% methanol). Destaining was performed overnight in 7%

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glacial acetic acid, 45% methanol.

2. Silver staining method

The gel was washed thoroughly, preferably overnight, in at least 5 changes of DDW, then stained in ammoniacal silver nitrate solution, freshly prepared as follows. 2.8 ml ammonia AR solution (specific gravity 0.88), and 3.75 ml 1M NaOH was made up to 100ml with DDW. 8 ml of freshly-prepared 20% silver nitrate solution were added dropwise with stirring, and the solution made up to 200 ml with DDW. The gel was stained for 15 minutes with rapid stirring, after which it was extensively rinsed in DDW through 3 changes over 30 minutes. Staining was developed in formaldehyde/citric acid solution (0.5ml 40% formaldehyde, 50 mg citric acid to 1 litre DDW). Bands developed over a 10 minute period after which the reaction was stopped by immersion in tap water.

Immunostaining of nitrocellulose

After transfer, the nitrocellulose filter was washed in TRIS-buffered saline (TBS), 3 changes, 10 minutes each. Non-specific protein-binding sites were blocked by incubation in 3% milk protein solution (Cadbury's Marvel) in TBS for 30 minutes. The filter was then washed in TBS, and if necessary could be stored at $-20^{o}C$ in a sealed polythene bag at this stage. Incubation with 5ml of the appropriate primary antibody took place in a sealed polythene bag, at the appropriate dilution (most commonly

1 in 100) in TBS containing 5% heat-inactivated neonatal calf serum (HINCS) and 0.5% Tween 20. After 2 hours incubation at room temperature, it was washed in excess TBS/HINCS/Tween, 3 changes over 15 minutes. The filter was then incubated with the appropriate biotinylated secondary antibody 1 in 100 in TBS/HINCS/Tween for 1-2 hours and washed as before.

Horseradish peroxidase detection system

The filter was then incubated with 5 ml biotinylated preformed complex of streptavidin-peroxidase (Amersham) 1 in 100 in TBS for 30 mins. After final washing in TBS (3 changes) the reaction was developed with diaminobenzidine (DAB) solution (as in section 2.1.3). When complete (usually within 5 minutes), the reaction was terminated by washing in DDW. Nitrocellulose filters were then dried and stored in the dark in a sealed polythene bag.

Alkaline phosphatase (Bluegene) detection system

The filter was incubated with 5 ml AP-streptavidin (BRL), 1 in 1000 in TBS pH 7.4 for 15 minutes, in a sealed polythene bag. After washing in TBS pH 7.4 (3 changes, 15 minutes), the filter was washed in TBS pH 9.5 containing 50mM MgCl$_2$, for 15 minutes. It was then incubated in a sealed polythene bag containing 5ml of fresh development buffer prepared according to the manufacturer's instructions (BRL). After 30 minutes incubation at room temperature in the dark, the reaction was terminated by

washing in DDW. In practice, staining of bands was
usually complete within 5 minutes. Stained filters were
then dried and stored in the dark in sealed polythene
bags.

^{125}I-Protein A autoradiographic detection system

The ^{125}I-Protein A autoradiographic detection system was
adopted particularly for tracing p21-ras in immunoblots
from human colorectal tissues, and was used for its
increased sensitivity over the enzyme-linked detection
systems. Immunostaining of nitrocellulose filters was
slightly altered from the method described above in order
to conform more closely with the method of DeBortoli et
al (1985). This method differed in using an overnight
incubation of the nitrocellulose filter with Y13-259 at 1
in 200 dilution at 4 ^{o}C, and replaced TBS/HINCS/Tween with
NTE-NP40 (50mM TRIS pH 7.4, 150mM NaCl, 2mM EDTA, 0.1%
NP40). Washing and incubation stages were carried out in
an ice-cold shaking water bath. The secondary antibody
(unconjugated goat anti-rat IgG - Sigma) was applied at 1
in 500 dilution in NTE-NP40 containing 3% BSA for 90
mins. After washing in NTE-NP40 (2 changes, 10 mins),
^{125}I-protein A (Amersham), specific activity
approximately 30 mCi/mg, 30ul in 30ml NTE-NP40 + 3% BSA
was added and incubated for 1 hr. After washing twice in
NTE-NP40 as before, the filters were air-dried, wrapped
in cling-film and exposed to X-ray plates overnight or
for 3 days.

2.1.3 IMMUNOHISTOCHEMISTRY

2.1.3.1 Use of monoclonal antibody Y13-259 to p21ras

Monoclonal antibody Y13-259

Y13-259 is a monoclonal antibody prepared from rats bearing tumours induced by the Harvey strain of the murine sarcoma virus (Furth et al, 1982). It precipitates p21 protein species encoded by both Harvey and Kirsten strains of the virus, and reacts with Ha, Ki and N-ras p21 products of the cellular oncogene. It reacts with the products both of the normal and mutationally-activated ras gene.

The hybridoma cell line was a gift from Dr D.A. Spandidos. The antibody was prepared from culture supernatants by ammonium sulphate precipitation as described by Furth et al (1982). The protein concentration of such preparations was measured by the Mancini radial immunodiffusion method, and was generally around 2.5 mg/ml (J. Going, personal communication). Aliquots of 200ul were prepared and stored at -70°C. Care was taken to ensure that aliquots were frozen and thawed only once before use for immunohistochemistry.

Fixation conditions and tissue processing

The following fixatives and fixation conditions were used for post-fixation of cryostat sections and cytocentrifuge

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preparations in an effort to optimise immunostaining while retaining good morphology of cells and tissues:

1. Acetone (100%), 15 minutes, room temperature.
2. Formaldehyde (4% in PBS), 15 minutes, room temperature.
3. Glutaraldehyde (0.1% in 0.1M sodium cacodylate pH 7.4), 15 minutes, room temperature or 4 $^{\circ}$C.
4. Methanol (100%), 15 minutes, room temperature.

Paraffin sections of FHO5T1 tumours and human colorectal tissues were prepared according to routine histological methods. Tissues were fixed in 4% formaldehyde for 24 hours, dehydrated through graded alcohols, cleared in chloroform and wax-impregnated at 56 $^{\circ}$C over a 20 hour processing schedule. 5 micron paraffin sections were cut, mounted on glass slides and air dried at 56 $^{\circ}$C.

In an attempt to unmask antigens by proteolytic digestion, dewaxed rehydrated sections were incubated in 0.1% trypsin, 0.1% calcium chloride in 10 mM TBS pH 7.6 for 15 minutes at 37 $^{\circ}$C before applying antibody.

PLPD fixation

Periodate-lysine-paraformaldehyde-dichromate (PLPD) fixative was prepared using a modification of the method described by McLean and Nakane (1974). It contains 5mM sodium metaperiodate, 37.5mM lysine-HCl, 1% paraformaldehyde, 18mM phosphate buffer pH 7.4 and 5%

potassium dichromate. It was freshly prepared from stock on the day of use (see Appendix 3). Tissue blocks approximately 5mm thick were fixed for a minimum of 18 hours at 4°C in the dark. They were then washed in running tap water overnight to remove dichromate pigment. Tissue blocks were then dehydrated, cleared and embedded in paraffin wax as above, and sections cut and dried at 56°C.

Second stage antibodies

For the indirect immunoperoxidase technique, horseradish peroxidase (HRP)-conjugated sheep anti-rat Ig (Amersham) and HRP-conjugated rabbit anti-rat Ig (Dako) were used, with very similar results. For the peroxidase anti-peroxidase (PAP) method, unconjugated rabbit anti-rat Ig and monoclonal rat PAP-complex (Sera Lab) were used. For the streptavidin-biotin-peroxidase (ABC) method, the secondary antibodies employed were biotin-conjugated rabbit anti-rat Ig (Vector) or biotin-conjugated goat anti-rat Ig (Sigma). Faint anti-human activity encountered in the latter antibody was abolished by absorption with acetone-dried human liver tissue, and human immunoglobulins (Cohn fraction II, Sigma). For immunogold silver staining, 5nm gold particle conjugated rabbit anti-rat IgG (Janssen) was used.

Indirect immunohistochemical methods

Cytocentrifuge preparations, acetone-fixed cryostat

sections or rehydrated PLPD-fixed paraffin sections were washed in TRIS-buffered saline pH7.6 (TBS) for 5 mins. Non-specific binding of primary antibody was blocked by application of 10% normal human serum in TBS (NHS/TBS) for 5 mins. Monoclonal antibody Y13-259 was applied at concentrations varying from 5ug/ml to 100ug/ml in NHS/TBS. In general, 25ug/ml was most satisfactory for tissue sections (a dilution of 1 in 100 of purified culture supernatant) whilst 5ug/ml was used for cytocentrifuge preparations. The antibody was applied for a minimum of 1 hour (lengthier incubations including overnight incubation conferred no appreciable advantage). After washing in TBS and blocking as above, the appropriate secondary antibody was applied, diluted 1 in 50 in NHS/TBS for 1 hr. After thorough washing in TBS, the final peroxidase-conjugated reagent (where appropriate) was applied 1 in 50 in TBS for 30 minutes. In the case of the ABC streptavidin-biotin complex method, this was preformed complex of biotinylated streptavidin-HRP (Amersham) and was applied 1 in 200 in TBS for 15 minutes. After thorough washing the colour reaction was developed with DAB solution (1mg/ml diaminobenzidine in 50mM TRIS-HCl pH 7.6, containing 10mM imidazole, activated with 1ul/ml 100 vols H_2O_2 immediately prior to use). The reaction was allowed to develop for up to 2 mins after which it was stopped by washing in tap water. Thereafter, sections were counterstained in haematoxylin, dehydrated through graded

alcohols, cleared in xylene and mounted.

Immunogold silver staining

Paraffin sections of PLPD- and formaldehyde-fixed tissues were incubated with primary and gold-conjugated secondary antibodies as described above. They were then washed thoroughly in deionised distilled water, and treated with 250ul of silver enhancement solution (equal volumes of IntenSE 2 enhancer and initiator solutions prepared immediately before silver enhancement, according to the manufacturer's instructions (Janssen)). The slides were allowed to develop for up to 15 minutes, the reaction being monitored visually by microscope. They were then washed in tap water, counterstained, dehydrated and mounted as previously.

Controls

Positive controls were included in early staining runs, and negative controls in all runs. Positive controls consisted of cytocentrifuge preparations of FHO5T1 cells fixed in acetone, or acetone-fixed frozen sections of FHO5T1 mouse tumours. For negative controls, each slide was accompanied by one in which the primary antibody was substituted by NHS/TBS and the remainder of the procedure carried out as above.

2.1.3.2 Monoclonal antibody RAP-5

The monoclonal antibody RAP-5 is one of a series of

murine monoclonals raised against a synthetic octapeptide composed of amino acids 10 - 17 of the mutated Ha-<u>ras</u> oncogene. Its originators described optimal reactivity in immunohistochemical techniques with formaldehyde-fixed tissues (Thor et al, 1984). RAP-5 was donated as ascites fluid by Dr J. Schlom. Immunoblotting and immunohistochemistry with RAP-5 were performed essentially as described above. For immunoblotting, RAP-5 was applied at dilutions of 1:100 and 1:1000. For immunohistochemistry, titration experiments indicated optimal dilutions to be in the range between 1:10,000 and 1:20,000. The streptavidin-biotin HRP complex method was used as above; the secondary antibody was biotin-conjugated sheep anti-mouse IgG (Amersham) used at 1:50 dilution. Cytocentrifuge preparations of CHL and FHO5T1 cells were prepared as described above, and post-fixed in either acetone or 4% formaldehyde for 15 minutes. Paraffin sections of formaldehyde-fixed FHO5T1 tumours and human colorectal tissues were used for immunohistochemistry.

2.2 RESULTS

2.2.1 <u>ras</u> <u>expression</u> <u>in</u> <u>genetically</u> <u>manipulated</u> <u>cells</u>

2.2.1.1 Immunoblotting for p21 with Y13-259

Protein extracts from FHO5T1 and CHL cell lines were separated by SDS-PAGE, transferred to nitrocellulose and probed with monoclonal antibody Y13-259. Results are illustrated in Fig. 2.1. Y13-259 detects a single protein band of molecular weight approximately 21kDa present in extracts of FHO5T1 cells but either absent or detectable at much reduced quantity in equivalent extracts from CHL cells. Approximately equal amounts of protein were loaded on each track of the gel, determined either by formal protein concentration assay, or by demonstration of approximately equal loading of gels by kenacid blue staining.

The single 21kDa protein band was detectable when Y13-259 was used at 1:100 and 1:1000 dilution. Protein extracts from FHO5T1 tumours growing in immune-deprived mice showed a major single band at the same position, but there were also a number of fainter bands of higher molecular weight proteins. The major protein was detectable using either horseradish peroxidase or alkaline phosphatase detection systems, at approximately equal intensity. A formal comparison of the sensitivities of the two detection systems was carried out.

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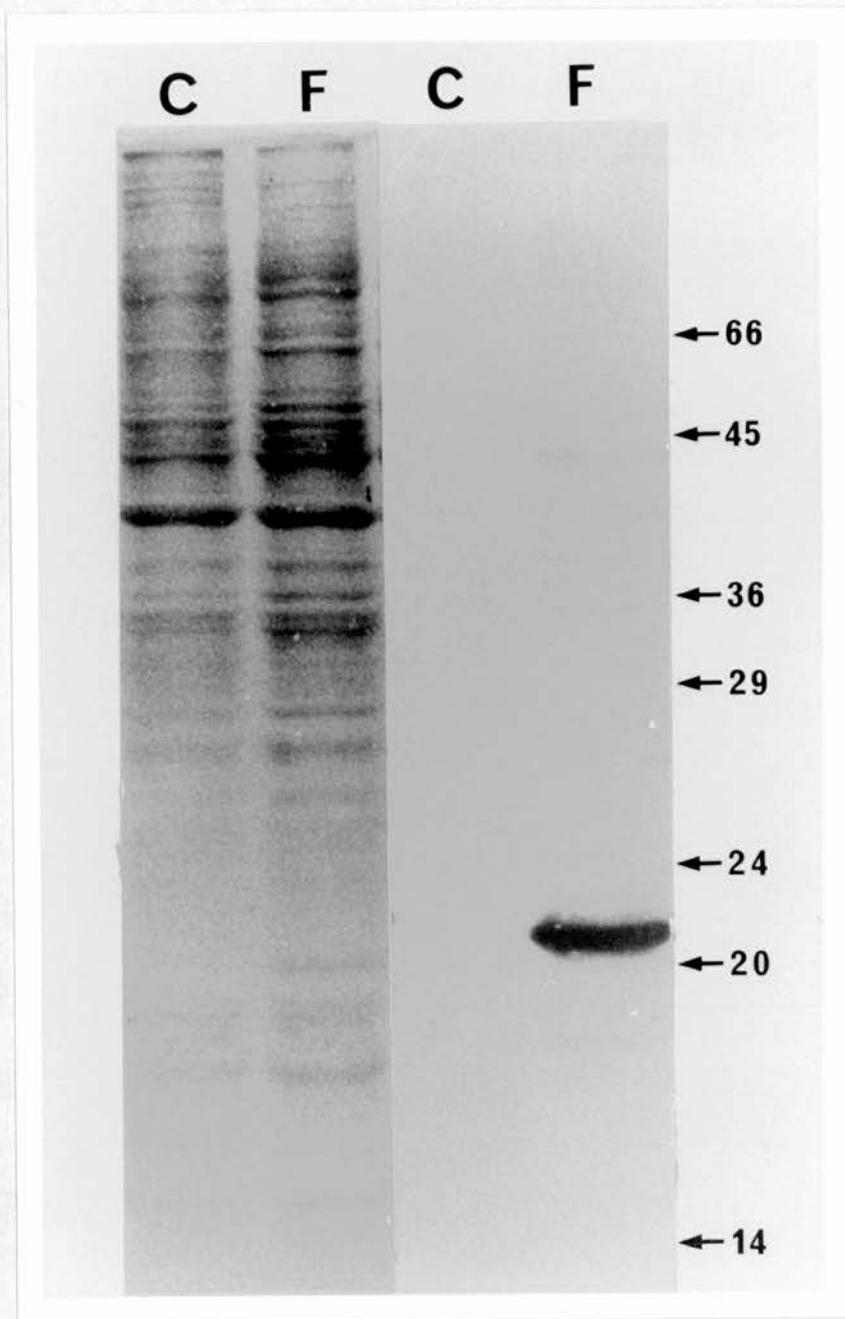
Fig. 2.1 Electrophoresis of proteins from FHO5T1 cells
(F) and CHL cells (C). The left panel shows the original
polyacrylamide gel stained with kenacid blue, indicating
similarly loaded tracks. The right panel shows an
immunoblot stained with Y13-259 at 1 in 100 dilution,
detected by the alkaline phosphatase method. Mobility of
molecular weight markers is indicated. A single band of
21 kDa appears in the FHO5T1 extract, but is absent from
the CHL extract.

Comparison of HRP and AP detection systems

Duplicate sets of doubling dilutions of FHO5T1 protein extract were separated on the same polyacrylamide gel and transferred to nitrocellulose. The nitrocellulose was cut in two, and the immunodetection systems were compared. The results are shown in Fig. 2.2. There was no significant difference in the sensitivity of the two systems. Both detected p21 in undiluted protein extract and 1:2 dilution, but not at 1:4 or 1:8. The 'cut-off' at 1:4 was equally complete in both systems. In further experiments, the AP system was usually selected, as the stained filters showed less non-specific background, were not light-sensitive, and were easier to photograph.

Immunoblots with RAP-5

Immunoblots of protein extracts of FHO5T1 and CHL cell lines were prepared as described and probed with the murine monoclonal antibody RAP-5, at 1:100 and 1:1000 dilutions. The results are shown in Fig. 2.3. Multiple protein bands were detected at the higher concentration, but no specific band was detectable in the 21 kDa region, where there was no difference observed between CHL and FHO5T1 protein extracts.

2.2.1.2 Immunohistochemistry

Fixation conditions for immunohistochemistry with Y13-259 are critical. Early experiments employing the fixatives in common use for immunochemical techniques demonstrated

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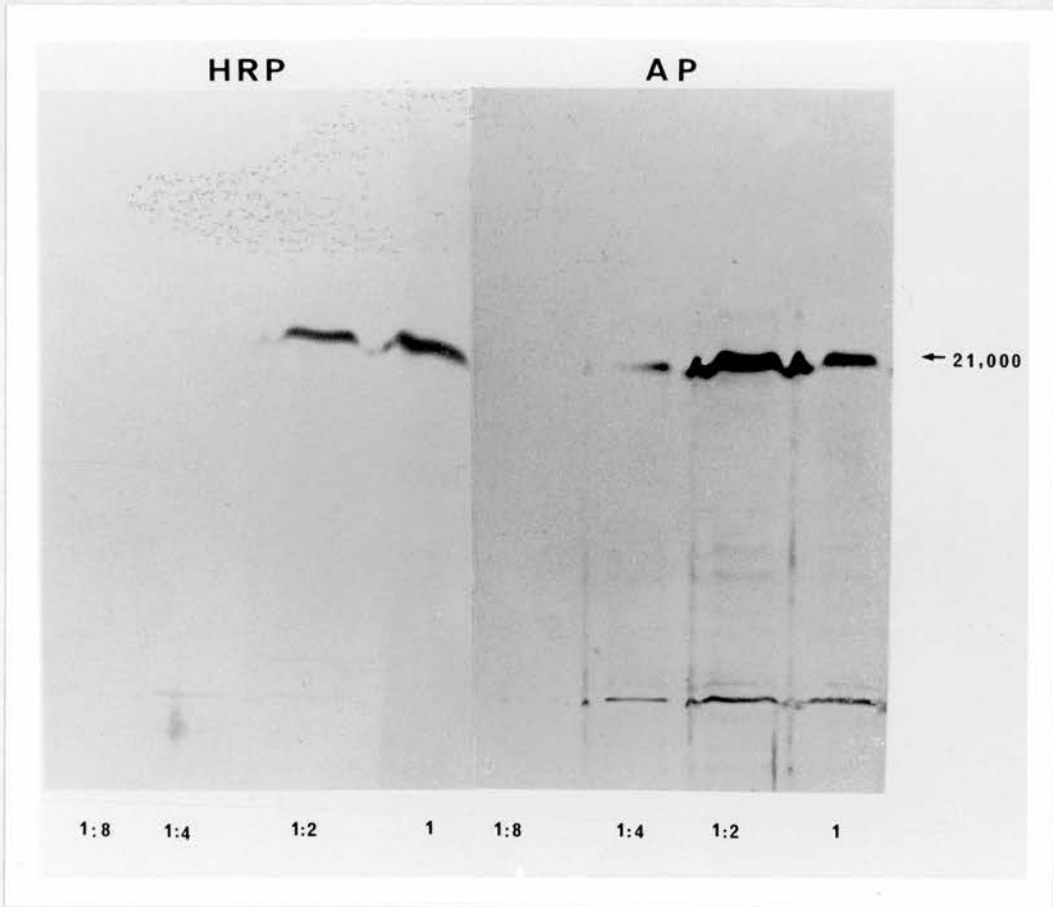
Fig. 2.2 Comparison of horseradish peroxidase (HRP) and
alkaline phosphatase (AP) detection systems. Doubling
dilutions of a protein extract of FHO5T1 cells were run
on PAGE and transferred to nitrocellulose in duplicate.
The immunoblots were stained with Y13-259 at 1 in 100
dilution, and HRP and AP detection systems compared. A
single band of 21 kDa is detected with both systems, but
a threshold is observed in which the band is barely
detectable at 1 in 4 dilution, and is absent at 1 in 8.
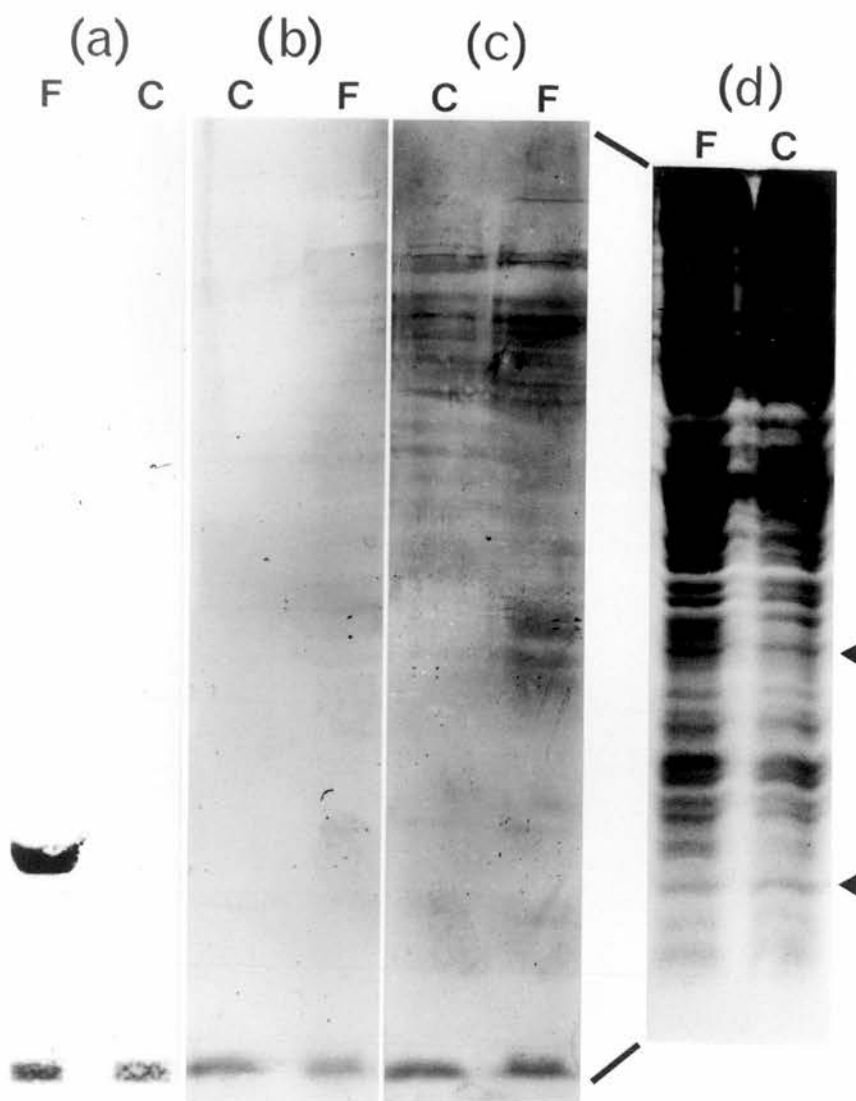
Fig. 2.3 Comparison of Y13-259 and RAP-5 in the detection
of ras p21 product. Proteins from FHO5T1 (F) and CHL (C)
were separated by PAGE. In (a) an immunoblot is stained
with Y13-259 at 1 in 100 dilution. The 21 kDa band is
present in FHO5T1 but absent from CHL. In (b), RAP-5
staining of identical extracts is shown at 1 in 1000
dilution, and (c) at 1 in 100 dilution. (d) shows
similarly loaded tracks in the original PAGE gel stained
with kenacid blue. The positions of 14 kDa and 24 kDa
marker proteins are indicated by arrowheads.

that the aldehyde-based fixatives formaldehyde and glutaraldehyde almost completely abolished immunoreactivity with Y13-259. This was true of cytocentrifuge preparations of cell lines, paraffin sections of agar-embedded cell pellets, and sections of FHO5T1 tumours from immune-deprived mice. Proteolytic digestion of such preparations by trypsin failed to restore useful amounts of immunoreactivity. It would appear that Y13-259 is therefore not suitable for use with routinely processed biopsy material. Similarly, methanol and ethanol were unsatisfactory fixatives. Acetone fixation was satisfactory in preserving immunoreactivity with Y13-259, but often at the expense of very poor morphological preservation of the tissues for histology.

Using acetone post-fixation, all cells of the FHO5T1 cell line showed strong immunoreactivity with monoclonal antibody Y13-259, both in cytocentrifuge preparations (Fig 2.4) and in frozen sections of agar-embedded cell pellets. Staining was completely absent from negative controls in which Y13-259 was omitted from the immunohistochemical method, and replaced by normal serum. Staining of FHO5T1 cells was readily obtained using the less sensitive indirect immunoperoxidase technique, but its intensity was amplified by the use of the ABC method. In contrast, staining was absent from the great majority of the non-transfected parental cell strain CHL. A small

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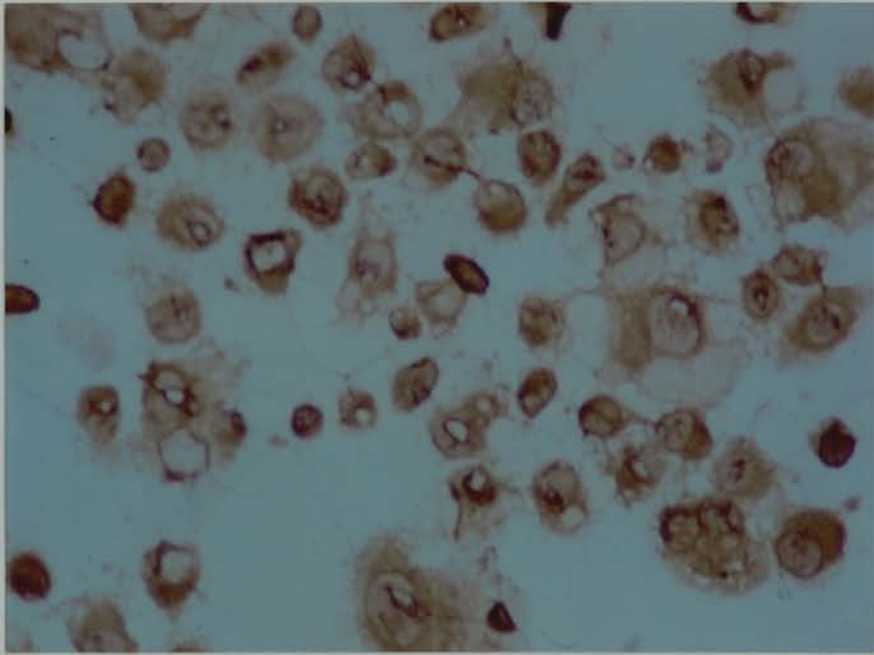
Fig. 2.4 Acetone-fixed cytocentrifuge preparation of
FHO5T1 cells stained with Y13-259. All cells show strong
immunoreactivity.
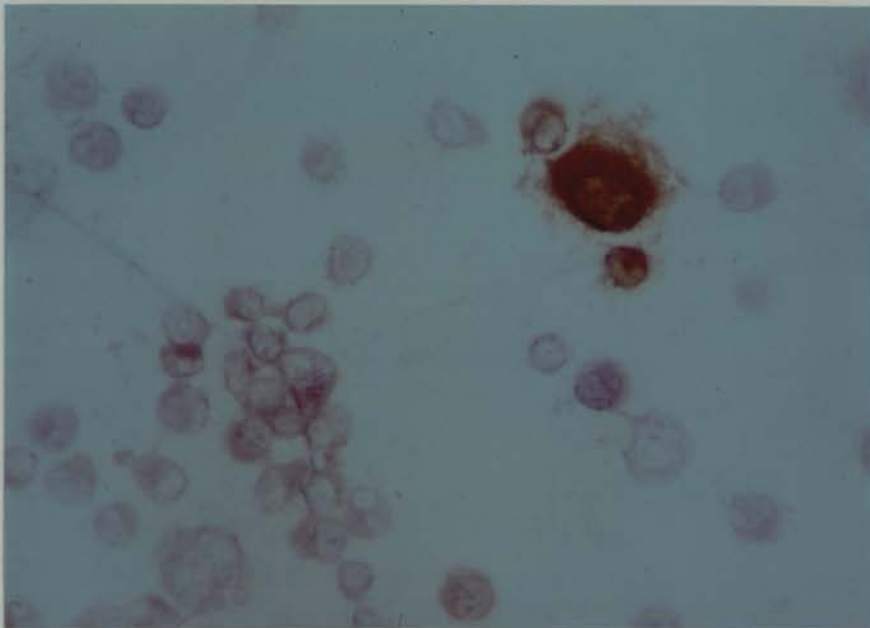
Fig. 2.5 Acetone-fixed cytocentrifuge preparation of CHL
cells stained with Y13-259. The great majority of cells
are negative, but a small proportion shows strong
staining.

minority, variable between 5% and 15%, showed strong specific staining which was absent from negative controls (Fig. 2.5).

Positive staining of FHO5T1 cells from cell lines in culture in general showed a membrane-associated distribution of stain, seen in frozen sections of cell pellets, although some cytoplasmic stain was seen in some cells in most cases, and staining was not as crisp as seen in PLPD-fixed specimens (see below). Membrane staining was not seen in acetone-fixed frozen sections of FHO5T1 tumours growing in immune-deprived mice. In these tumours, strong staining of all tumour cells was observed, showing a cytoplasmic distribution. This was in marked contrast to the adjacent normal murine tissues such as skeletal muscle which showed almost complete absence of staining, thus serving as a valuable form of in-built negative control in these sections (Fig 2.6).

PLPD fixation

The specialised fixative periodate-lysine-paraformaldehyde-dichromate (PLPD) was initially used in an effort to solve some of the problems encountered with acetone-fixed frozen sections. The FHO5T1 positive control system was used to investigate the properties of this fixative, both with respect to the pattern and cellular distribution of immunoreactivity with Y13-259, and to the preservation of optimal cellular and tissue

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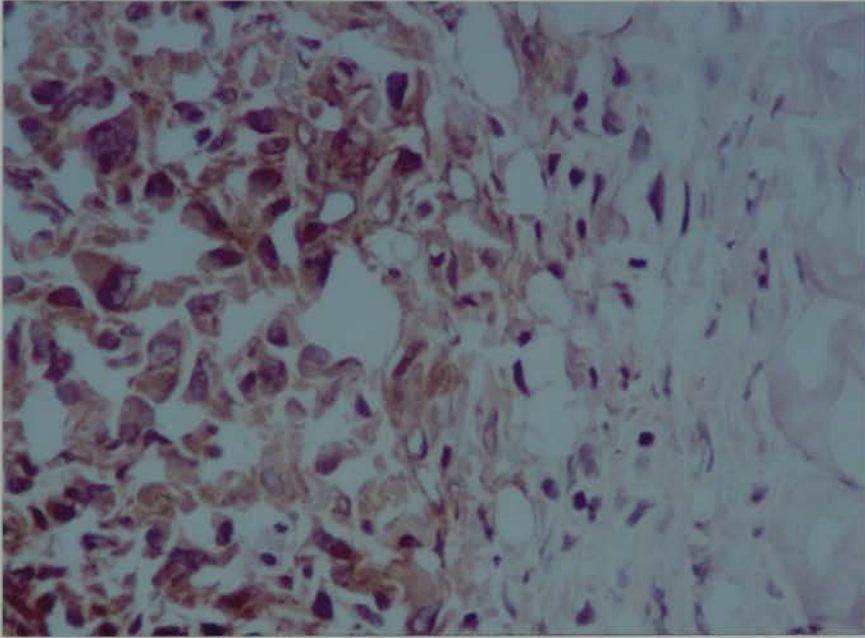
Fig. 2.6 Acetone-fixed frozen section showing margin of
FHO5T1 tumour in immune-deprived mouse; section stained
with Y13-259. The tumour cells show strong
immunoreactivity, whilst adjacent fibrous tissue and
skeletal muscle are negative.

morphology. Paraffin sections of agar-embedded pellets of
FHO5T1 and CHL cells fixed in PLPD were stained with Y13-
259 using the ABC immunohistochemical technique as
previously applied to frozen sections. As before, all
FHO5T1 cells showed strong staining, whilst CHL cells
showed almost no staining. The FHO5T1 cells showed a
distinctly cell-membrane associated pattern of staining
(Fig 2.7). Although this pattern of staining had been
previously observed in frozen sections of similar agar-
embedded preparations, the definition was much superior.
Paraffin sections of FHO5T1 tumours in immune-deprived
mice also showed good retention of immunoreactivity with
Y13-259, with all the tumour cells showing strong
staining. Such staining was often clearly linear cell-
membrane associated, but usually some cytoplasmic
staining was also observed (Fig. 2.8). Such membrane-
associated distribution had not been previously seen in
frozen sections of such tumours. Background staining was
minimal with PLPD-fixed FHO5T1 tumours, and histological
preservation was greatly superior to that of acetone-
fixed frozen sections, and in many ways comparable with
formaldehyde-fixed paraffin sections. However, shrinkage
artefact was slightly more prominent, and chromatin
clumping often occurred in the cell nuclei which stained
densely haematoxyphilic. PLPD can therefore not be
regarded as an ideal substitute for formaldehyde.
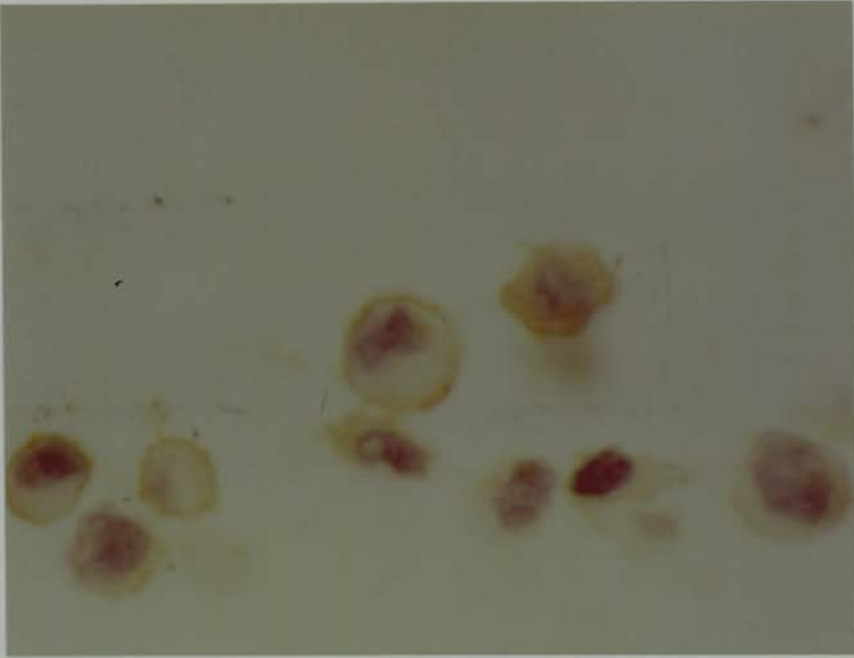
Fig. 2.7 Paraffin section of PLPD-fixed agar-embedded
pellet of FHO5T1 cells stained with Y13-259. Distinct
membrane-associated staining is present.
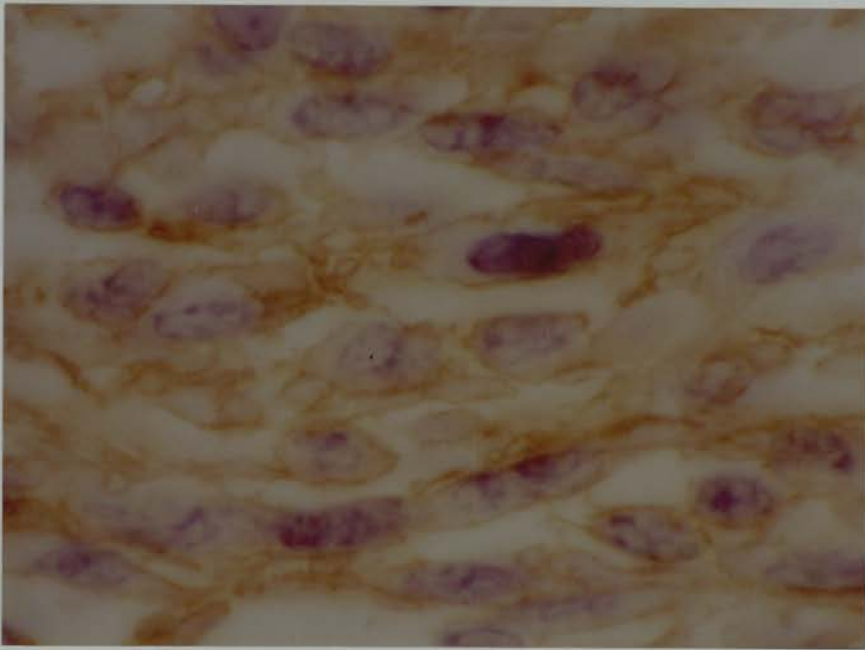
Fig. 2.8 PLPD-fixed paraffin section of FHO5T1 tumour in
immune-deprived mouse. Cell-membrane associated staining
is obtained with Y13-259.

2.2.2 ras expression in human colorectal tissues

2.2.2.1 Immunohistochemistry with Y13-259

Frozen section technique

In contrast to the FHO5T1 positive control system, Y13-259 showed generally low levels of specific staining in human colorectal tissues, and it was necessary to employ the more sensitive ABC immunohistochemical detection system. Fixation conditions for human tissues did not vary from those found to be optimal in the control system, and acetone post-fixed frozen sections of fresh (or stored frozen) colorectal tissues were required.

Several methods of immunohistochemical staining were compared in an attempt to optimise the sensitivity and specificity of the technique. The details of the methods are outlined in section 2.1.3.1. For each technique, a range of concentrations of primary and secondary antibodies was used, and the influence of variation of incubation times was investigated. The conditions described in section 2.1.3.1 are optimal.

The indirect method, in which a peroxidase-conjugated secondary anti-rat IgG antibody is used, was unsatisfactory, as rather faint staining was obtained which was insufficiently distinct from the background levels of staining to be meaningful. This was in contrast to the situation in the FHO5T1 system, in which this method was perfectly adequate.

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The non-amplified streptavidin-biotin system, in which a biotinylated anti-rat IgG secondary antibody was followed by incubation with a simple peroxidase-conjugated streptavidin stage, similarly produced insufficiently distinct staining for reliable assessment.

The peroxidase-anti-peroxidase (PAP) technique, in which an unconjugated anti-rat IgG secondary antibody provides a bridge between the primary antibody and a peroxidase-anti-peroxidase complex, was even less satisfactory, in that background levels of staining were high, and no specific staining could be observed, even after overnight incubation with the primary antibody.

Most satisfactory was the amplified streptavidin-biotin-complex (ABC) technique, in which a biotinylated secondary antibody is followed by reaction with a pre-formed complex of streptavidin-biotin-peroxidase. With ABC, specific staining could be obtained which was of higher intensity than that of any of the other methods.

A recurring problem in acetone-fixed frozen sections of human colorectal tissues, but which did not arise in the FHO5T1 control system in either cytocentrifuge preparations or sections of mouse tumours, was that of high levels of background staining of stromal and other tissues. In early attempts this was so high as to render assessment of specific staining impossible, and various experimental strategies were employed in an effort to reduce it. The normal serum of a variety of animal

species was used as a blocking agent prior to incubation of sections with both primary and secondary antibody. Sera of swine, sheep, goat, rabbit and human were compared, and human serum was found to significantly reduce background staining. Absorption of the primary antibody with acetone-dried human liver tissue was also felt to offer some reduction in background levels. Despite these measures, background staining varied to some extent between staining experiments, and in some cases remained unacceptably high. Staining of such cases was repeated until it was judged that a reliable result was obtained.

Staining patterns in human colorectal tissues

Frozen sections of human tissues were all obtained from surgical resection specimens, the majority for neoplastic disease, but some for inflammatory or diverticular disease. Normal colonic mucosa from resections for non-neoplastic conditions showed very similar staining patterns to uninvolved mucosa from surgical resection margins and from directly adjacent to colorectal neoplasms. Staining was generally of low intensity and uniformly distributed throughout the cytoplasm of cells at all levels of the crypt and at the epithelial surface (Fig. 2.9). In many cases, such staining was of low intensity, but judged to be specific.

Adenocarcinomas showed a variable staining pattern. In
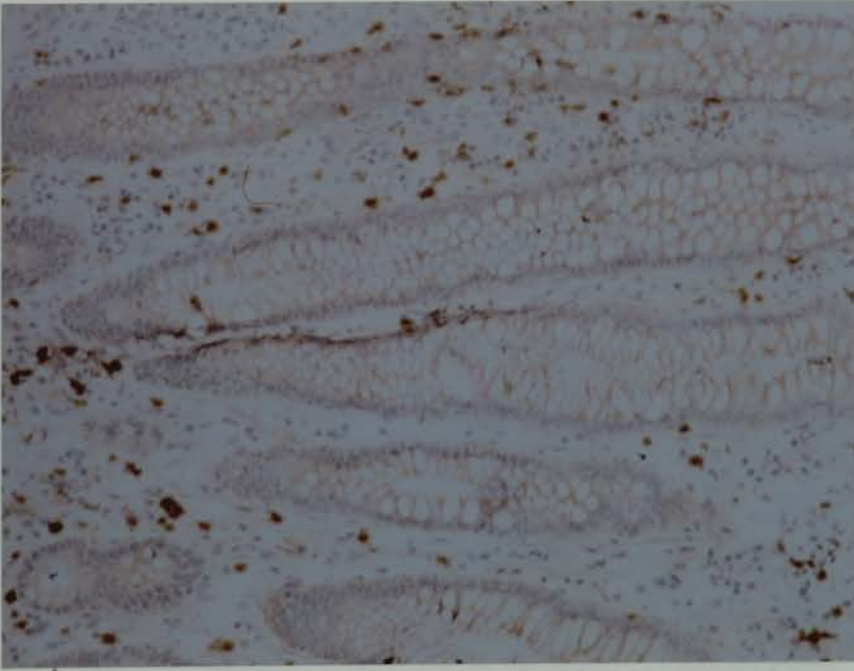
Fig. 2.9 Acetone-fixed frozen section of normal colonic
mucosa stained with Y13-259. Faint but specific
immunoreactivity is seen in all epithelial cells
throughout the crypt. (Macrophages in the lamina propria
show endogenous peroxidase activity).
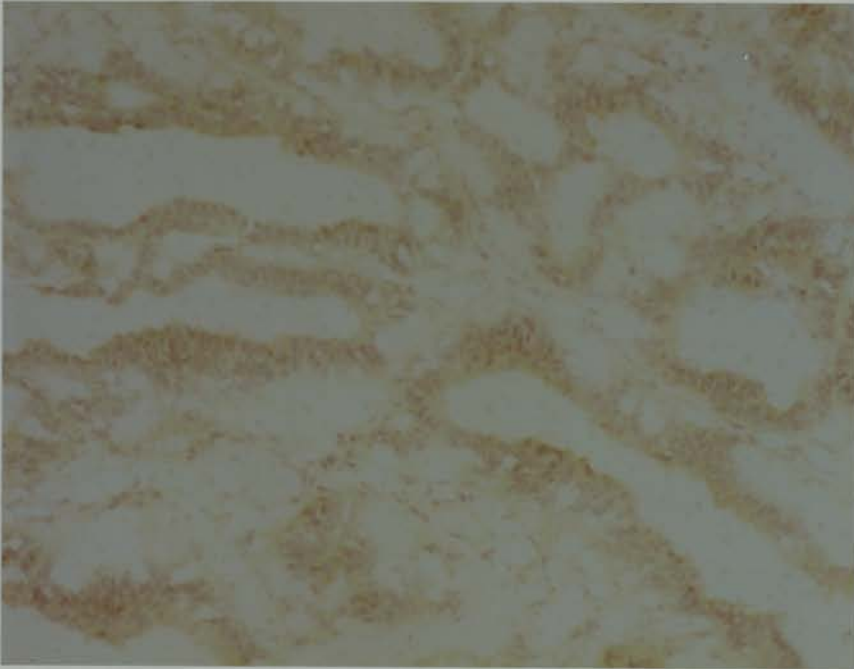
Fig. 2.10 Acetone-fixed frozen section of colonic
carcinoma stained with Y13-259. This case shows faint
staining of carcinoma cells.

the majority, staining was negative or indistinguishable from background levels. A proportion showed specific staining of moderate intensity (Fig. 2.10), and a small number stained with high intensity. All carcinoma cells showed staining of similar degree within the same section, and the distribution was cytoplasmic.

Adenomas showed the highest intensity of staining (Fig. 2.11). This was cytoplasmic in location, and more intense than that seen in the adjacent uninvolved mucosa, although a sharp interface was never seen, and the greatest intensity of staining was usually seen at the centre of the adenoma.

Frozen section series

While recognising that immunohistochemistry can not be regarded as a quantitative technique, the differences in staining intensity described above were striking, and a simple 3-stage grading system was devised. Staining which was similar to the level of background was designated as negative or equivocal (+/-); faint specific staining was designated moderate (+); intense staining was awarded ++. A small series of assorted colorectal tissues was stained with the Y13-259 ABC technique and assessed in this way. Grading was performed independently by two observers, and in more than 75% of cases, there was agreement in assessment of staining intensity. Where different assessments were made, the differences did not

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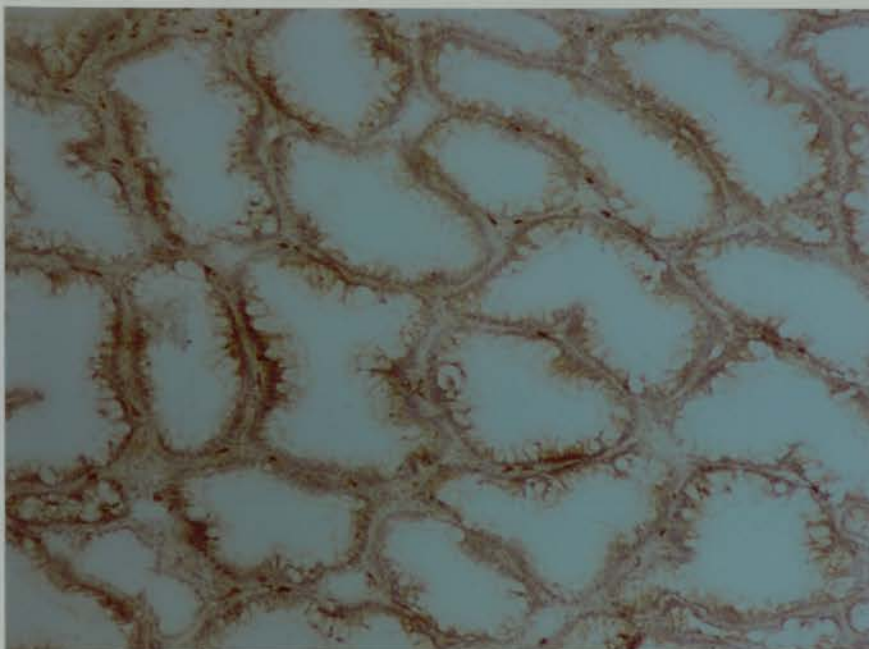
Fig. 2.11 Acetone-fixed frozen section of tubular
adenoma. Strong staining of epithelial cells is seen with
Y13-259.
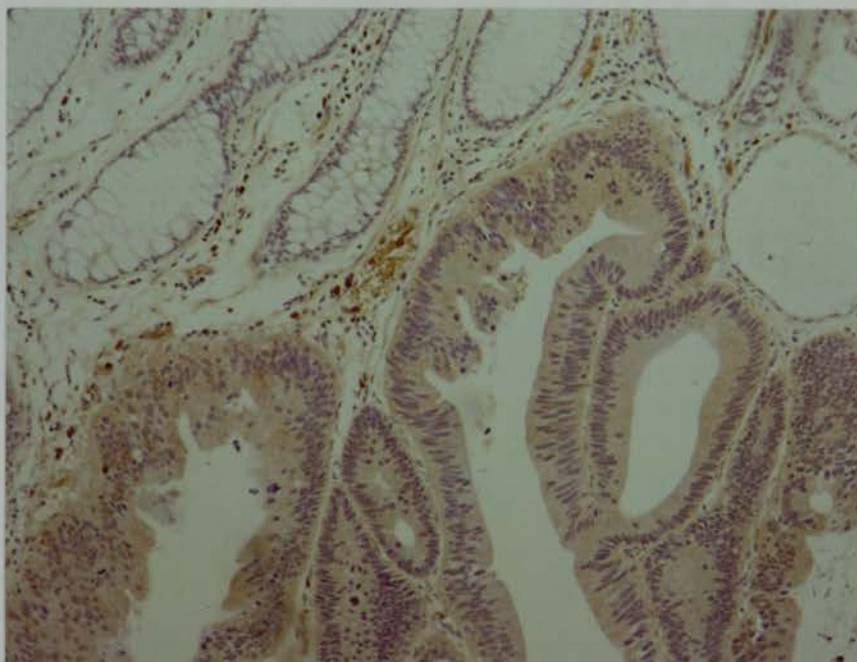
Fig. 2.12 PLPD-fixed paraffin section of the interface
between an adenoma and non-neoplastic colonic mucosa,
stained with Y13-259. The cells of the adenoma show
strong immunoreactivity, whilst non-neoplastic mucosa is
negative.

affect the statistical significance of the results. The results shown in Table 2.1 are a consensus. The intra-observer variability of the author was tested at an interval of several months from the first assessment and consistency was achieved in more than 80% of cases.

TABLE 2.1

Tissue:	Number of cases - staining intensity with Y13-259		
	+/-	+	++
Carcinomas	14	10	2
Adenomas	0	2	5
Normal adjacent to carcinoma	8	9	1
Normal adjacent to adenoma	2	4	1
Normal non-neoplastic	4	3	0

Table 2.1. Adenomas show a significantly greater intensity of staining compared to carcinomas (p<0.01) and all normals (p<0.002)(Four-fold Table Test). Carcinomas showed no significant difference in staining intensity from normal mucosa.

There was no correlation found between staining intensity and histological differentiation, Dukes' stage and site of carcinomas, and histological subtype, degree of dysplasia and site of adenomas.

PLPD-fixed paraffin sections

Paraffin sections of human colorectal tissues fixed in PLPD showed specific immunoreactivity with Y13-259 which together with the superior definition of staining pattern and tissue morphology represented a major improvement on the frozen section method. Furthermore, background staining which had been a continual problem was very significantly diminished in comparison with acetone-fixed frozen sections. For this reason, consistency of staining between experiments was much improved. Best results were obtained with fresh tissue trimmed to thin blocks and placed immediately in fixative, but results were very satisfactory with tissues which had been stored at $-70\,^{\circ}C$ for up to 3 years and then placed in the fixative. Immunoreactivity remained in tissues which had been fixed for 6 weeks in PLPD and subsequently processed to paraffin. No diminution in immunoreactivity was evident in PLPD-fixed paraffin-embedded tissue blocks stored at room temperature for several months.

The pattern of staining in colorectal tissues was in general similar to that described in frozen sections. In normal mucosa, staining was faint or absent; when present, it titrated out rapidly (see titration experiments below). In contrast adenomas showed strong specific staining. This was cytoplasmic in distribution, and convincing membrane-localised staining was not observed. However there was a sharp transition between the

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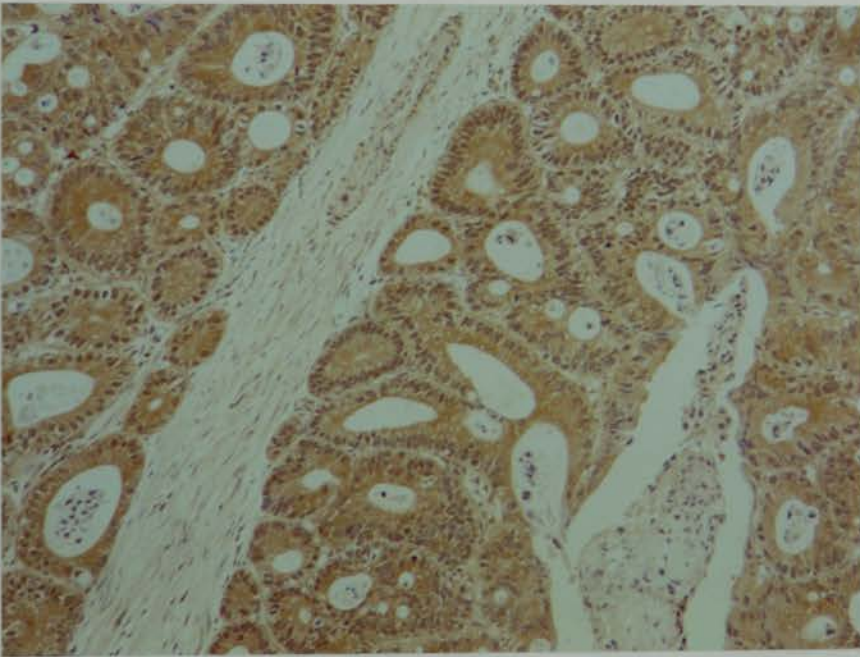
Fig. 2.13 PLPD-fixed paraffin section of adenocarcinoma
of colon. All carcinoma cells show immunoreactivity with
Y13-259, clearly distinguished from the stroma which
shows very low levels of staining.
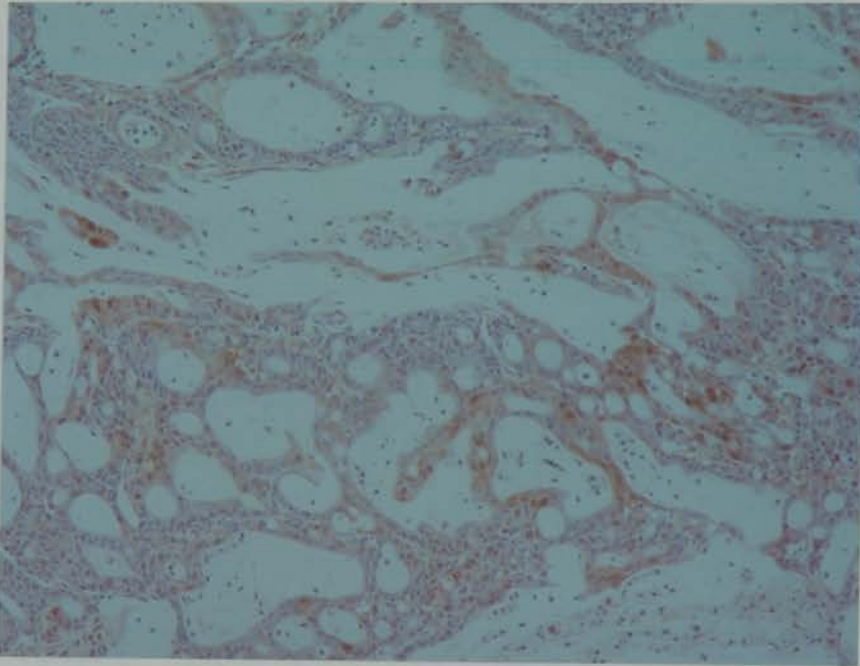
Fig. 2.14 PLPD-fixed paraffin section of rectal
adenocarcinoma. Heterogneity of staining of tumour cells
is seen, and some cells show nuclear staining.

positively-staining cells of the adenoma and the negative cells of the adjacent uninvolved mucosa (Fig 2.12). The principal difference in staining reaction between PLPD-fixed material and frozen sections was in the carcinoma group. Almost all carcinomas showed specific immunostaining which was cytoplasmic in distribution, with occasional cases in which nuclear staining was observed. Membrane staining was not observed. Staining was usually quite intense, and clearly distinguished from the low level of background staining (Fig. 2.13). In a few cases, heterogeneity of tumour cell staining was seen, the majority of cells showing staining but a small proportion negative (Fig 2.14). This was an uncommon pattern, and in most cases all carcinoma cells were positive.

Titration experiments

The improvement in reproducibility of staining intensity between experiments afforded by PLPD-fixed material allowed a more quantitative approach to the assessment of differences in staining between normal, adenomas and carcinomas. In a limited series of cases, the primary antibody Y13-259 was applied at a range of dilutions from 1 in 10 to 1 in 500. Sections were assessed independently by two observers, and for each case the highest dilution was recorded at which specific staining could still be observed. In practice it was found that a sharp threshold is generally observed, and agreement was reached in more

than 75% of cases. In cases of disagreement, a consensus was arrived at, and the results are shown in Table 2.2.

TABLE 2.2

			Dilution of Y13-259				
Tissue	Total	Neg	1:10	1:25	1:50	1:100	1:500
Normal	15	7	2	5	1	0	0
Adenoma	15	0	0	0	3	7	5
Carcinoma	19	0	0	4	10	4	1

Adenomas show positive staining to higher dilutions of Y13-259 than carcinomas and normal mucosa (p<0.01). Carcinomas show staining to higher dilutions than normal mucosa (p<0.01) (Chi square test).

2.2.2.2 Immunoblotting for p21 in colorectal tissues
Alkaline phosphatase detection system
Immunoblots of protein extracts from human colorectal tissues were prepared as above, but the AP detection system was insufficiently sensitive to detect p21 ras with Y13-259 with any degree of reliability. Occasionally, faint bands were obtained at approximately 21 kDa in some specimens, but in most cases were absent. A range of higher molecular weight protein bands were detected which were absent from extracts of FHO5T1 and CHL cell lines. Their nature is uncertain, but they may include histone proteins and immunoglogulins. Detection

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of the smaller amounts of p21 present in spontaneous
human tumours required modification of the technique.

Immunoprecipitation of p21 from tumour extracts was
attempted using a modification of the technique of
Gallick et al (1985). Briefly, this method involved
reaction of protein lysates with Y13-259, incubation with
rabbit anti-rat IgG, followed by treatment with
Staphylococcal protein A. Precipitates were then washed,
and immunoblotting carried out as above. The technique
proved unsuccessful, and was not pursued.

^{125}I-Protein A detection system

The ^{125}I-protein A method was adopted for use with
extracts of human colorectal tissues because of its
reported increased sensitivity over enzyme-linked
methods. Initial experiments followed the method of
DeBortoli et al (1985), as described in section 2.1.2,
but this was subsequently modified to increase the
amounts of protein loaded on each track to up to 1 mg.
Using this method, bands were present in the 21 kDa range
in samples which had yielded no detectable p21 by the AP
detection system.

Protein extracts of FHO5T1 cell lines and tumours showed
diminution in amounts of immunoreactive p21 when stored
at -70 ^{o}C for more than a few weeks, although in some
cases p21 could be detected in reduced amounts after more

than 12 months. Extracts of human tissues required to be used within a few days of preparation, as p21 was undetectable in samples of extract stored for longer. Freshly prepared extracts of tumours which had been stored as frozen whole tissues showed retention of p21 indistinguishable in amount from fresh tumours.

In protein extracts of human colorectal tissues, whether from normal mucosa, adenoma or carcinoma, bands were detectable in the 21 kDa region. There was some variation in the intensity between different samples, but consistently greater intensity was not found in extracts of any particular tissue type. Protein extracts from human colorectal tissues showed p21 bands as doublets, in contrast to the single band observed in FHO5T1 extracts. Some variation in intensity of inividual components of the p21 doublet was noted, but a consistent pattern was not found to correlate with any particular tissue type. Results of a typical experiment are shown in Fig. 2.15.

2.2.2.3 Immunohistochemistry with RAP-5 in colorectum
Paraffin sections of formaldehyde-fixed human colorectal tissues were stained using RAP-5 with the ABC method. The pattern of staining differed significantly from that seen with Y13-259. Although normal mucosa generally showed low levels of staining, its presence and intensity was variable between cases and between different fields of the same case. Adenomas and carcinomas showed positive

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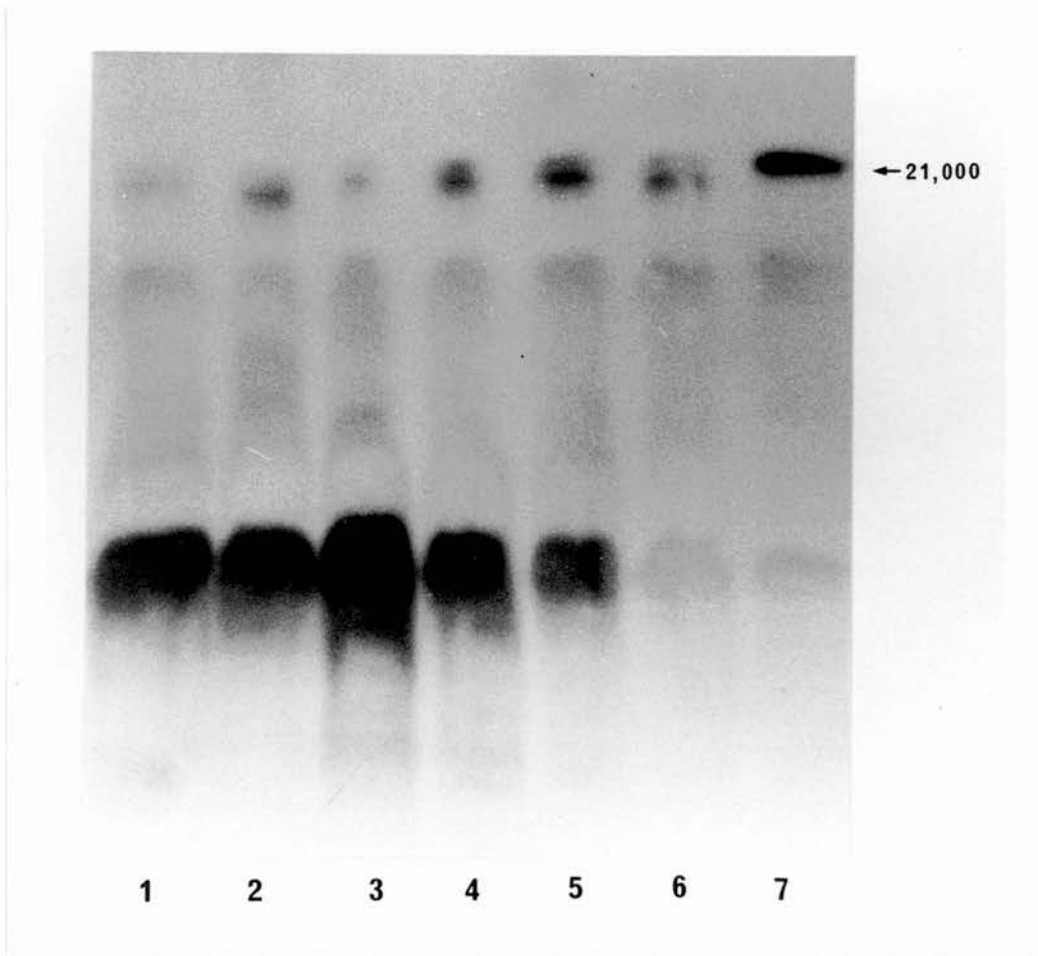
Fig. 2.15 Immunoblot of protein extracts of human
colorectal tissues, separated by PAGE and detected on
nitrocellulose by Y13-259 with ^{125}I-protein A. Tracks
loaded with equivalent amounts of the following protein
samples: 1 - adenoma, 2 - carcinoma (same patient); 3 -
carcinoma; 4, 5 and 6 - normal mucosa, adenoma and
carcinoma respectively (same patient); 7 - extract of
mouse FHO5T1 tumour (positive control for p21 ras). The
marker indicates the 21 kDa position. A single p21 band
is detectable in the extract of FHO5T1 tumour; p21 bands
are present in all human colonic tissues, a faint doublet
appearance discernible in several specimens.

staining, and although there was often a considerable degree of heterogeneity of staining within individual lesions, there was no differential staining between infiltrative and non-infiltrative areas. Relatively high levels of staining occurred in stroma and muscularis propria, and serosal cells were consistently strongly positive. A range of other human tissues including normal skin, bronchial mucosa, cartilage, smooth and striated muscle and lymphoid tissue also stained positively with RAP-5.

2.3 DISCUSSION

Profound phenotypic changes follow the transfection of transcriptionally enhanced mutationally-activated Ha-ras oncogene into early-passage rodent fibroblasts. A contact-inhibited non-tumourigenic diploid (or near-diploid) cell strain (CHL) is transformed to a cell type (FHO5T1) giving rise to rapidly-growing infiltrative metastasising aneuploid tumours (Spandidos and Wilkie, 1984). Quantitative RNA dot-blot analysis indicates such transfectants to have up to 50-fold elevation in Ha-ras mRNA transcript compared with the parental cells, representing approximately 0.8% of total cellular mRNA (Wyllie et al, 1987). The observation that T24 Ha-ras transfectants derived from different parental cells (such as rat fibroblast line 208F) acquire a similar range of aggressive properties is in support of the proposal that the constellation of phenotypic changes is a consequence of elevated levels of ras expression, and not merely a coincidental feature of individual clones studied. The genetic manipulation which engendered the FHO5T1 cell line is of course an artificially-contrived situation, and there may be few naturally-occurring analogues amongst spontaneously-occurring human neoplasms. This does not diminish from the fundamental interest in this system as a means of investigating phenotypic changes due to ras expression, or from its value as a positive control system for immunohistochemical and other studies

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of _ras_ p21 expression. One of the strengths of the system is the opportunity it affords to study known _ras_-expressing cells both in pure population in cell culture, and growing as authentic malignant tumours in animals.

The monoclonal antibody Y13-259 was raised in rats bearing tumours induced by the Harvey strain of the murine sarcoma virus (Furth et al, 1982) and it has been shown to immunoprecipitate human p21 _ras_ products of Ha, Ki and N-_ras_ genes (Furth et al, 1987). Its specificity for p21 has been documented in a variety of different reports using different techniques (e.g. Gallick et al, 1985; Mulcahy et al, 1985) and the epitope it recognises seems to be located in the region of constancy of the three p21 species, probably amino acids 70-81 (Lacal and Aaronson, 1986). Using the FHO5T1 test system, the present study has provided further confirmation of the specificity of Y13-259 for the _ras_ gene products by immunoblotting and immunohistochemistry.

The major problem with the use of Y13-259 in immunohistochemistry has been the apparent lability of this epitope under conditions of strong aldehyde fixation. In the experience of the writer and that of others (Kerr et al, 1985; Ghosh et al, 1986), formaldehyde fixation completely abolishes immunoreactivity of paraffin sections of human tissues, and no useful staining is restored after proteolysis by trypsin. Some workers appear to have found differently

(Furth et al, 1987; Chesa et al, 1987), but in neither of these reports is sufficient detail provided of the fixation conditions used with human tissues.

Most immunohistochemical studies with Y13-259 have involved the use of acetone-fixed frozen sections. As confirmed in this study, best results are obtained with a highly sensitive immunohistochemical detection system. Although elevations in ras transcription have been reported in a number of human tumours, levels do not generally approach the artificially high values of the FHO5T1 system. Mutational activation of ras may not result in large increases in transcription, and it is perhaps not surprising therefore to find lower levels of p21 in human tumours.

Acetone-fixed frozen sections have yielded results regarded as generally trustworthy, but there are many disadvantages, some of which have already been mentioned. An ideal solution to the problem would be to identify another antibody to ras p21 of similarly restricted specificity to Y13-259 but whose reactivity is retained in formaldehyde-fixed tissues. Reports of the monoclonal antibody RAP-5 seemed promising in this regard (Horan Hand et al, 1984; Thor et al, 1984). It was raised by immunisation of a mouse with a synthetic octapeptide containing amino acids 10 - 17 of the mutated human Ha-ras oncogene, linked to thyroglobulin. The hybridoma was

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originally selected on the basis of differential binding to the peptide reflecting the mutated gene product compared to that of the non-mutated homologue. It was subsequently found that RAP-5 binds to cells of normal tissues and tumours (such as breast carcinoma) in which mutated ras would not be expected. This has been assumed to be due to cross-reaction of the antibody with the non-mutated p21 product, but this assumption has not been properly validated. Important claims were made for RAP-5, notably its putative preferential binding to infiltrative areas of carcinomas in breast and colon. This pattern was reported to be present only in formaldehyde-fixed tissues (Horan Hand et al, 1984; Thor et al, 1984). The results of the present study failed to confirm the claims made for RAP-5. Its inability to differentiate between FHO5T1 and CHL cells, together with the wide range of proteins detected on immunoblotting and the wide range of cell types stained by immunohistochemistry cast serious doubt on the specificity of RAP-5 for any ras-related protein. This work demonstrates the necessity of stringent testing of antibodies for use in immunohistochemistry, and illustrates the value of an unequivocal positive control system.

Apart from Y13-259, there are no widely available antibodies to p21 of acceptable specificity, so consideration was given to altering fixation conditions in order to retain immunoreactivity while optimising

morphology. These requirements are to some extent in conflict. The quality of preservation of morphology of a tissue is at least in part dependent on the degree of cross-linkage between tissue constituents induced by a fixative, whilst preservation of antigenicity is inversely proportional to the number of cross-links and the speed at which they are introduced. Paraformaldehyde (methanol-free formaldehyde) has been found to provide good morphological preservation, and although antigenic denaturation occurs, it is less than that induced by formaldehyde, and it can permit adequate retention of immunoreactivity when used at optimal concentration. McLean and Nakane (1974) conceived a fixative for immuno-electron microscopy in which periodate and lysine were added to paraformaldehyde. The theoretical basis for this fixative (PLP) lies in the ability of lysine to cross-link carbohydrate moieties oxidised by the periodate, thus providing better preservation of the conformational structure of glycoprotein antigens. Potassium dichromate was a later addition included for its membrane-stabilising properties (Holgate et al, 1986).

Membrane localisation of p21 distribution was suggested by the staining pattern of frozen sections of agar-embedded FHO5T1 cultures, and confirmed with similar PLPD-fixed preparations, where localisation of staining was much more precise. Membrane staining can also be readily identified in sections of PLPD-fixed FHO5T1 mouse

tumours, although cytoplasmic staining is also present and is often the predominant pattern. In contrast, the localisation of stain in PLPD-fixed human tissues has not been observed in the cell membrane. Cytoplasmic staining predominates, but on occasion staining is also seen in the cell nucleus. Other authors have also commented on the differences in staining distribution between ras-expressing cells in tissue culture, which showed membrane staining, and tissue sections where diffuse cytoplasmic staining was observed (Bizub et al, 1987; Brown et al, 1987). Indeed the membrane staining seen in PLPD-fixed mouse tumours in the present study is believed to be the first description of membrane distribution of p21 in tissue sections of tumours.

The cytoplasmic distribution of staining of human tumour tissue may be an artefact of tissue sectioning as suggested by the above authors, but there are possible alternative explanations. Difficult to exclude is the suggestion that perioperative ischaemia or hypoxia may result in an alteration in the cellular distribution of p21 before the tissue is fixed. In mouse FHO5T1 tumours handled in such a way as to simulate the conditions of handling for human resection specimens, there was an impression of redistribution of staining to the cytoplasm from the membrane, but this was hardly conclusive. Against this autolysis hypothesis is evidence which indicates that once p21 is situated in the plasma

membrane, it is securely anchored there and very difficult to remove in vitro either by proteolysis, salt extraction, chelating and reducing agents, or detergents (Grand et al, 1987). These authors have also provided evidence that, at least for the product of N-ras, even when produced in vast excess, all post-translationally modified p21 locates in the plasma membrane. This is in keeping with the observation of the present work that the p21 of FHO5T1 cells in culture locates only in the plasma membrane.

Although it is possible that an artefact of tissue sectioning or autolysis explains the cytoplasmic distribution of staining, it may be authentic. ras polypeptides are synthesised as cytosolic precursor molecules, pro-p21 ras (Shih et al, 1982). Post-translational modifications occur before attachment to the inner face of the plasma membrane, and the critical step is covalent palmitylation occurring at the conserved cysteine residue at position 186 (Chen et al, 1985). After this step, p21 is rendered highly insoluble, and becomes firmly attached to the inner face of the plasma membrane. It does not span the full thickness of the lipid bilayer - only the C-terminal 4 amino acids are integrated. It is possible that a derangement of the post-translational modification of p21 may have the consequence of cytoplasmic solubility and distribution of the protein; p21 over-expressed in E. coli and not

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acylated after translation behaves as a soluble cytoplasmic protein (Lacal et al, 1984).

The observation of nuclear staining in some PLPD-fixed sections of human tissues may also be a genuine reflection of p21 distribution in these cases, but if so, its significance is quite uncertain. More likely, this pattern is also artefactual. A possible explanation is the observation by Grand et al (1987) and confirmed in this study that Y13-259 appears to cross-react in immunoblots with a protein believed to be of histone origin.

Immunohistochemical staining is fundamentally a qualitative technique, and when assessing results in individual cases, ideal criteria should only take account of the presence or absence of specific staining in the section. Nonetheless there is no doubt that real differences in the intensity of specific staining do occur which are likely to reflect true differences in the amount of antigen present. In devising the simple scoring system used for assessing staining of frozen sections, which essentially records whether specific staining, if present, is faint or strong, it was considered that a valid comparison could be obtained of the relative proportions of p21 present in different tissues. It is clearly important that such scoring is reliable and reproducible, and the high degree of inter- and intra-

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observer agreement achieved confirms that consistent results are obtainable by this method.

The principal result of the frozen section series is the observation of consistently high levels of p21 expression in adenomas, in contrast to the generally lower levels in normal mucosa and most carcinomas. In the context of the adenoma-carcinoma sequence, this suggests that ras expression is elevated in the earlier stages of the process, but that expression diminishes again as adenomas evolve into carcinomas. However, concern about the validity of the scoring system used led to attempts to confirm these findings using a titration technique of increasing dilutions of Y13-259 on serial paraffin sections of tissues fixed in PLPD. As this fixation technique became available later in the study, in only a minority of cases were both techniques applied to the same individual tissues. The titration method was not applied to frozen sections as the high background staining made assessment of specific staining impractical at high dilutions of primary antibody.

Titration of Y13-259 concentration in the series of PLPD-fixed colorectal tissues confirmed the major finding of high expression of ras in adenomas compared with low levels in normal mucosa. This approach has also revealed expression of ras at intermediate levels in the great majority of carcinomas - a finding confirmed by the high

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proportion of carcinomas staining positively in the series stained using Y13-259 at the higher concentration of 1 in 25. The similarity in results to the frozen section series is illustrated by considering the percentages of each tissue type showing positive staining at 1 in 100 dilution of Y13-259.

TABLE 2.3

	FS Series % ++	PLPD Series % +ve at 1 in 100
Normal	6	0
Adenomas	71	80
Carcinomas	8	26

ras activation may occur early in the carcinogenic process in the colorectum, but high levels of expression may be a marker rather than a cause of the active proliferation of neoplastic cells. Cytokinetic data indicate that adenomas have a shorter cell cycle time and a higher proportion of cells in cycle than the normal mucosa (Bleiberg and Galand, 1976; Deschner and Lipkin, 1976). Furthermore, adenomas have a shorter cell cycle time than carcinomas and normal mucosa. The carcinogenic process in colon may require that the cells of an adenoma achieve a certain altered proliferative potential, a marker of which is increased ras expression, and which once achieved renders them vulnerable to the action of a

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further carcinogenic stimulus or stimuli which converts the lesion to an invasive carcinoma. Once this has acted, the requirement for ras expression is supplanted, and levels fall. In keeping with this hypothesis are the results obtained by Gallick et al (1985) who observed higher levels of p21 detectable by immunoblotting in colorectal carcinomas at an early pathological stage (Dukes' stage A) compared to more advanced lesions which had metastasised.

The main purpose of immunoblotting in this study was validation of antibody specificity and confirmation of the presence of p21 in human colorectal tumours. Additional points of interest have emerged from its application to colorectal tissues. p21 was detected as a doublet band in all human colonic tissues, whether normal or neoplastic. This is in keeping with the findings of Furth et al (1987) in a range of normal tissues, and DeBortoli et al (1985) in a series of breast carcinomas. It is most likely that the doublet band represents the product of different members of the ras gene family (Ha, Ki and N-ras) migrating at slightly different rates. Although the p21 proteins of normal and mutationally activated ras genes also show slightly different electrophoretic mobilities, this explanation is less likely as the same pattern was seen in normal and neoplastic tissues. The single band detected in extracts of FHO5T1 cells represents the mutated Ha-ras gene

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product, present in vast excess over other <u>ras</u> gene products in these cells.

Like immunohistochemistry, immunoblotting is essentially a qualitative technique. However, when using the ^{125}I-Protein A detection system, it might have been anticipated that if there were significant differences in amounts of p21 between samples, these differences would be revealed as bands of varying intensity. The finding of p21 bands of only slightly varying intensity in normal mucosa, adenomas and carcinomas was not the expected result in view of the immunohistochemical findings, but only a small number of cases have been investigated. Similarly the observation of variation in the intensity of one or other component of the doublet bands in different specimens is intriguing, especially in the light of recent reports of <u>ras</u> deletions in colonic carcinomas. These findings await confirmation in a larger series of cases.

CHAPTER 3

IMMUNOHISTOCHEMICAL DETECTION OF C-MYC ONCOPROTEIN

SUMMARY

c-myc expression in colorectal tissues was studied by immunohistochemistry, principally using the monoclonal antibody Myc1-9E10. Antibody specificity was investigated by application of immunohistochemistry to myc-expressing cell lines, and by immunoblotting of protein extracts of cell lines and colorectal tissues. Immunoblotting identified a single major protein band in both extracts which is believed to represent the c-myc oncoprotein, but its electrophoretic mobility was unexpectedly rapid, indicating molecular weight of 40-45 kDa, and the explanation of this is uncertain. Immunohistochemistry showed positive staining in cell lines known to transcribe c-myc at artificially high levels, but positive staining of similar pattern was also present in an unmanipulated progenitor cell line. This is believed to indicate the endogenous c-myc expression expected in proliferating cells in culture. Positive staining was observed in tissue sections of all human colorectal tissues. Conditions of fixation had an important effect on staining distribution, with rapid fixation in formaldehyde required for optimal results. A series of 50 colorectal resection specimens for carcinoma, fixed in this way, showed differences in distribution of immunoreactivity between normal mucosa, adenomas and carcinomas. Nuclear staining predominated in normal mucosa, but carcinomas showed diffuse cytoplasmic

location of staining in all cases. Adenomas showed an intermediate pattern, with both cytoplasmic and nuclear staining patterns present. Cytoplasmic staining was significantly more frequent in adenomas than in normal mucosa (p<0.01). The results indicate that differences in the cellular processing of the c-myc oncogene product may occur during the evolution of colorectal neoplasms.

3.1 MATERIALS AND METHODS

3.1.1 Cell lines

A cell line was used as a positive control in which c-myc expression was augmented by transfection with the high expression plasmid vector pMCGM1 which contains the entire c-myc gene linked to Moloney virus LTR sequence (Spandidos, 1985). The cell line RFMCGM1 (referred to subsequently as RFM) is a derivative of the 208F rat fibroblast line, transfected with pMCGM1. RFM cells show variable elevation of c-myc mRNA transcript levels up to 18-fold relative to the parental 208F line (Wyllie et al, 1987). The cell lines were maintained in culture as described in section 2.1.1, and cytocentrifuge preparations and cell pellets in agar prepared also as described previously. 208F cells, although said to be non-tumourigenic, engendered slowly-growing tumour nodules when injected subcutaneously into immune deprived mice. Approximately 50% of inoculations of RFM gave rise to aggressive fibrosarcomas under similar conditions. Such tumours were not suitable as controls for immunohistochemistry because the anti-p62-c-myc antibody Myc1-9E10 (see below) is of mouse origin, and the immunohistochemical technique requires anti-mouse IgG as a secondary antibody. Cross-reactions with endogenous mouse IgG resulted in unacceptably high levels of background staining.

3.1.2 Human colorectal tissues

Methods of collection and handling of resection specimens
of human colorectal tissues have been described in
section 2.1.1. For immunohistochemistry, frozen sections
and paraffin sections were used, and the effects of
different fixatives compared. For frozen sections, post-
fixation in acetone or formaldehyde was carried out as in
section 2.1.3.1. Paraffin sections of formaldehyde-fixed
or PLPD-fixed tissues were also used. Special efforts
were made to minimise the delay in placing fresh tissues
in the appropriate fixative.

3.1.3 Immunohistochemistry

Antibodies to the p62-c-myc protein product

The monoclonal antibodies Myc1-9E10 and CT14 are two
members of a series of six monoclonals isolated from mice
immunised with synthetic peptide immunogens whose
sequences are derived from that of the human c-myc gene
product p62. Both were raised against the C-terminus
peptide residues 408-432. Unlike CT14, 9E10 fails to
immunoprecipitate native p62-c-myc, but it is a sensitive
probe for the protein on immunoblots, and binds to it
with high affinity (Evan et al, 1985). Both antibodies
have been used in immunohistochemical studies and 9E10
has been reported to show a higher degree of sensitivity
for the p62 protein than CT14 (Sundaresan et al, 1987).
Myc1-9E10 and CT14 were prepared from hybridoma culture

supernatants by ammonium sulphate precipitation, and stored as aliquots of 200 ul at $-70\,^{\circ}$C. The polyclonal antibody anti-p62-c-<u>myc</u> was prepared by immunisation of rabbits by whole recombinant p62-c-<u>myc</u> protein expressed in bacteria (Watt et al, 1985), and was donated by Dr R. Watt of Smith, Kline and French, Philadelphia.

Immunohistochemical technique

Immunohistochemical staining was performed using the indirect (peroxidase-conjugated secondary antibody) or the ABC (streptavidin-biotin peroxidase complex) technique. In principle, the methods are exactly as described previously (section 2.1.3.1); details of the method vary as follows:

1. Endogenous peroxidase activity was blocked before application of the primary antibody by incubating the dewaxed rehydrated paraffin sections in 3% hydrogen peroxide in methanol for 15 minutes, followed by washing in TBS.

2. When using the murine monoclonal antibodies 9E10 and CT14, non-specific binding was blocked by application of 10% normal rabbit serum in TBS. When using the rabbit polyclonal anti-p62-c-<u>myc</u> antiserum, 10% normal swine serum was applied.

3. All primary antibodies were applied at a dilution of 1 in 100 for 1 hour.

4. For 9E10 and CT14, the secondary antibody was biotin-conjugated rabbit anti-mouse IgG (Dako), applied

at a dilution of 1 in 400 in 10% normal rabbit serum. For polyclonal anti-p62-c-<u>myc</u>, biotin-conjugated swine anti-rabbit IgG (Dako) was applied also at 1 in 400 dilution.

5. In some experiments, Amersham ABC complex was replaced by Dako ABComplex, freshly prepared immediately before use according to the manufacturer's instructions.

In all experiments, a negative control was provided for each section in which the primary antibody was omitted and replaced by 10% normal rabbit serum.

Immunoblotting

Protein extracts of cell lines and human colorectal tissues were prepared as described in section 2.1.2, and SDS-PAGE separation and transfer to nitrocellulose carried out as previously. Immunodetection was carried out using the alkaline phosphatase detection system as previously, the only difference being the species-specificity of the secondary antibody (anti-mouse IgG for 9E10 and CT14, anti-rabbit IgG for polyclonal anti-p62-c-<u>myc</u>).

3.2 RESULTS

3.2.1 Immunoblotting using Myc1-9E10

Protein extracts of the 208F and RFM cell lines were
separated on PAGE alongside extracts from colorectal
tissues and transferred to nitrocellulose. Using the AP
detection system, a major single band was detectable in
RFM cells, at approximately 40-45 kDa. This band was also
present at approximately equal intensity in 208F cell
extracts, also as a single major band. Colorectal tissues
showed a similar major band in this region , but multiple
bands of lower intensity were also present at both lower
and higher molecular weights. Although a faint band was
present in the 60-65 kDa range, this was of considerably
lower intensity than the band at 40-45 kDa, and was
absent from extracts of the known myc-expressing cell
line RFM (Fig. 3.1).

3.2.2 Immunohistochemistry

Cell lines

The 208F and RFM cell lines were used as a control
system to identify the optimal conditions of fixation and
tissue handling for immunohistochemical detection of the
c-myc oncoprotein. Fixation conditions were found to have
a significant effect on the ability to demonstrate

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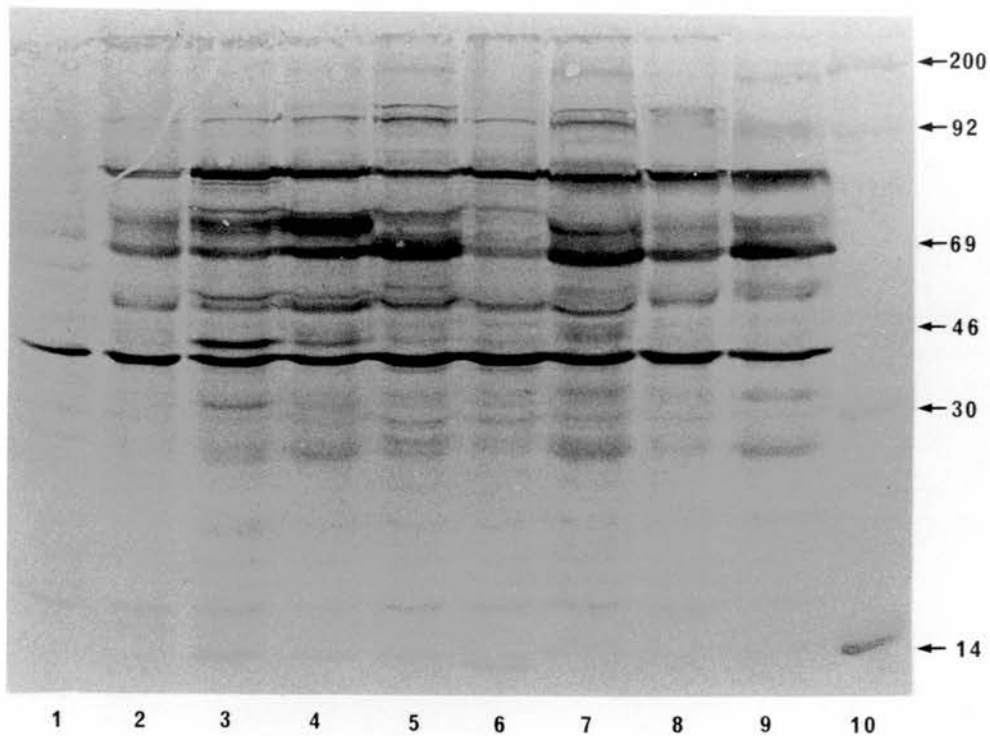
Fig. 3.1 Immunoblot of protein extracts of RFM cell line
and human colorectal tissues, separated by PAGE and
detected on nitrocellulose with Myc1-9E10 with alkaline
phosphatase detection system. Tracks were loaded with
equivalent amounts of protein extracts from the following
specimens: 1 - RFM myc-expressing cell line in culture;
2, 3 & 4 - carcinoma, adenoma and normal mucosa
respectively from same patient; 5, 6, 7 & 8 - carcinomas
from separate patients; 9 - normal mucosa; 10 - molecular
weight markers (Amersham, 'Rainbow Markers').

positive staining. The mouse monoclonal antibody 9E10 was
the anti-myc primary antibody used in most experiments.
Despite the observation that positive staining was
demonstrable using 9E10 with the indirect technique
(peroxidase conjugated secondary antibody), the ABC
streptavidin-biotin-complex technique was used because of
its increased sensitivity. Immunohistochemical staining
was performed on cytocentrifuge preparations of 208F and
RFM cell lines fixed in acetone or formaldehyde. All
cells of both lines showed positive staining when fixed
in acetone but were negative when formaldehyde-fixed.
Staining was most frequently localised diffusely
throughout nucleus and cytoplasm, but in some
preparations, exclusively nuclear staining of granular
pattern was obtained. The reason for this divergent
pattern of staining in apparently similar preparations
was never identified. Allowing for the fact that there
were some morphological differences between the two cell
lines, no discernible difference was seen in staining
pattern between 208F and RFM. Staining was absent from
negative controls.

The staining with 9E10 was compared with that obtained
using the two other anti-p62 antibodies, CT14 and
polyclonal antip62-c-myc. These reagents, produced in
very different ways, resulted in a closely similar
distribution of immunoreactivity in acetone-fixed
cytocentrifuge preparations. No significant difference in

pattern or intensity of staining was demonstrated between 208F and RFM cell lines. Negative controls showed no staining.

Paraffin sections of formaldehyde-fixed agar embedded cell pellets were stained using the three anti-myc antibodies. Positive staining was obtained with 9E10 in sections from both cell lines. No difference in pattern or intensity of staining was observed between them, both showing diffuse immunoreactivity distributed throughout nucleus and cytoplasm of all cells. Exclusively nuclear staining was never observed in these preparations. Morphological preservation was not good in these preparations and accurate subcellular localisation of staining was not possible. Staining with CT14 and polyclonal anti-myc was significantly fainter but of similar pattern.

Human colorectal tissues

A variety of fixation conditions were compared using frozen and paraffin sections of human colorectal tissues, and differences were observed in the quality, localisation and intensity of immunoreactivity. The antibody 9E10 was the principal antibody used in this part of the study. Paraffin sections of formaldehyde-fixed tissues showed retention of immunoreactivity, and positive staining was obtained with sections of

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routinely-handled resection specimens of colorectal tissues from the files of the Biopsy Service. However, when these sections were compared with those from a series of colorectal resection specimens which had been specially handled to optimise fixation and minimise delay in placing tissue blocks in formaldehyde, the latter group showed a very clear improvement in terms of the intensity and localisation of staining pattern. The improvement was particularly seen in sections of normal mucosa, in which distinct nuclear localisation was seen in a significant proportion of epithelial cells, whilst others were quite negative. This contrasted with the pattern in normal mucosa of routinely-fixed tissues where although positive staining was still observed, its distribution was most frequently diffusely cytoplasmic, and differences in staining between individual cells in the mucosa were much less clearly defined.

Frozen sections of colorectal tissues, post-fixed in acetone, also retained some immunoreactivity, but were significantly less satisfactory in three respects. The pattern of immunostaining was poorly localised, being diffusely cytoplasmic or sometimes concentrated at the adluminal aspect of the surface epithelium (Fig. 3.2). The staining was significantly fainter than in formalin-fixed sections, and morphological preservation was poor. Paraffin sections of tissues fixed in PLPD showed complete loss of immunoreactivity. These differences in

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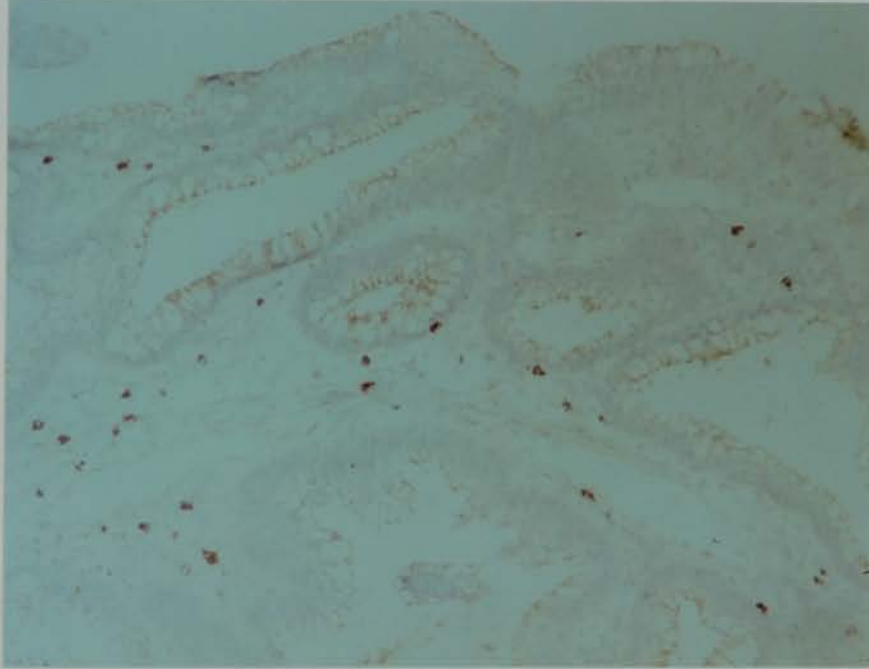
Fig. 3.2 Acetone-fixed frozen section of colonic adenoma
stained with Myc1-9E10. Staining is faint and variable,
and localised to the adluminal aspect of the glandular
epithelium. (Endogenous peroxidase activity is seen in
macrophages in the lamina propria).

staining patterns were shown to be a consequence of fixation, in that tissue blocks from the same specimen fixed in formaldehyde or PLPD, or subjected to frozen section showed the different patterns of staining described.

It was concluded that optimal fixation for immunohistochemistry with 9E10 required minimum delay in placing adequate-sized tissue blocks in formaldehyde. Under such conditions, the theoretically predicted nuclear localisation of immunoreactivity was consistently obtained in a proportion of cells in sections of normal mucosa.

Resection specimens

A series of 50 colorectal adenocarcinomas were stained for p62-c-myc using monoclonal antibody 9E10. In all cases, the resection specimens were specially handled for optimal fixation as above; the average time interval between resection and adequate fixation was estimated to be approximately 30 minutes. Blocks of carcinoma were selected to include adjacent uninvolved mucosa where possible, and in 25 cases, blocks from the mucosa of the resection margin were examined. When present, co-existent adenomas from the resection specimens were sampled, and in Dukes' stage C carcinomas, examples of involved lymph nodes were used.
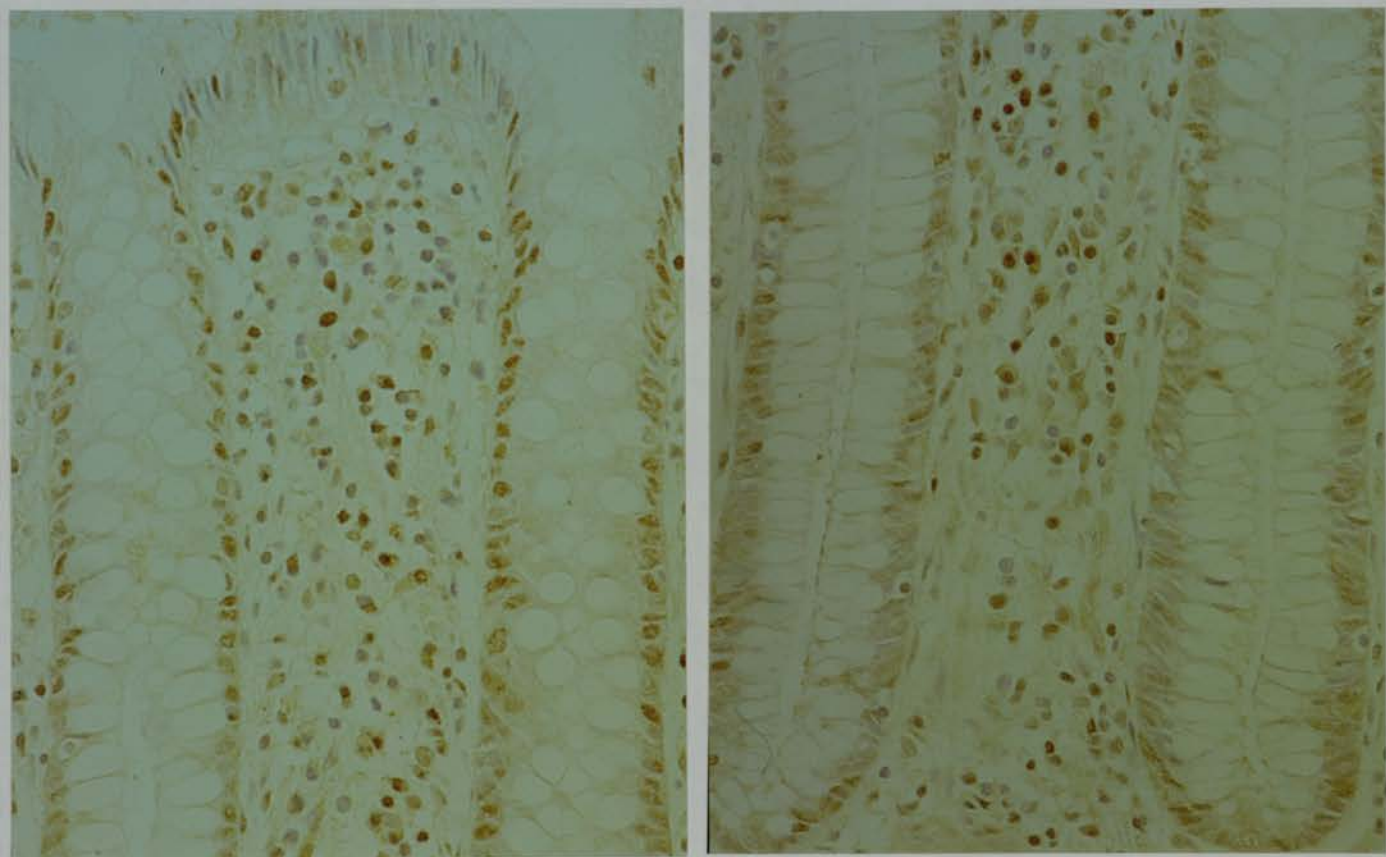
Figs. 3.3a & b. Normal colonic crypts from mucosa of
resection specimen rapidly fixed in formalin, stained
with Myc1-9E10. In the superficial part of the crypts,
most cells show nuclear staining, but on the surface
epithelium, a proportion of nuclei are negative. At the
crypt base, a proportion f cells show staining, but some
are negative. There is almost no cytoplasmic staining
throughout.

Specific staining was always obtained in normal mucosa, and in the majority of cases, a consistent pattern of distribution was observed (Figs. 3.3a & b). The pattern was similar in mucosa adjacent to adenomas and carcinomas, and from resection margins of specimens. Areas of exclusively nuclear staining of colonic epithelial cells were observed in the great majority of cases (93%), and in 39% of cases, exclusively nuclear staining was the pattern of localisation in more than 90% of positively-staining cells. The proportion of cells showing such nuclear staining was lower at the bases of the crypts than in the intermediate (maturation) zone, where the majority of cells showed staining. Nuclear staining was also usually seen in a proportion of cells on the surface epithelium, variable up to approximately 50% of cells, while neighbouring surface cells which were morphologically indistinguishable were negative. However in many cases, cytoplasmic staining of surface epithelial cells occurred, and when this pattern was seen, all cells at the surface were stained (Fig. 3.4). Cytoplasmic staining was present in 61% of blocks of normal mucosa, but in only 7% was this pattern present in more than 90% of positively-staining cells. Basal and intermediate cells of the crypt only infrequently showed cytoplasmic staining.

Adenomas showed a variable pattern of staining distribution, but positive staining was observed in all

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cases. In 12 of 19 cases (63%), both nuclear and cytoplasmic localisation of staining were observed in different parts of the lesion (Fig. 3.5), but in 2 cases, more than 90% of positively-staining cells showed nuclear staining only, while in 5 cases, cytoplasmic staining was similarly predominant. When exclusively nuclear staining predominated, a proportion of cells were seen which showed no staining, but when cytoplasmic staining was predominant, all cells appeared to be stained. No correlation was identified between the histological type of the adenoma and the pattern of staining. The numbers are small, but the impression was gained that nuclear staining was more frequent in small adenomas, whilst cytoplasmic staining was commoner in the larger lesions. Areas of severe dysplasia in adenomas showed cytoplasmic staining.

Primary carcinomas all showed positive staining, although there was some variation in the intensity in different areas of the same tumour, and between different tumours. No attempt was made to quantify such differences. There was no obvious correlation with the degree of differentiation or any other histological property of the tumour. In all carcinomas, staining was predominantly cytoplasmic in distribution, and present in all cells (Fig. 3.6). Exclusively nuclear staining was only rarely observed in some areas of some carcinomas, and was never seen in more than 10% of positively-staining cells. A
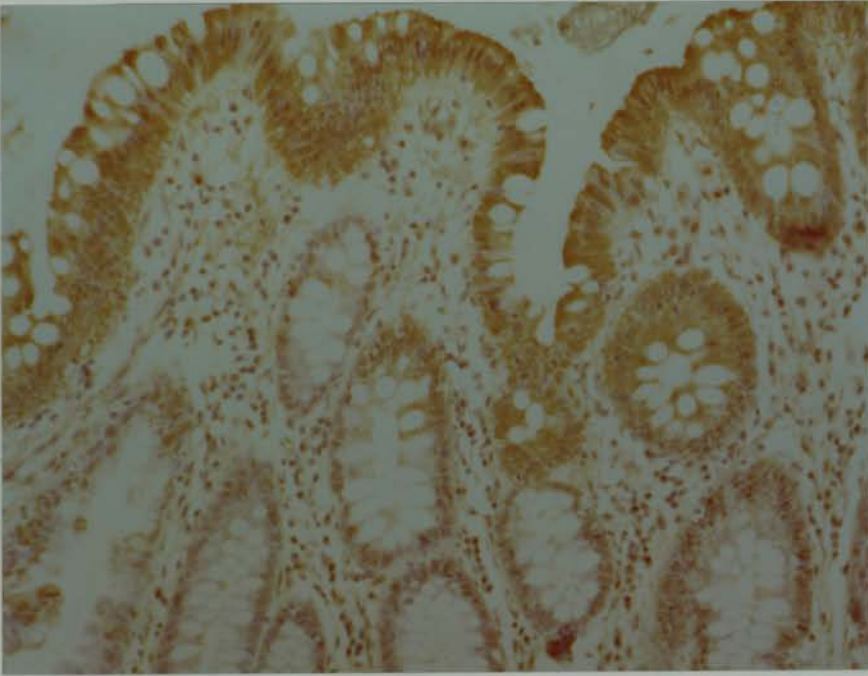
Fig. 3.4 In some cases, cytoplasmic staining occurred of epithelial cells at the surface of normal mucosa.
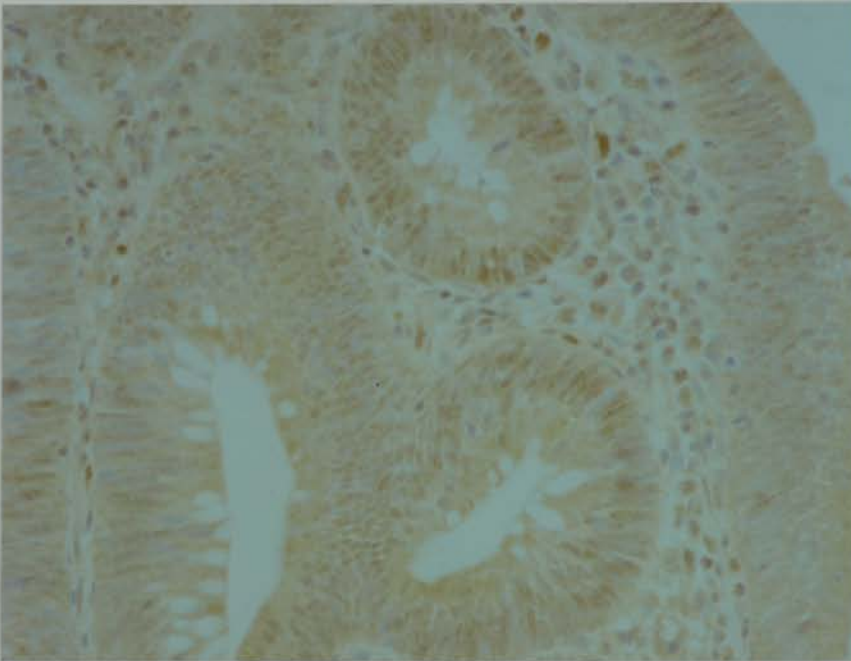
Fig. 3.5 Adenoma of colon stained with 9E10. Both nuclear and cytoplasmic staining are present in epithelial cells; a proportion of the nuclei are negative.
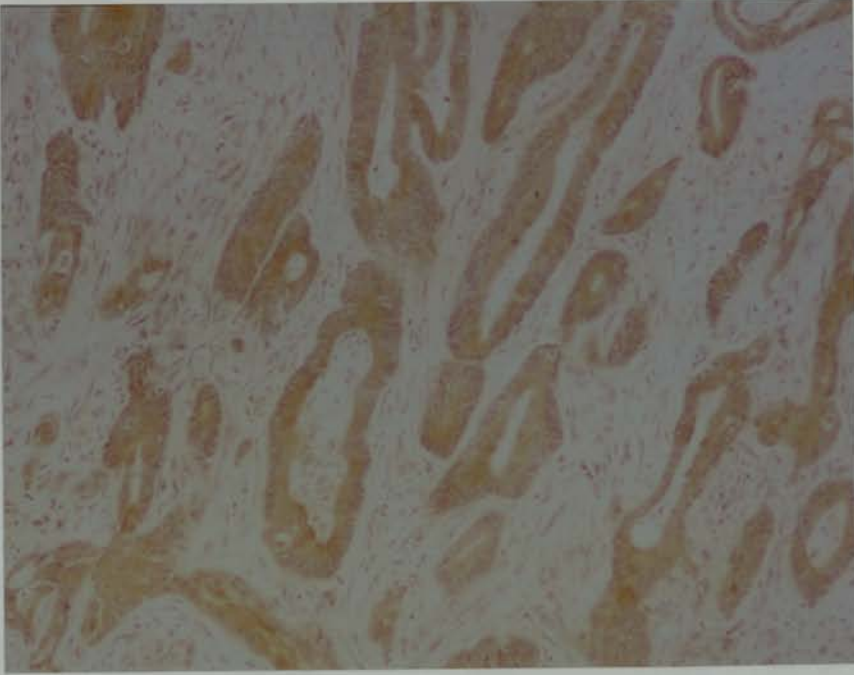
Fig. 3.6 Infiltrating adenocarcinoma of colon stained
with 9E10. All cells of the adenocarcinoma show staining
which is diffusely localised throughout the cytoplasm.
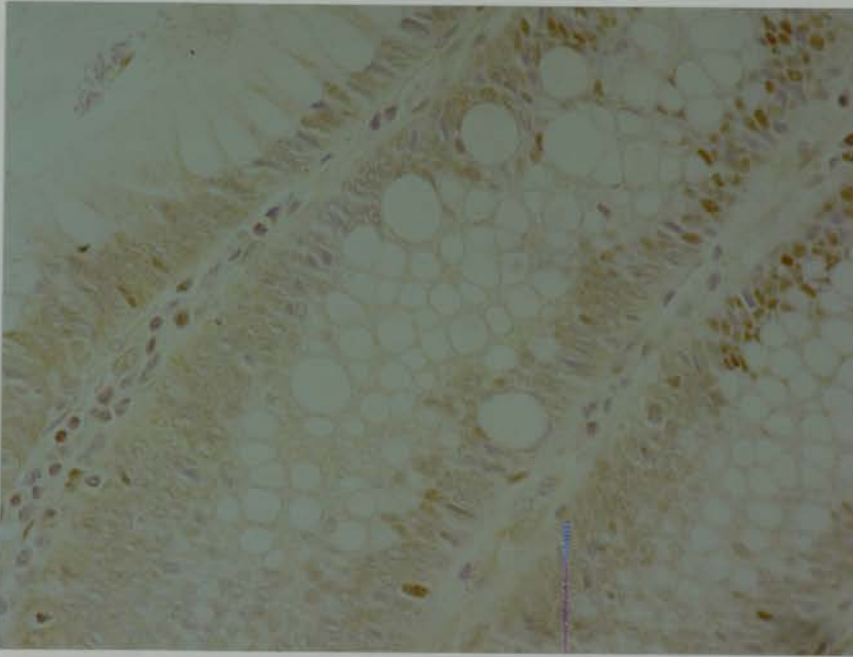
Fig. 3.7 Interface between non-neoplastic colonic mucosa
(top) and carcinoma. Distinct nuclear staining is present
in normal mucosa; cytoplasmic staining is present in
carcinoma cells.

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clear difference in the cellular distributin of staining occurred at the interface between normal mucosa and carcinomas. Normal mucosa showed nuclear staining, whilst the carcinoma showed cytoplasmic staining (Fig. 3.7). Cytoplasmic staining of all carcinoma cells was seen in all cases of metastatic carcinoma in lymph nodes.

An attempt was made to assess the differences in staining distribution in different tissues in a semi-quantitative way. 3 categories of staining distribution were defined as follows:

   1. N>C - exclusively nuclear staining present in 90% or more of positively-staining cells.

   2. C>N - cytoplasmic staining present in 90% or more of positive cells.

   3. N=C - areas of exclusively nuclear and of cytoplasmic staining both present in different parts of the section, neither pattern accounting for 90% or more of positive cells.

Table 3.1 shows the results of staining of each tissue assessed in this way.

TABLE 3.1

	N>C	N=C	C>N	Total
Normal (margin)	9	14	2	25
Normal (adjacent to to carcinoma)	17	22	2	41
Normal (adjacent to to adenoma)	7	9	2	18
Adenoma	2	12	5	19
Carcinoma	0	0	50	50
Lymph node metastasis	0	0	10	10

Carcinomas show a highly significant predominance of the cytoplasmic staining pattern compared with normal mucosa and adenomas. Metastatic carcinoma in lymph nodes shares this pattern. Adenomas show a significantly higher frequency of cytoplasmic staining when compared with all normal mucosae taken together ($p < 0.01$ - Chi squared test). There is no significant difference in distribution of staining pattern between normal mucosa adjacent to either adenomas or carcinomas compared with that derived from surgical resection margins.

3.3 DISCUSSION

3.3.1 <u>Specificity</u> <u>of</u> <u>Myc1-9E10</u> <u>for</u> <u>p62-c-myc</u>

The monoclonal antibody Myc1-9E10 was shown by its originators to recognise an epitope of the human c-<u>myc</u> protein p62 (Evan et al, 1985), and although it did not immunoprecipitate detectable amounts of native c-<u>myc</u> protein from detergent extracts of Colo 320 HSR cells (a cell line derived from a human colonic tumour in which the c-<u>myc</u> oncogene is amplified) it was shown to be a sensitive probe for p62-c-<u>myc</u> in immunoblotting experiments, recognising a single protein band of deduced molecular weight 62 kDa. Subsequent reports have described the use of 9E10 in immunohistochemical studies (Sundaresan et al, 1987; Jones et al, 1987), and although it has not found such extensive use as the antibody 6E10, which is yet another member of the same group of antibodies produced by Evan et al (1985), it has been reported to produce patterns of immunostaining consistent with the predicted distribution of p62, and similar to the patterns obtained with other anti-p62 antibodies.

Immunoblotting investigations carried out in this study have provided an unexpected result concerning the protein recognised by this antibody. It was shown that 9E10 binds to a protein present in extracts of both RFM and 208F cell lines. The protein forms a single homogeneous band, and other bands are very much fainter, or absent in some

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preparations. The molecular weight of this protein, deduced from its electrophoretic mobility on SDS-PAGE, is in the region 40-45 kDa. This finding has been repeated in separate experiments with separately-prepared extracts of the cell lines. A protein of identical electrophoretic mobility is also present in protein extracts of human colorectal tumours, although in these preparations, several other prominent bands occur. The explanation for the apparently anomalous mobility of the protein is not certain, and it has not yet been possible to compare the pattern obtained with other anti-myc antibodies on the same protein extracts. It is possible that the epitope recognised is unrelated to the p62 protein, but the originators of this antibody (Evan et al, 1985) have described reactivity with a single protein band in the 60-65 kDa region in extracts of myc-expressing cells. However, anomalous electrophoretic mobility for the c-myc protein has been described previously, and it has been noted that the precise proportions of acrylamide and bis-acrylamide in the gel can have an important effect on the rate of migration of the c-myc protein (Evan et al, 1985). There is also one report of a 48kDa myc-related protein detected by PAGE (Giallongo et al, 1983), which may perhaps be explained by such technical considerations.

The 208F and RFM cell lines have proven to be considerably less satisfactory as positive controls for

immunohistochemistry than the analogous ras-expressing system described in chapter 2. Immunohistochemistry was unable to show clear-cut differences in staining pattern between 208F and RFM cells, despite evidence from quantitative RNA dot blots on the same cultures of an increase of between 6 and 20 fold of c-myc transcript in the transfected RFM cell line. There are several alternative explanations. Firstly, c-myc expression is to be expected in all cells in cycle, and relatively small quantitative differences in p62 would be detected poorly by immunohistochemistry. Secondly, the 208F cell line cannot be regarded as approximating to normal early passage cells - the cells are aneuploid, and although reputedly non-transformed, consistently gave rise to slowly-growing nodules of low-grade tumours on inoculation into immune-deprived mice (Wyllie et al, 1987). The very similar patterns of immunohistochemical staining obtained with the two other anti-myc antibodies (CT14 and polyclonal anti-p62-c-myc), whose specificity for p62 has been adequately documented by their respective originators (Evan et al, 1986; Watt et al, 1985) supports the proposal that 9E10 indeed recognises an epitope of p62 in both 208F and RFM cells.

3.3.2 Cellular localisation of immunoreactivity

Nuclear localisation of immunostaining for c-myc protein is predictable on theoretical grounds. p62 has been shown by immunofluorescence and cell fractionation studies to

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localise to the cell nucleus (Hann & Eisenmann, 1984; Beimling et al, 1985), and more recently it has been shown to have precisely the same pattern of distribution within the nucleus as that of small nuclear ribonucleoprotein particles (Spector et al, 1987). Although in vitro, p62 shows non-specific binding to DNA, in vivo the greater proportion of the protein does not appear bound, as DNA'ase treated nuclei retain p62 immunoreactivity within the nuclear matrix (Spector et al, 1987). The association of p62 with its nuclear binding target appears to be dependent on a relatively weak and reversible ionic interaction, as p62 can be quantitatively extracted from isolated nuclei in relatively low salt concentration (Evan & Hancock, 1985). It has emerged that experimental conditions of extraction are critical - a temperature-dependent irreversible process of insolubilisation of p62 occurs in isolated nuclei at 37°C, in which p62 coprecipitates with a number of other proteins (Evan & Hancock, 1985). This explains previous descriptions of limited extractability of p62 from the nucleus (Eisenmann et al, 1985).

During mitosis, myc proteins become dispersed throughout the cytoplasm, and have no association with condensed chromosomes (Windqvist et al, 1984). Apart from this situation, however, only a small proportion (approximately 5%) of p62 is found in the cytoplasmic fraction of cultured cells subjected to detergent lysis

at low ionic strength (Evan et al, 1985). The c-myc protein is synthesised in the cytoplasm in around 7 minutes, but is rapidly transported to the nucleus (Beimling et al, 1985). It has a short half life of around 15 - 20 minutes, but the manner of its inactivation or degradation is unknown. The protein is present in more than one form within the cell, and there are phosphorylated and non-phosphorylated forms. Phosphorylation appears to occur identically in transformed and non-transformed cells, and lack of phosphorylation does not prevent nuclear localisation. Recombinant bacterially-expressed p62 (which is non-phosphorylated), when micro-injected into the cytoplasm of quiescent fibroblasts, is transported to the cell nucleus within 15 minutes, where it localises with the small nuclear ribonucleoprotein particles (Spector et al, 1987). A specific amino acid sequence directing transport of p62 to the nucleus has not been identified, but it seems unlikely that such a sequence if it exists is abnormal in transformed cells as p62 appears to be qualitatively normal in most tumours in which c-myc is elevated.

In the present study, under conditions of rapid fixation in formaldehyde, nuclear localisation of staining was consistently demonstrated in paraffin sections of normal colonic mucosa from surgical resections, but cytoplasmic staining was present to a variable extent in similar

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tissues in which fixation had been delayed. It seems probable that an artefactual redistribution of p62 occurs to explain this observation. Under conditions of tissue hypoxia, such as occur in a surgical resection specimen during the operation and after removal, ionic changes occur in cells, including a significant fall in the intracellular pH. This may be sufficient to interrupt the non-covalent association of p62 with its nuclear target, with consequent redistribution of the protein throughout the cytoplasm. Rapidity of fixation is therefore believed to be of importance with respect to the cellular localisation of p62. It is also likely to be important with respect to the presence of intact p62 itself. p62 is a very labile protein with a short half life, and within 3 hours of a stimulus inducing cellular quiescence and differentiation, the protein is virtually undetectable by immunoblotting of cell extracts using 9E10 as probe (Evan et al, 1985).

Recently, Loke et al (1988) have reported an immunohistochemical study which indicates that variations in fixation procedure can lead to significant and sometimes paradoxical differences in subcellular distribution of the c-myc protein product in cell lines and normal mouse tissues. In cytocentrifuge preparations of the myc-expressing cell line HL60, it was found that a redistribution of immunoreactivity occurred from nucleus to cytoplasm with increasing length of time in certain

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fixatives (acetone or formaldehyde). In paraffin sections of normal mouse tissues fixed in formaldehyde, nuclear staining was lost and occurred in the cytoplasm. Whilst the findings of this study differ in detail quite significantly from those of the present study, they emphasise the fact that fixation conditions are very important in immunolocalisation of myc product, and that consistency of technique is essential.

The consistent observation of cytoplasmic localisation of immunoreactivity in surgically resected carcinomas is less easily explicable purely on the basis of artefactual redistribution of p62. That such redistribution does occur to some extent is suggested by the fact that significant areas of nuclear staining were never seen in carcinoma cells in resection specimens, whereas a proportion of endoscopically-biopsied carcinomas (in which fixation in formaldehyde can be assumed to be almost instantaneous) sometimes showed distinct nuclear localisation of staining. However, it was consistently noted in resection specimens that distinct nuclear staining occurred in normal mucosa directly adjacent to carcinoma showing cytoplasmic staining. This would seem to exclude a fixation effect, and implies a significant difference in the cellular handling of p62 between normal mucosa and carcinomas. It is relevant to this hypothesis that adenomas from the same resection specimens showed a significantly higher frequency of cytoplasmic staining

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than normal mucosa, but less than carcinomas. It is also worth noting the implications of these observations of altered distributions of p62 for data provided by a recently developed flow cytometric assay which quantifies the protein in isolated cell nuclei derived from archival paraffin blocks of pathological material (Watson et al, 1985).

Different fixatives may have a different effect on the ionic interaction between p62 and its nuclear ligand, and this may explain some of the paradoxical patterns of staining seen in cell lines and tissues with different fixatives. Diffusion and disappearance of p62 during fixation may explain the faint or absent staining in acetone-fixed and PLPD-fixed tissue sections. Formaldehyde fixation clearly does not denature the epitope recognised by 9E10, but staining is absent from cytocentrifuge preparations of cell lines because in these, the cell membrane is intact and impermeable, and p62 consequently inaccessible to antibody. Paraffin sections of the same cell lines fixed in formaldehyde show strong staining. Acetone fixation of cytocentrifuge preparations results in permeabilisation of the cell membrane to antibody which can enter the cell and react with p62.

The cytoplasmic location of staining in 208F and RFM cells is difficult to explain. Fixation delay is not the

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explanation, as the cell lines were freshly harvested and rapidly fixed. It is possible that cytoplasmic immunoreactivity is a true reflection of p62 distribution in these cell lines.

3.3.3 p62 distribution in colorectal tissues

There are a number of published reports describing patterns of immunohistochemical staining for p62-c-myc in gastro-intestinal tissues including colorectal mucosa, adenomas and carcinomas (Stewart et al, 1986; Ciclitira et al, 1987; Sundaresan et al, 1987; Jones et al, 1987). In all of these studies, the monoclonal antibody Myc1-6E10 was used, a different member of the series of antibodies raised by Evans et al (1985). In the study of Jones et al, 9E10 was noted to give identical patterns of staining to 6E10, and Sundaresan observed identical staining patterns with CT14 and 6E10. Immunohistochemical techniques of varying sensitivity were employed in these studies, but all describe results of staining with formaldehyde-fixed paraffin embedded tissue sections. As far as can be judged, no special procedures were taken for rapid fixation in any of these studies.

There is a considerable lack of uniformity in the results described in these reports, although there are some points of broad agreement. In the normal mucosa, Stewart et al (1986) observed staining to be maximal in the transition zone of the crypts between the crypt base and

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the surface epithelium. Staining was cytoplasmic in location, an effect attributed solely to the fixation procedure. Jones et al (1987) observed rather similar, but faint staining of maturing crypt cells and surface epithelium, but cells at the crypt bases were negative. Sundaresan et al (1987) described exclusively nuclear staining of the proliferative (basal) zone, mixed nuclear and cytoplasmic staining in the maturation (transition) zone, and exclusively cytoplasmic staining in the mature (surface) zone. Ciclitira et al (1987) described very weak staining of occasional superficial enterocytes in normal mucosa which was otherwise negative.

A somewhat closer measure of consensus was achieved in descriptions of the patterns of staining obtained in colorectal carcinomas. Stewart, Jones and Sundaresan all observed positive staining in all carcinomas which was predominantly or exclusively cytoplasmic in location. In their study of adenomas and carcinomas of patients with familial adenomatous polyposis, Sundaresan and associates claimed to be able to identify proliferative, maturation and mature zones in these lesions, analogous to those of the normal crypt. It is difficult to accept their suggestion of increasingly cytoplasmic localisation of staining with increasing cellular maturation, as they provide no indication of how these zones were identified, nor whether the cytokinetics of cells in their zones bear any relation to those of the normal crypt.

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The results of the present study support the assertion of increased c-myc expression in colorectal carcinomas, as all cells showed staining in carcinomas, compared with only a proportion of cells in the normal mucosa. There is also reason to accept in part the observation of Jones et al (1987) that the cytoplasmic distribution of staining can in some circumstances be attributed to fixation conditions. It appears however that there is a real difference between normal mucosa and carcinomas in the manner of association of the c-myc protein with the nucleus, and further support for this comes from the significantly increased frequency of cytoplasmic staining in adenomas compared with normal mucosa. This suggests that the change may occur early in the process of colonic carcinogenesis.

CHAPTER 4

RNA HYBRIDISATION TECHNIQUES

SUMMARY

Nick translated labelled whole plasmid DNA probes
containing oncogene sequences were used in an attempt to
develop an in situ hybridisation technique for the
autoradiographic detection of mRNA transcripts of
oncogenes in cell lines and human colorectal tissues.
Analysis of total RNA in extracts of cells and tissues
showed evidence of considerable RNA degradation in human
tissues, but despite this, the presence was confirmed of
hybridisable oncogene mRNA transcripts by dot blot
hybridisation on nitrocellulose. For in situ
hybridisation experiments, sections of cell pellets or of
human colorectal tissues were subjected to a range of
fixation conditions, pre-hybridisation treatments
including proteolysis and acetylation, and post-
hybridisation conditions. Specific hybridisation was
never demonstrated with any probe. Background grain
counts were significantly decreased by acetylation of
sections. Non-specific adherence of probes to cells in
tissue sections, provisionally identified as macrophages,
was abolished by fixation in ethanol-acetic acid. The
possible reasons for the failure of the method are
discussed. It was concluded that in situ hybridisation
techniques for demonstrating oncogene mRNA transcripts in
tissue sections have many problems and disadvantages
compared with immunohistochemical methods for detection
of oncogene proteins.

4.1 MATERIALS AND METHODS

4.1.1 DNA probes

The following DNA plasmid probes were used in RNA dot blotting and in-situ hybridisation techniques:

a) PT24-C3 (Shih et al, 1982), containing a 6.3 kbp insert of the mutationally activated T24 human Ha-ras-1 gene.

b) PSP3K (McCoy et al, 1983), containing a 640bp sequence of the 3' exon of Ki-ras-2 in SP65 vector.

c) PMC41C (Watson et al, 1983), containing an 8.4 kbp sequence including the entire human c-myc gene.

d) PHR281 (Erickson et al, 1981), containing a 6.7 kbp sequence of human ribosomal DNA.

e) PBR322 (Bolivar et al, 1977) - plasmid DNA without inserts.

All probes were used as nick-translated whole plasmid DNA, heat denatured by boiling for 5 minutes immediately before use.

4.1.2 Nick translation of DNA probes

Nick translation was carried out using minor modifications of the method described by Rigby et al, (1977). Probes were labelled with ^{32}PdCTP (preferred for dot blotting) or ^{35}SdCTP (for in situ hybridisation experiments). Nick translation using ^{3}H-dCTP yielded DNA probes of significantly lower specific activity, and was considered unsuitable for in situ hybridisation

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experiments. 0.5ug probe DNA was incubated with 10 ng/ml of DNA'se I, 1% stock E. coli polymerase I and a mixture of labelled dCTP and unlabelled dATP, dGTP and dTTP. The labelling reaction was allowed to occur at $14\,^{o}C$ for 3 hr in 0.05M TRIS pH 8.0. It was stopped by the addition of 0.2M EDTA. Excess tRNA was added, and the unincorporated nucleotides removed by Sephadex G50 chromatography. The labelled DNA was precipated by standard methods (Maniatis, 1982). Specific activity of ^{32}P and ^{35}S-labelled probes varied from approximately 5×10^{7} to 5×10^{8} cpm/ug DNA.

4.1.3 RNA extraction from cell lines and tissues

RNA was extracted from cell lines , experimental tumours and human colorectal tissues using a modification of the method described by Chirgwin et al, (1979). Sterile glassware and equipment were used throughout, and where possible, stages were performed on ice. 1-2 g of frozen tissue, or approximately 10^{7} cells were used. Tissues or cells were homogenised in a glass homogeniser in guanidium isothiocyanate buffer until dissolved. After centrifugation to remove solid particles, the supernatant was ultracentrifuged on a caesium chloride gradient overnight at 38krpm. The resulting pellet was extracted through chloroform-butanol and the aqueous phase containing RNA was ethanol precipitated and recovered by centrifugation. RNA concentration was measured by assessing optical density at 260 and 280 nm. The quality

of the extracted RNA was assessed by running samples on a
0.8% agarose gel containing 18% formaldehyde (prepared by
standard methods - Maniatis, 1982), which was stained by
ethidium bromide and photographed in ultraviolet light.

4.1.4 <u>RNA</u> <u>dot</u> <u>blotting</u>

Nitrocellulose filters were soaked in sterile DDW and
taken through graded SSC solutions to 20xSSC (1xSSC is
0.15 M NaCl, 0.025M trisodium citrate). RNA samples were
adjusted in concentration to 2.5ug/ul (and in some semi-
quantitative experiments doubling dilutions from that
concentration). Samples were boiled for 10 mins and 2ul
applied to each dot location of the filter. Denatured
unlabelled probe DNA (1 ng) was used for positive
hybridisation control. Filters were baked at 80 $^{\circ}$C for 1
hr. For hybridisation, filters were soaked in 20xSSC,
then incubated with 10 ml pre-hybridisation buffer (50%
formamide, 5xSSC, 5xDenhardt's solution, 50 mM sodium
phosphate pH 6.5. 1% glycine, 250ug sheared calf thymus
DNA, 0.1% SDS) in a sealed polythene bag at 42 $^{\circ}$C for 1
hr. This was drained and replaced by 10 ml hybridisation
fluid, essentially the same as pre-hybridisation fluid,
containing 0.5 ug nick translated DNA probe, denatured by
boiling for 5 mins immediately before use. Incubation was
carried out overnight at 42 $^{\circ}$C. The filter was then washed
in 3 changes of 2xSSC, 0.1% SDS at room temperature,
followed by 3x15 minute changes of 0.1% SSC at 50 $^{\circ}$C. The
filter was then blotted dry, wrapped in cling film, and

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placed next to two pre-sensitised X-ray plates, and kept at -70 °C. Plates were developed at 2 and 5 days.

4.1.5 In situ hybridisation experiments

Frozen sections of the following tissues and preparations were cut, ensuring that thawing did not occur at any time:

a) agar pellets of CHL and FHO5T1 cell lines, prepared as described in section 2.1.1.

b) FHO5T1 tumours from immune-deprived mice, with corresponding normal mouse tissues - mounted together to give a composite block.

c) human colorectal tissues, consisting of carcinomas, adenomas and normal colonic mucosa.

Sections were mounted on gelatine-coated slides and immediately fixed in one of the following fixatives:

a) 0.1% glutaraldehyde in 0.1M cacodylate buffer, 20 minutes, on ice.

b) 1% or 4% buffered formaldehyde, 20 minutes, room temperature.

c) 4% freshly prepared paraformaldehyde, 20 mins, room temperature.

d) 3:1 ethanol-acetic acid, 20 mins, room temperature.

After fixation, sections were washed in PBS for 10 mins, and were either dehydrated through graded alcohols for storage at -70 °C, or were taken directly to the next pre-hybridisation stage.

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A variety of pre-hybridisation treatments were compared with control untreated sections. Such experiments used multiple serial frozen sections cut from the same tissue block. Pre-hybridisation protocols were as follows:

a) Proteolytic digestion. Sections were incubated in self-digested Pronase (10ug/ml) in 50mM Tris pH 7.4 at 37 °C for 10 mins, or with Proteinase K (1.5ug/ml) in the same buffer containing 3mg/ml calcium chloride, for 10 mins at 37 °C For Proteinase K, the reaction was stopped by the addition of glycine (2mg/ml in PBS). Sections were then post-fixed in either 4% paraformaldehyde or 0.1% glutaraldehyde.

b) Acetylation. Sections were immersed in 0.1M triethanolamine buffer pH 8.0 to which acetic anhydride was added within seconds of use, to 0.25%. Slides were incubated for 10 mins at room temperature, then washed in PBS.

c) Pre-hybridisation with unlabelled sheared salmon sperm DNA. Sections were incubated overnight in hybridisation buffer (see below) containing excess (1mg/ml) sheared heat-denatured salmon sperm DNA, at 42 °C. Thereafter, sections were washed twice in 2xSSC at room temperature for 2 hrs, then taken to the hybridisation stage.

Hybridisation conditions

The protocol for hybridisation was slightly modified from the method of Akam (1983). Up to 1ug of nick translated

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DNA probe was added to 300 ul of hybridisation buffer (50% deionised formamide, 0.6M NaCl, 10mM TRIS pH 7.2, 1mM EDTA, 0.02% Ficoll, 0.02% PVP, 1mg/ml BSA, 0.1 mg/ml salmon sperm DNA) and boiled for 10 mins immediately prior to use. 8ul of the mixture was placed on the section, and sealed under a cover slip with cow gum. Sections were incubated overnight at 42 ^{o}C. The coverslip was then removed, and sections exposed to one of a variety of post-hybridisation protocols:

a) wash in 4 changes of post-hybridisation buffer (as hybridisation buffer, omitting probe and salmon sperm DNA), at 37 ^{o}C, over 24 hr.

b) wash in 2xSSC, 2hrs, room temperature; then in 6xSSC, 25% formamide, 39 ^{o}C, 2x10 mins; then overnight in 2xSSC at 4 ^{o}C.

c) wash in 2xSSC, 2 hr, room temperature; then 1 hr in 0.1xSSC at 60 ^{o}C; then overnight in 2xSSC at 4 ^{o}C.

Sections were then dehydrated through graded alcohols, and dipped in Ilford K2 emulsion for autoradiography. After drying, sections were placed next to pre-sensitised X-ray plates (which allowed a rapid visual screening of results). X-ray plates were developed after 3 days. Autoradiographs were developed after variable periods up to 3 weeks, and were then stained with haematoxylin and eosin.

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4.2 RESULTS AND DISCUSSION

Total RNA was extracted from cell lines, experimental mouse tumours and freshly-frozen human colorectal tissues, and in order to confirm the presence of RNA hybridisable to the DNA oncogene probes, dot blot hybridisation was carried out. RNA samples were first run on a denaturing agarose gel, and particularly in the case of human colorectal tissues, there was evidence of a considerable degree of RNA degradation having occurred. Although two major bands were usually discernible, representing 18S and 28S ribosomal RNA components, a continuous smear of RNA was present indicating degraded fragments of a wide range of sizes within the samples. Despite this, the extracts contained RNA in a sufficiently intact form to allow specific hybridisation on nitrocellulose, confirming the presence in the tissues examined of transcripts complementary to probe DNA sequences.

The results of one such dot blot hybridisation experiment is shown in Fig 4.1. RNA from human colorectal tissues and controls were spotted onto 4 identical nitrocellulose filters and probed with PT24C3 (Ha-ras), PSP3K (Ki-ras), PMC41C1 (c-myc) or PHR28-1 (human ribosomal sequence). The latter is a ubiquitous sequence in all human cells, and its transcript universally abundant. It served as a positive control to confirm the presence of RNA in a

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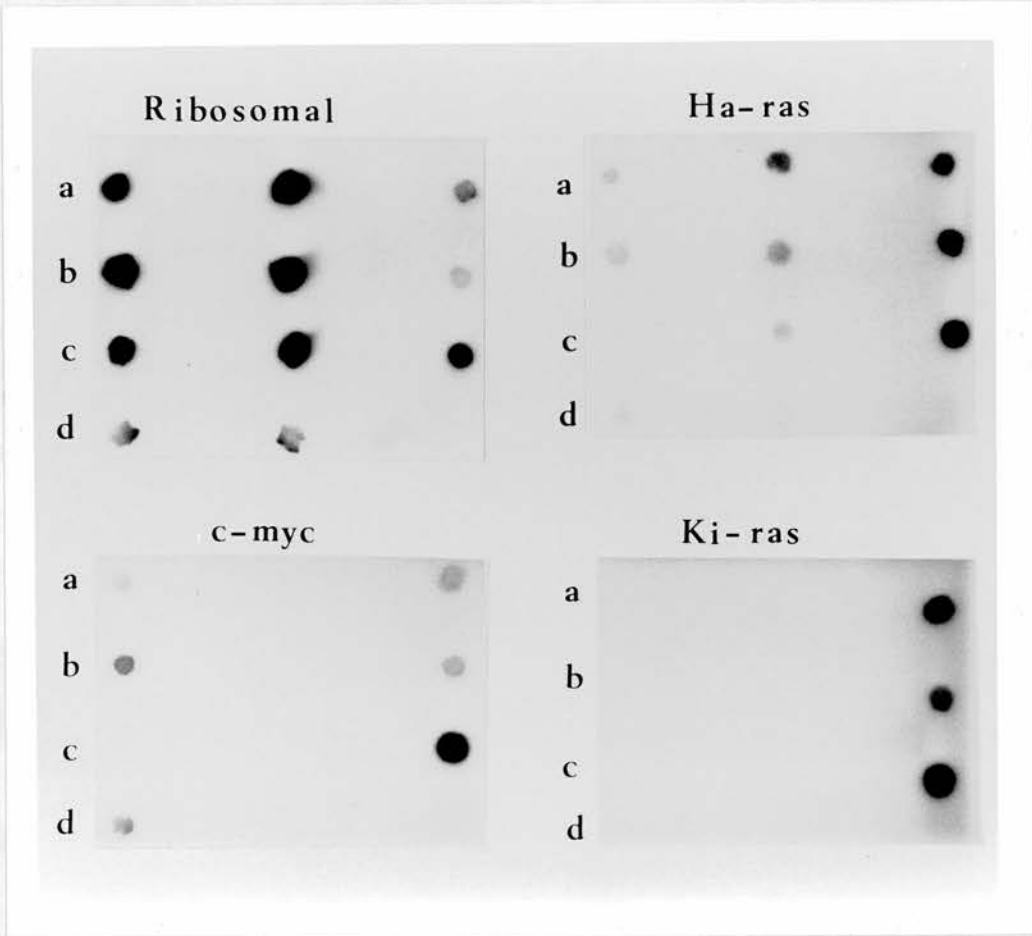
Fig. 4.1 RNA dot blots of human colorectal tissues and
controls. 4 identical blots are shown, probed with [32]-P
labelled nick-translated DNA probes containing sequences
of human ribosomal DNA, Ha-ras, Ki-ras or c-myc (see
text). Column 1 - RNA dots of normal mucosa (a), adenoma
(b) and carcinoma (c) from same patient; (d) - normal
mucosa from patient with diverticular disease. Column 2 -
RNA dots of (a) - FhO5T1 cells in culture; (b) - FHO5T1
tumour in mouse; (c) - CHL cells in culture; (d) - normal
mouse liver. Column 3 - dots of probe DNA as positive
controls - (a) - PSP3K (Ki-ras); (b) - PT24C3 (Ha-ras);
(c) - PMC41C1 (c-myc).

hybridisable form on the filters, and to confirm approximately equal amounts of RNA in each dot location.

Quantitative differences in RNA can not be reliably assessed using this experimental design, whose purpose was confirmation of the presence of oncogene transcript only. Nonetheless it is clear that there are differences in the dot intensities of different samples probed with the same probe. Investigation of such differences was not pursued in this study. Differences in the intensity of hybridisation of individual samples with different probes are due to an even wider range of potential variables and were not pursued.

In situ hybridisation to tissue sections was performed with human colonic tissues in which dot blot hybridisation had confirmed the presence of transcript. Similarly, hybridisation was attempted with sections of agarose pellets of FHO5T1 cell line, and FHO5T1 tumour, for which more quantitative data were available on abundance of Ha-ras transcript. Despite the use of a range of experimental conditions encompassing the major published methods, specific hybridisation was never demonstrated.

The rationale for the various strategies is as follows. In many instances, relatively high levels of background grain counts were present on autoradiographs, and it was

possible that a low level signal was being obscured. The radioactivity responsible for this background was not removed by extensive post-hybridisation washes of varying stringency as described above. Pre-hybridisation with heat-denatured salmon sperm DNA was designed to block non-specific adherence of DNA to tissue sections. Its effect was deleterious however, as background grain counts were increased significantly, and this approach was abandoned.

Most published methods for in situ hybridisation to tissue sections include a pre-hybridisation stage of proteolysis. The justification for this treatment is that any RNA present in the tissue section may be physically unable to hybridise with probe DNA as it is obscured by cellular proteins. The effects of Proteinase K and Pronase were compared with untreated control serial sections. There was no consistent difference in labelling of tissues, with no specific hybridisation demonstrable. Morphological preservation was poorer in treated sections, and a recurring technical problem was floating of sections.

The possibility was considered that non-specific background was due to probe DNA binding to local concentrations of basically charged proteins, via a charge effect. To investigate this, sections were incubated with acetic anhydride, a procedure designed to

acetylate basic groups, removing the charge effect when hybridisation is subsequently performed (Hayashi et al, 1978). Sections treated in this way showed a small but significant decrease in background activity, but specific hybridisation was still not detectable. The acetylation stage was retained in further experiments.

An early observation in human colorectal tissues submitted to in situ hybridisation techniques was of binding of probe DNA to a population of cells, tentatively identified as macrophages, in the lamina propria of normal mucosa and in the stroma of tumours (Fig. 4.2). Binding was non-specific in that it occurred with all DNA probes, including 'irrelevant' DNA plasmids such as PBR322 which contains no human sequences. Treatment of sections with RNA'se prior to hybridisation had no effect on binding, confirming that adherence was not due to hybridisation with RNA. Acetylation of sections before hybridisation also had no effect, and similarly proteolysis had no observable effect. The only experimental condition which abolished non-specific binding of this type was fixation of sections in ethanol-acetic acid after the pre-hybridisation stages.

There are many possible explanations for the failure of the methods of in situ hybridisation which were used in this study, and it is likely that a combination of different factors is responsible. Firstly, although

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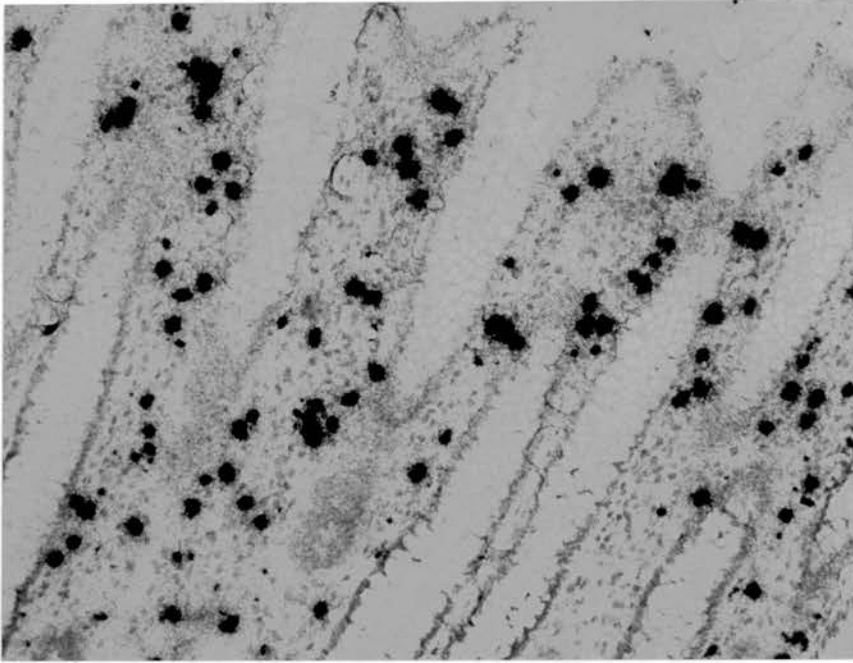
Fig. 4.2 Non-specific binding of ^{35}S-labelled DNA probe
PBR322 to cells in the lamina propria (probably
macrophages) of normal colonic mucosa. Binding is
unaffected by pre-treatment of sections with RNA'ase.

hybridisable RNA was demonstrated in extracts of tissues by dot blot hybridisation, the procedure of RNA extraction by its very nature involves a many-fold concentration of RNA from the relatively low levels in the tissues. It is likely that the number of copies of mRNA oncogene transcript in the tissues is sufficiently low that the sensitivity of the method is surpassed. The inability to detect ribosomal RNA, which is abundant, comprising approximately 70% of total cytoplasmic RNA, is difficult to explain on this basis, but colleagues have subsequently shown heavy labelling of rRNA in frozen sections of a variety of tissues. Their results emphasise two additional features:

(i) RNA is 'smeared' out of the tissue by the act of sectioning, appearing as an asymmetric 'shadow' conforming to the path of the microtome blade, and

(ii) the rRNA probe used in the present work had an affinity substantially less than optimal.

It is certain that copy number of oncogene transcript is considerably lower than that of rRNA. Whilst quantitative data are not available for human colorectal tissues, Wyllie et al, (1987) have shown that even in the highly ras-expressing cell line FHO5T1, Ha-ras transcript accounts for only approximately 0.8% of total RNA.

Degradation of RNA occurs very readily by endogenous or exogenous nucleases, and there was evidence of degradation having occurred in the RNA extracts,

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particularly those prepared from human colorectal tissues. In collecting human clinical specimens, particularly surgical resections of the large intestine, there is inevitably a period of peroperative hypoxia and ischaemia, followed by a variable delay until tissue samples are selected and frozen. During this period, RNA degradation occurs with a resulting diminution in the quantity of hybridisable RNA transcript in the tissues. Although all possible steps were taken to minimise delay in tissue handling, it is likely that a considerable degree of degradation occurred during this stage. The problem is more readily dealt with in cell lines and animal tumours, and the failure to demonstrate specific hybridisation in these preparations suggests that other factors are also important.

Fixation of tissue sections is necessary for a number of reasons. The structure of tissues must be preserved for subsequent morphological examination, and fixation inactivates all enzymatic activity (including nucleases) in the tissues, thus preventing endogenous degradation of RNA. Fixation, however, may also affect the ability of RNA to undergo hybridisation. This may involve direct chemical modification of RNA itself, or RNA may be physically obscured by proteins cross-linked in the fixation process, rendering it inaccessible for hybridisation.

Proteolytic treatment aims to overcome this potential problem by cleaving proteins and exposing the underlying RNA for hybridisation. The treatment is empirical and can be difficult to control, but its effectiveness has been emphasised in a number of publications. Post-fixation after protease treatment is said to prevent loss of mRNA during the hybridisation stage (Jilbert et al, 1986). A potential problem of contamination of enzyme preparations by small quantities of RNA'ses can be avoided by 'self-digestion' of the protease preparation before use.

The procedure of nick translation of probes yielded labelled DNA with specific activities of between 5×10^7 and 5×10^8 cpm/ug DNA. ^{32}P was used for dot blot hybridisation, but high penetration of its emission gave poor localisation on autoradiography and ^{35}S was used, which allowed generation of labelled probes of similar specific activity, but with improved localisation. Specific activity of labelled DNA of the levels achieved are satisfactory for dot blot hybridisation, but may be insufficiently high to allow hybridisation to be detected at the cellular level in tissue sections. More recently developed methods of DNA labelling, such as oligonucleotide primer labelling allow higher specific activities of DNA probes to be achieved, and may increase the sensitivity of the technique. Biotin labelling of DNA by nick translation or other techniques is another approach; after hybridisation, sensitive enzyme-linked

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detection systems can be used.

One of the effects of nick translation is a reduction in the single-stranded length of the labelled probe. The effect is principally a function of the DNA'se I concentration. Under the conditions used in this study, DNA of a wide range of different sizes is produced. Probe length is likely to have an important effect on the signal produced in in situ hybridisation, with two conflicting influences in operation. Shorter probes would be expected to have improved access to hybridisable RNA, but longer probes will show increased bound radioactivity per hybridised mRNA molecule. This theoretical conflict is reflected in the literature - Brahic & Haase (1978) observed an increased signal with shorter probes; Gowans et al, (1981) found little difference, and Hafen et al (1983) described a stronger signal with longer probes. In this study, it was assumed that the range of sizes of probe generated by nick translation would minimise the influence of this variable.

The rate and precision with which complementary nucleic acid sequences hybridise is dependent on several factors including the probe concentration, sequence complexity of the probe, temperature, solvent concentration and salt concentration. Hybridisation conditions were selected which were similar to those considered optimal for RNA dot blot hybridisation on nitrocellulose filters. Ample

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scope would have been present for optimising these conditions had specific hybridisation been demonstrated in tissue sections, but in its absence, the experimental conditions were not varied in this study.

Double stranded DNA probes, nick translatd and heat-denatured to single strands, have obvious disadvantages for detection of specific sequences. Only one strand of the duplex is complementary to the sequence being sought, while its partner will tend to exert competitive inhibition of the desired reaction. A labelled single-stranded probe has theoretical advantages in this regard, although in practice, colleagues using such single-stranded RNA probes (generated by the SP6 or Riboprobe system) for in situ hybridisation (K. Rose, A.H. Wyllie) have met with only limited success.

In conclusion, the techniques used in this study for the detection of oncogene transcripts by in situ hybridisation were found to have many problems and disadvantages compared with immunohistochemistry. In the context of the qualitative demonstration of oncogene expression in tissues, mRNA transcripts and oncogene protein products are equally acceptable as targets for specific probes. Immunohistochemistry using specific antibodies to oncoproteins was therefore the method selected in this study.

CHAPTER 5

DISCUSSION

5.1 The ras oncogene in human colorectal neoplasia

There is agreement from the work of several investigators that expression of the ras oncogene is increased in colorectal carcinomas compared with non-neoplastic colonic mucosa (Spandidos & Kerr, 1984; Slamon et al, 1984; Gallick et al, 1985; Pulciani et al, 1985). The immunohistochemical findings of the present study confirm this, but provide additional evidence to show that ras expression tends to be higher still in pre-malignant colorectal adenomas. This observation has not been previously recorded as few studies have specifically examined ras expression at the adenoma stage. However, Spandidos & Kerr (1984) demonstrated elevated levels of ras mRNA transcripts in both adenomas and carcinomas, and interestingly, some of the highest levels occurred in adenomatous lesions. Gallick et al (1985) examined ras expression in carcinomas by immunoblotting techniques, and found that the highest levels of ras p21 protein occurred in cancers at an early clinical stage (Dukes' stage A), whereas more advanced lesions which had produced metastases tended to have lower levels. Despite this, no clear relationship has emerged between ras expression and clinical or prognostic parameters. Kerr et al (1986) found no correlation between levels of ras mRNA and prognosis, and in the series of carcinomas examined by immunohistochemistry in the present study, again no relationship could be shown between ras expression and

any clinical or pathological features of the tumours.

There are a number of studies claiming to examine p21 _ras_ expression and localisation in colorectal tumours by immunohistochemistry, but most have employed the monoclonal antibody RAP-5 (Horan Hand et al, 1984; Thor et al, 1984; Czerniak et al, 1987; Michelassi et al, 1987). The conclusions from these studies are at variance with the results reported herein; RAP-5 staining is reported to increase with increasingly malignant phenotype during the process of colonic carcinogenesis. Evidently, the pattern of staining with RAP-5 is of interest and may have biological significance, but as discussed in chapter 2, it is doubtful whether it bears any relation to _ras_ expression.

Immunochemical studies with other antibodies are scanty. Kerr et al (1985) using Y13-259 obtained broadly similar patterns of staining to the results of this study, although increased expression in adenomas was less obvious. In an immunoblotting study of a range of human tumours, Tanaka et al (1986) used polyclonal antibodies raised to synthetic peptides of p21. Single p21 bands were detected in normal colonic mucosa in 7 cases, and in 3, expression was elevated in carcinomas relative to the corresponding normal mucosa. The electrophoretic mobility of the p21 detected in these carcinomas was the same as that of the normal mucosa, and it was concluded that the

codon 12 mutation was absent in these cases, as this would have given a slower migrating protein. These findings differ slightly from the immunoblotting data of the present study (chapter 2) in which doublet bands of p21 were found in extracts from both normal mucosa and carcinomas. This is probably a reflection of the different antibodies used.

Increased expression of ras in carcinomas seems not to be a consequence of gene amplification. Pulciani et al (1985) examined DNA from a series of unmanipulated primary human tumours including 31 colorectal carcinomas and 13 adenomas, and in no case was amplification of ras genes detected. Similar results were reported by Alexander et al (1986), Yokota et al (1986) and Meltzer et al (1987), and the collective experience of these studies also indicates that major ras gene rearrangements are also uncommon.

However an interesting finding in these studies was the identification of allelic deletion of Ha-ras occurring in a small proportion of cases. The significance of this is uncertain, and whilst it may represent random loss of chromosomal material in genetically unstable tumours, it may be indicative of a more specific event in initiation or progression. A deletion of Ha-ras itself is unlikely to be a primary event, but it may point to involvement of another gene or genes in close proximity on chromosome 11

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whose deletion leads to development of malignancy.

The paradigm of this mechanism which has received most attention is the hereditary form of retinoblastoma, in which susceptibility to the tumour is inherited through a germline mutation, and the neoplasm develops after a somatic change (usually deletion) of the homologous allele. It appears that loss or functional inactivation of both alleles of a normal regulatory gene allows development of malignancy (Klein & Klein, 1985). Almost nothing is yet known about the functions of such genes (sometimes referred to as 'anti-oncogenes'), but their existence is not in doubt, and their role is coming under intense scrutiny at present. As discussed in section 1.4.3 (chapter 1), the recent mapping of the FAP gene to chromosome 5q21 (Bodmer et al, 1987) and the demonstration of allelic loss at this locus in 20% of sporadic cancers (Solomon et al, 1987) indicates that this mechanism may operate on chromosome 5 in a significant proportion of cases of colorectal cancer. Similar events involving different genes also occur on other chromosomes, notably chromosome 17p (Fearon et al, 1987), but probably also less frequently on chromosome 11.

ras genes appear to achieve transforming potential most commonly by the acquisition of activating mutations of codons 12 or 61, and recent evidence suggests that such

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mutations of Ki-ras occur in up to 40% of colorectal cancers (Bos et al, 1987). In this study, 6 of the carcinomas examined had residual adenomatous elements, and in 5 of these, the activating mutation was also present in Ki-ras in the adenomatous areas. Forrester et al (1987) also reported Ki-ras mutation to be present in a similar proportion of colorectal carcinomas, and also in 7 out of 8 villous adenomas. This is compelling evidence that mutational activation of Ki-ras is a critical early event in the carcinogenic process in the colon.

In vitro, mutational activation of ras is not always required to confer the transformed phenotype, but although the normal ras gene can be artificially driven by powerful retroviral LTR sequences to effect transformation, this is a relatively ineffective way of activating ras. In primary cultures, the cooperation of another cotransfected oncogene such as myc is required to cause transformation (Land et al, 1983). In contrast, the mutated ras gene much more readily induces properties of the malignant phenotype, properties which are nonetheless still modulated by the level at which ras is expressed. Mutated ras genes driven by their own cellular promoters fail to transform primary embryo fibroblasts, whist consistently inducing transformation in established fibroblast cell lines. However, when driven by powerful retroviral enhancer elements, they transform both

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(Barbacid, 1986).

Mutational activation of ras has no effect on its inherent rate of expression (Tabin et al, 1982), and as ras amplification or rearrangements are uncommon in colorectal tumours, the elevated expression of ras in colorectal adenomas and carcinomas may be secondary to some other change in the neoplastic cells. The nature of such a change is a matter for speculation, but the influence of other expressed oncogenes may be important. It is clear from the simplicity of the process by which ras genes become activated that the full spectrum of the malignant phenotype cannot be conferred in this single step. There is good evidence from in vitro experiments that single oncogenes, even in activated form, have limited powers of transformation, whereas a number of examples are known of different oncogenes cooperating to produce oncogenic transformation. This is in keeping with the nature of human cancer, a slowly-developing multi-stage disease.

In vitro models show that ras may cooperate with the myc oncogene to induce malignant properties in cells, but before considering the nature of such cooperation, it is appropriate to examine what has emerged from this study on the individual contribution of the c-myc oncogene in the process of colorectal neoplasia, and how this relates to the work of other investigators.

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5.2 c-myc and colorectal carcinogenesis

It is clear from a variety of sources that c-myc expression is increased in colorectal carcinomas relative to normal mucosa (Slamon et al, 1984; Erisman et al, 1985; Calabretta et al, 1985; Alexander et al, 1986; Yokota et al, 1986; Sikora et al, 1986; Stewart et al, 1986; Jones et al, 1987). RNA dot blotting performed in 29 colon carcinomas by Erisman et al (1985) revealed 5-40 fold elevation of c-myc transcript compared to normal mucosa in 72% of cases, but Southern blotting showed absence of amplification or major rearrangements affecting the gene in any case. Amplification of c-myc has been detected by other groups however, but at low frequency. Yokota et al (1986) described 3-8 fold amplification in 2 out of 32 colon cancers; 2-5 fold amplification was reported in 2 of 9 carcinomas by Alexander et al (1986), and Meltzer et al (1987) reported amplification in 3 out of 45 carcinomas. However, again no major rearrangements involving the c-myc locus were detected in any of these studies. Rothberg et al (1985) noted that elevated expression of c-myc occurred significantly more frequently in tumours arising in the left colon than those of the right, and suggested that this may relate to differences in epidemiology and clinical behaviour between tumours at these two sites.

Immunohistochemical studies (previously discussed in

section 3.3.3) also provide evidence for increased expression of the p62 c-myc oncoprotein in colon carcinomas, and in this regard at least, are in agreement with the findings of the present study. The biological significance of increased myc expression is a matter for some debate. It has been pointed out by Calabretta et al (1985) and Torelli et al (1987) that the increase in myc expression in colonic carcinomas is accompanied by a parallel increase in expression of other cell-cycle related genes, including those for histones, whose expression is restricted to the S phase. Increased histone gene expression has therefore been cited as evidence of an increased growth fraction in tumours, and it was suggested that increased c-myc expression is a non-specific reflection of the greater proportion of cycling cells. It may, however, be erroneous to regard c-myc as a cell-cycle related gene in the same sense as the histone genes. There is no direct correlation between c-myc expression and passage through the cell cycle - for example, Campisi et al (1984) have described constitutive high expression in quiescent chemically transformed cells in culture, and more recently, Erisman et al (1988) have shown constitutively high c-myc mRNA and protein levels in cell lines derived from human colorectal carcinomas. Normal cells do show increased c-myc transcripts in the first cycle following growth stimulation (Kelly et al, 1983), but levels return to basal values in subsequent cycles. Indeed c-myc mRNA transcript levels have been

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shown to be invariant throughout the cycle in actively proliferating cells in culture (Hann et al, 1985; Thompson et al, 1985). Rather than being associated with proliferation per se, high levels of c-myc expression appear to be associated with the ability of the cell to enter the cell cycle from the G_0 phase. However, as mitogenic stimulation of asynchronously growing cell cultures induces elevation of c-myc expression, a G_0-arrested cell population is not required for this elevation of c-myc expression to occur, and Erisman et al (1988) have argued that activation of expression may be a direct response to growth factor-receptor interaction. In keeping with this suggestion is the observation that serum (ie growth factor) depletion of asynchronous cultures for short periods leads rapidly to decreased c-myc expression. Continual presence of growth factors may be required to sustain basal levels of c-myc expression. In the light of such suggestions, it can be argued that the enhancement of c-myc expression in colorectal carcinomas is not a consequence of increased cell proliferation in tumours, but a primary change specific to the tumour, disrupting normal control of proliferation, perhaps by preventing exit to the G_0 phase.

Until more is understood about the molecular function of the protein product of c-myc, and the mechanisms by which c-myc expression regulates cell proliferation, the

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significance of increased expression is likely to remain a matter for speculation. The same applies to the interpretation of the altered patterns of p62 c-myc localisation between the nucleus and cytoplasm observed in this study. It is clear that complex events occur at several levels in the regulation of c-myc expression which may influence the amounts and localisation of the protein product. The rapid synthesis and transport of p62 to the nucleus, and its short biological half life suggest that minor changes in the rate of synthesis, transport to the nucleus or subsequent breakdown could lead to relatively major changes in the quantity of p62 present in the cytoplasm at any one time. The main conclusion which can be drawn from the immunohistochemical results herein is that some change occurs in the cellular processing of the p62 protein during the adenoma-carcinoma sequence leading to its accumulation in the cytoplasm in carcinomas. The nature of the change is entirely unknown.

5.3 Cooperation of c-myc and ras oncogenes

Neither the activated cellular ras nor myc oncogenes can transform normal cells singly, but in cooperation they can achieve full transformation in vitro (Land et al, 1983). Similar cooperation has been described between a number of other oncogenes, and it appears that the two cooperating genes may each elicit a limited range of

cellular responses that act in distinct but complementary ways. Weinberg (1985) has emphasised that although up to 40 different oncogenes are known, there is a much smaller number of pathways governing cellular proliferation, and that it may be possible to classify oncogenes according to a limited range of functional properties. He has divided oncogenes into two groups, 'nuclear' or 'cytoplasmic' according to the localisation of their gene product, and such a simple classification correlates quite well with what is known of the functions of their proteins, and the effects produced when the oncogenes are introduced into cell cultures by transfection. 'Nuclear' oncogenes (such as c-myc) confer immortalisation in culture and reduced serum requirements, but are poor at inducing anchorage-independent growth. 'Cytoplasmic' oncogenes (such as ras) tend not to induce immortalisation, but promote anchorage-independent growth and may induce growth factor secretion. In most instances, activation of oncogenes from both nuclear and cytoplasmic groups appears to be necessary to effect transformation in vitro. This framework is certainly an over-simplification, as there are several reported exceptions to this scheme. It is also important to note that these studies have involved fibroblasts rather than epithelial cells which may behave quite differently. Nonetheless, the concept of cooperating oncogenes is an attractive one, and is consistent with the multistage process of human carcinogenesis.

The detailed mechanisms involved in oncogene cooperation remain obscure, but a number of observations may be of relevance. Cells transfected with the c-myc oncogene show enhanced transcription of a number of resident cellular genes as well as introduced genes (Kingston et al, 1984). It has also been reported by Wyllie et al (1987) that expression of certain endogenous cellular oncogenes (abl and fos) can be substantially elevated in myc and ras transfectants. Interestingly, in the transfected cell lines with tumourigenic properties, elevated expression of oncogenes of both nuclear and cytoplasmic groups occurred, even in cells transfected with a single extraneous oncogene. This is consistent with the proposal that activation of oncogenes of both types is important in the induction of transformation.

There is direct evidence of trans-activation of transcription of certain oncogenes by other oncogene proteins derived from the adenovirus early region 1A. When introduced into cells, the cellular promoter activities of endogenous c-fos and c-myc genes are stimulated, and their transcription shows significant elevation. On the other hand, transcription of Ha-ras is unaffected (Sassone-Corsi et al, 1987). It is interesting to note that segregation of behaviour is again seen between the 'nuclear' and 'cytoplasmic' oncogenes in this situation.

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The hypothesis that multiple oncogenes may exert their actions on a limited number of biochemical pathways to transformation receives support from study of transformation revertants. Zarbl et al (1987) selected revertants of v-fos-transformed fibroblasts, and found that these cells were resistant to subsequent transformation by several oncogenes (v-fos, v-Ha-ras, v-abl, v-mos). They proposed that mutation may occur in a gene or genes lying on a cellular pathway common to transformation by several oncogenes which need not necessarily share obvious structural similarities. Interestingly, these revertants were not totally resistant to transformation - other structurally quite dissimilar viral oncogenes could cause transformation of these cells. It seems likely that such oncogenes either use a different pathway, or act at a point downstream of the block.

Clearly the complexities of interactions between different oncogenes are difficult to unravel, especially in human tumours in vivo, and distinguishing primary and secondary events is a problem. However it may be reasonable to concentrate on the potentially fundamental role of c-myc in the process, as it appears that elevated levels of the myc product may perturb regulation of the cellular transcription apparatus. It has been suggested by Weinberg (1985) that activation of c-myc may induce transcription of a collection of cellular genes whose

products are critical for growth and differentiation.

5.4 Other oncogenes

Data on the involvement of other oncogenes in colorectal carcinogenesis are even scantier. Bolen et al (1987) have described 5-8 fold increased tyrosine kinase activity of the pp60-c-src oncogene product in all of 15 colorectal carcinomas, and even higher increases in cell lines derived from colonic cancers. This was not attributable to increased abundance of the protein, and it was suggested that the increased specific activity of the phosphotransferase may contribute to the genesis of colon tumours. In a study designed to detect oncogene amplification or allelic deletions of oncogenes that are heterozygous for restriction fragment length polymorphisms (RFLP), Meltzer et al (1987) used 11 different oncogene probes to detect alterations in DNA from a series of 45 colon carcinomas. In addition to identifying 3 cases of c-myc amplification (referred to above), c-erbB-2 amplification was detected in one case, and allelic deletion of c-myb was found in 4 cancers and 1 of 15 adenomas. No abnormalities were detected with the other oncogene probes. The study of Yokota et al (1986) was of similar design, and in addition to finding c-myc amplification in 2 out of 32 colon cancers, allelic deletion of c-myb was also reported in one case. It is in the nature of studies of this type that the experimental

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design is organised to detect a small number of specific abnormalities, and when 'screening' for alterations in a range of oncogenes in this way, the incidence is inevitably underestimated. For example, the RFLP technique can only identify allelic deletion in heterozygotes, and then only if length polymorphism is present in an oncogene cut by a particular restriction enzyme. Moreover, many rearrangements and most point mutations would be undetected by this method. The relative infrequency of abnormalities detected and their lack of uniformity suggests that they may be a consequence rather than a cause of carcinogenesis.

In conclusion, it is evident that deregulated expression of several oncogenes occurs in the process of colorectal carcinogenesis, and that alterations of ras and myc oncogene expression are particularly frequent. It is tempting to conclude that ras and myc in cooperation are responsible for important properties of malignant cells, and play a direct role in the early stages of colorectal carcinogenesis. However the malignant phenotype represents the integration of the individual actions and interactions of multiple genes, and it may be wrong to imagine that the individual action of any one gene should correlate very closely with behaviour. It is probable that there are multiple routes by which cells may achieve the malignant phenotype, and in a system of complex

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interactions between oncogenes and many other cellular genes, we are faced with the familiar problem of distinguishing the causes from the consequences of neoplastic change. However there is reason for optimism that some of the fundamental molecular mechanisms of carcinogenesis have now been identified, and the future holds the challenging prospect of defining in detail the molecular mechanisms by which normal cells acquire the properties of malignancy. We are now in a strong position to meet the challenge.

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PUBLICATIONS FROM THESIS

Parts of the work described herein have been published; photocopies of papers follow, (included by kind permission of Macmillan Press Ltd).

1. A description of immunohistochemical detection of ras p21 product in the FHO5T1 cell line and in colorectal tissues was published in the British Journal of Cancer, 1985, 52: 687-693, and selected for publication in abstract in The Year Book of Cancer, 1987, pp144-146.

2. A report of evaluation of monoclonal antibody RAP-5 by immunohistochemistry and immunoblotting was published in the British Journal of Cancer, 1986, 54: 877-883.

3. Frozen section immunohistochemistry results with monoclonal antibody Y13-259 in colonic tissues were the subject of an oral presentation to The Pathological Society of Great Britain and Ireland, in Cardiff in June 1985. An abstract was published in The Journal of Pathology, 146: 249A.

4. Immunohistochemical results with monoclonal antibody Myc1-9E10 in colorectal tissues were presented in poster form to The Pathological Society in Southampton in July, 1987. An abstract was published in The Journal of Pathology, 152, 234A.

5. A report of immunohistochemical demonstration of ras p21 product in PLPD-fixed paraffin sections has been accepted for publication in The Journal of Pathology, (1988), 155.

Immunohistochemical detection of the *ras* oncogene p21 product in an experimental tumour and in human colorectal neoplasms

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Summary The monoclonal antibody Y13 259 to the *ras* oncogene protein product p21 was used in an immunohistochemical study of *ras* expression in human colorectal neoplasms. The ability of the antibody to detect enhanced levels of *ras* expression was confirmed by its use with an experimental neoplasm known to express *ras* at high levels. Human colonic adenocarcinomas in general showed a similar staining intensity to that seen in normal mucosa. Adenomas however showed consistently high p21 expression as indicated by staining intensity. This suggests that elevated *ras* expression may be important in the development of adenomas, but that high levels need not be sustained in the conversion to invasive carcinoma.

Although the *ras* gene family has been studied longer than any other group of cellular oncogenes, there is still uncertainty over its role in authentic human carcinogenesis. *Ras* genes code for GTP-binding and GTP'ase enzyme activities of molecular weight 21,000 daltons, referred to as p21 (Shih *et al.*, 1979; Gibbs *et al.*, 1984; McGrath *et al.*, 1984; Sweet *et al.*, 1984), which appear to be located on the inner face of the plasma membrane (Willingham *et al.*, 1980), and are probably normally involved in the transduction of membrane-associated stimuli for cell proliferation (Bishop, 1983; Kamata & Feramisco, 1984). Activation of the genes by mutation has been detected in approximately 15% of a wide variety of spontaneous human tumours and transformed cell lines in culture (Der *et al.*, 1982; Parada *et al.*, 1982; Pulciani *et al.*, 1982; Varmus, 1984). Raised levels of *ras* transcript have also been identified in RNA extracted from several types of human tumour (Spandidos & Kerr, 1984; Slamon *et al.*, 1984; Spandidos & Agnantis, 1984; Spandidos *et al.*, 1985). Controversy remains, however, over whether *ras* activation is a primary event in carcinogenesis or appears during tumour progression. At a more pragmatic level, it is quite obscure whether the detection of *ras* expression in pre-malignant lesions or malignant tumours would provide useful diagnostic or prognostic information.

In this paper, we describe the use of the monoclonal antibody Y13 259 in detection of *ras* p21 in human colorectal neoplasms. We have confirmed the ability of the antibody to detect enhanced levels of *ras* expression by applying it in parallel to the experimental neoplasm FHO5T1, in which high levels of *ras* were achieved through genetic manipulation.

Materials and methods

Tissues

Tissues used included 21 adenocarcinomas of the colon and rectum, 6 benign tubular adenomas of the colon, and 7 specimens of colonic tissue resected for non-neoplastic conditions (diverticular disease or ulcerative colitis). The diagnosis in each case was confirmed on paraffin sections. Specimens were received fresh within minutes of resection in the operating theatre. They were examined immediately, samples of tissue taken (including where possible the tumour/normal interface), placed in plastic vials and 'snap' frozen in liquid nitrogen where they were stored until required. Frozen sections (6 μm) were cut, mounted on gelatine-coated slides, fixed in acetone at room temperature for 15 min and air dried. As controls for immuno-staining, two cell lines were maintained in culture as previously described (Spandidos & Wilkie, 1984): the transformed line FHO5T1, which contains the mutated T24 human Ha-*ras* oncogene inserted within a high expression vector, and its parental, untransformed Chinese hamster lung fibroblast strain, here called CHL. FHO5T1 cells were also grown as tumours in nude mice. Frozen sections of these tumours and normal mouse tissues were prepared exactly as above. The cultured cells were studied in cytocentrifuge preparations, or in frozen sections of pellets in low melting temperature agarose, fixed as above.

Antibodies

The monoclonal antibody to p21 designated Y13 259 was prepared from the hybridoma cell lines as previously described (Furth *et al.*, 1982). Secondary antisera used in a comparison of reagents and staining methods were horseradish-peroxidase (HRP)-conjugated rabbit anti-rat Ig (Dako), HRP-conjugated sheep anti-rat Ig (Amersham), biotin-conjugated rabbit anti-rat IgG (Vector), unconjugated rabbit anti-rat Ig and monoclonal peroxidase–anti-peroxidase complex (Sera Lab.).

Immunostaining

Sections were washed in TRIS-buffered saline, pH 7.6 (TBS) and non-specific binding blocked by application of normal human serum diluted 1 in 5 in TBS (NHS/TBS). Y13 259 diluted 1 in 100 in NHS/TBS was applied for 1 h. After washing in TBS and blocking as above, the appropriate secondary antibody was applied at a dilution of 1 in 50 in NHS/TBS for 1 h. Anti-human tissue activity was significantly diminished in secondary antibodies by absorption with acetone-dried human liver tissue and human immunoglobulins (Cohn fraction II, Sigma). Sections treated with HRP-conjugated secondary antibodies were washed in TBS and the reaction developed with DAB solution (1 mg ml^{-1} diaminobenzidine (BDH) in 50 mM TRIS-HCl pH 7.60, containing 10 mM imidazole, activated with H_2O_2 immediately prior to use. Sections incubated with biotinylated secondary antisera were washed in TBS and further incubated for 30 min with biotinylated preformed complex of streptavidin-HRP (Amersham) diluted 1 in 200 in TBS. After final washing, the reaction was developed as above. Negative control sections were included for each case, and positive controls (cytocentrifuge preparations of the FHO5T1 cell line) were used in each staining experiment.

Preliminary work comparing the indirect, PAP and streptavidin-biotin methods showed highest sensitivity with the latter method. In our hands, the staining shown with Y13 259 in human tissues is generally of relatively low intensity, and it is thus important to minimise non-specific background staining. The clear backgrounds and higher dilutions of primary antibody afforded by the amplified streptavidin-biotin method determined the choice of this method in the main study.

Results

Experimental neoplasm

The transformed cell line FHO5T1 showed strong reactivity of all cells with Y13 259, both in cyto-

centrifuge preparations and in frozen sections of cell pellets. In contrast, the great majority of cells of the parental strain from which it was derived (untransformed Chinese hamster lung fibroblasts, termed CHL), showed no reactivity. A small proportion however, approximately 5%, showed strong specific staining (Figures 1 and 2).

FHO5T1 cells inoculated into nude mice produced malignant tumours of fibrosarcomatous appearance (to be described in detail elsewhere). Frozen sections of such tumours showed strong specific staining of all tumour cells with Y13 259, whilst the adjacent murine tissues were negative (Figure 3).

The reactivity of Y13 259 with this tumour was completely lost in formalin-fixed paraffin-embedded sections, and was not restored in any measure by trypsinisation. Similarly, formaldehyde or glutaraldehyde fixation of cytocentrifuge preparations of the FHO5T1 cell line abolished antibody binding. Y13 259 is therefore not suitable for use with routinely processed biopsy material.

Human colonic tissues

Positive staining of the intensity of the FHO5T1 tumour was not seen in any of the human colonic tissues studied. In general, where positive staining was detected, it was of low intensity, despite use of the most sensitive peroxidase detection system available to us.

The results of staining of 21 colo-rectal adenocarcinomas, 6 colonic adenomas, their adjacent uninvolved mucosa where available and 7 cases of colonic resections for non-neoplastic conditions are shown in Table I. Staining of sections was independently assessed by two observers and graded as equivocal or negative ($+/-$); moderate

Table I Staining intensity of colonic tissues for Y13 259.

Tissue	Number of cases – staining intensity		
	$+/-$	$+$	$++$
Carcinomas	15	4	2
Adenomas	0	1	5
Normal adjacent to carcinoma	7	9	0
Normal adjacent to adenoma	2	4	0
Normal non-neoplastic	4	3	0

Adenomas show a significantly greater intensity of staining compared to carcinomas ($P<0.01$) and all normals ($P<0.002$). (Four-fold Table Test). Carcinomas show no significant difference in staining intensity from normal mucosa.

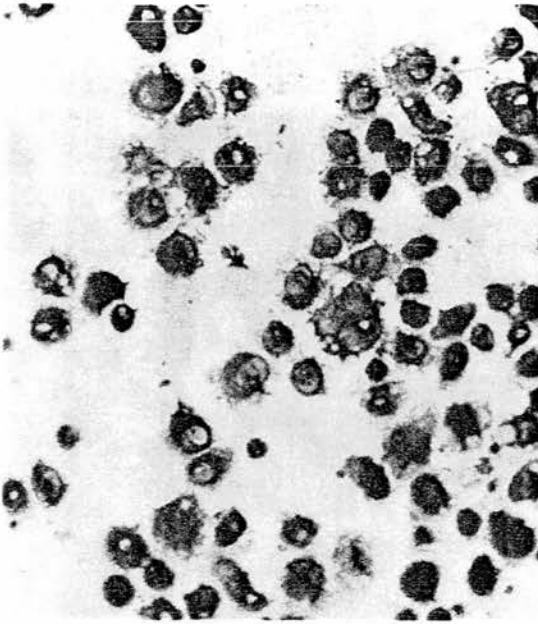**Figure 1** Transformed (FHO5T1) cells – cytocentrifuge preparation. All cells show intensely positive staining with Y13 259. (× 320).
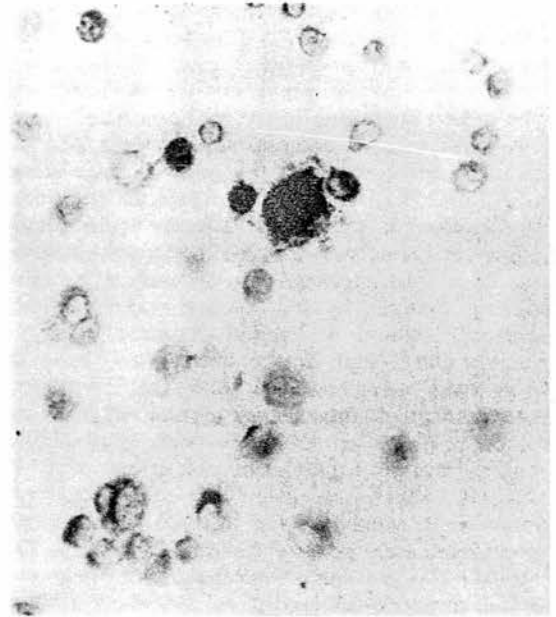

Figure 2 Parental untransformed Chinese hamster lung fibroblasts (CHL) stained with Y13 259. Most cells show no reactivity, but a small subpopulation show intensely positive staining. (× 320).
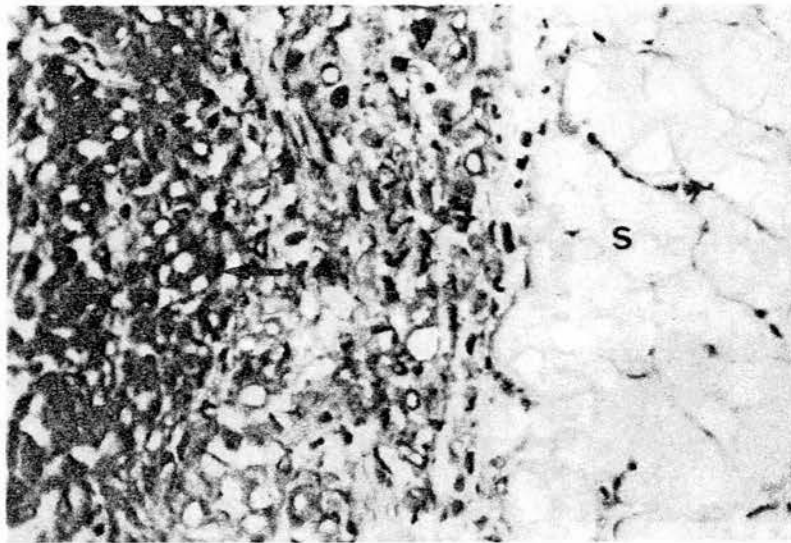

Figure 3 Frozen section of advancing edge of FHO5T1 tumour in nude mouse. Tumour shows uniformly intense staining (arrow); adjacent fibrous tissue and skeletal muscle (S) is negative. (× 320).

(+) or intense (+ +). Agreement between observers occurred in more than 75% of cases; where different assessments were made, such cases did not affect the statistical significance of the results. The Table represents a consensus.

Normal colonic mucosa showed equivocal or faintly positive reactivity of uniform distribution with Y13 259 (Figure 4). No significant difference was observed in the mucosa adjacent to neoplastic lesions compared with mucosa from non-neoplastic resections. Adenocarcinomas showed a variable staining pattern; in 15, staining was absent or equivocal, whilst 4 showed moderately strong staining and 2 were graded as intense (Figure 5). There was no correlation with the histological pattern, depth of invasion or clinical stage of the tumour. Where a difference was observed in staining pattern between the carcinomas and the adjacent mucosa, a sharp transition was not observed. In contrast, 5 out of 6 colonic adenomas showed intensely positive staining (Figure 6). The interface between normal and neoplastic epithelium in the adenomatous lesions did not show a sharp
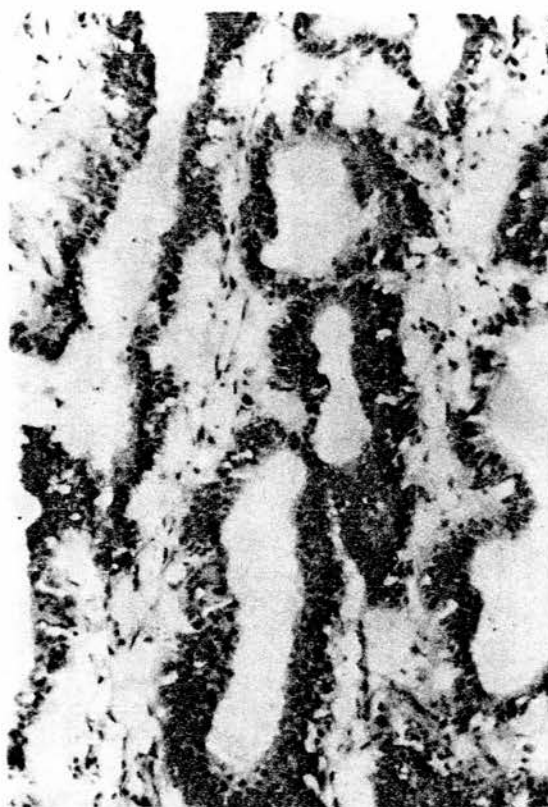

Figure 5 Adenocarcinoma of colon showing positive staining. (This case shows the most intense degree of staining; the majority of carcinomas are negative). (× 160)

Figure 4 Normal colonic mucosa shows only very faint staining. (Macrophages in the lamina propria show endogenous peroxidase activity). (× 160)

transition in reactivity; positive staining was often most intense at the centre of the adenomas.

Discussion

The monoclonal antibody Y13 259 is one of a series produced in rats bearing tumours induced by the Harvey murine sarcoma virus (Furth *et al.*, 1982). These authors showed it to precipitate p21 protein species encoded by both the Harvey and Kirsten strains of the virus. There is close homology of the protein products of viral and cellular *ras* genes. Capon *et al.* (1983*a*) have shown the viral and cellular p21 protein products to be identical in all but three out of 189 amino acid residues. Y13 259, traced on immunoblots by the streptavidin-biotin method, binds to a single protein band, with an apparent mol. wt of 21,000 on SDS-PAGE, present in substantial quantities in *ras*-transformed cells, and at much lower levels in
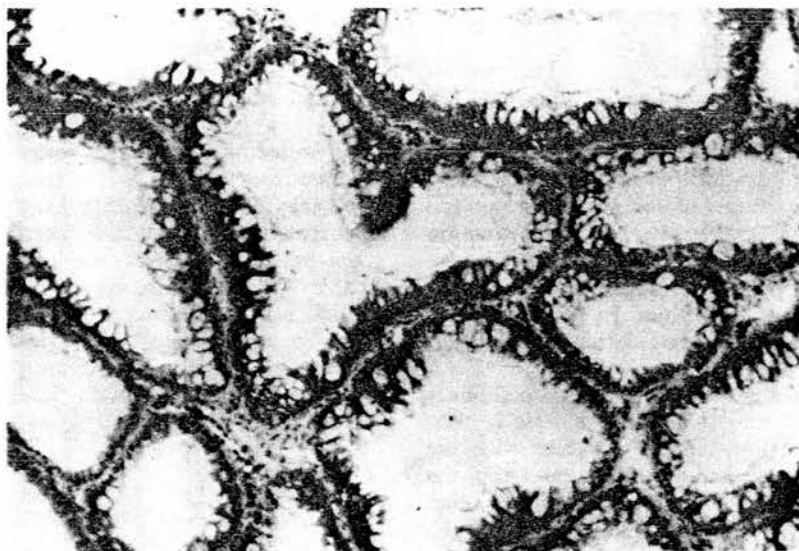
Figure 6 Tubular adenoma of colon showing intense staining with Y13 259. (× 160)

untransformed cells (D.A. Spandidos and T. Dimitrov, unpublished work). The antibody is thus potentially useful in the detection of c-*ras* oncogene expression in human tissues.

It was of great value in this study to have access to a known positive control in the form of the experimental neoplasm FHO5T1. This transformed cell line is known to express the mutated T24 Ha-*ras* oncogene at high levels; quantitation of oncogene mRNA in dot blots indicated 20–60 fold more Ha-*ras* message in the transformed cells relative to the untransformed parental fibroblasts (Spandidos & Wilkie, 1984). Whilst there is no formal proof of the specificity of Y13 259 for *ras* p21, we have established its greatly increased reactivity with *ras*-transformed cells over their untransformed parental cell strain.

Although increased transcription of activated *ras* genes has been observed in certain neoplasms (DeFeo *et al.*, 1981; Chang *et al.*, 1982; McCoy *et al.*, 1983), mutation at specific positions (12 or 61) in the amino acid coding sequence is the feature most consistently observed in *ras* activation (Capon *et al.*, 1983*a*; *b*). It was therefore not surprising to find that p21 expression in human tumours never attained the artificially high levels seen in the experimental tumour.

The presence of a subpopulation of CHL cells strongly positive with Y13 259 may be related to differential expression of *ras* at different stages in the cell cycle (Campisi *et al.*, 1984). It is less likely to represent contamination of CHL by FHO5T1 or spontaneous transformation of CHL cells, as the

proportion of *ras* positive CHL cells has not changed after further passage in continuous culture.

The principal result of this study is the consistently high level of p21 expression in adenomas, whereas the carcinomas in general showed lower staining intensity. As the great majority of colo-rectal carcinomas are believed to arise from adenomatous polyps (Morson & Dawson, 1979), it would seem that the elevated p21 expression diminishes significantly as the lesions evolve into invasive carcinomas.

An essentially similar conclusion was reached by Spandidos and Kerr (1984), who reported increased levels of RNA transcripts of Ki-*ras* and Ha-*ras* oncogenes in a series of colonic adenomas and adenocarcinomas, but noted higher expression of *ras* mRNA in some adenomas compared with corresponding carcinomas from the same patients. A recent study employing the same antibody Y13 259 with immunoblotting techniques (Gallick *et al.*, 1985) also reached the conclusion that p21 expression tended to be greatest in the earlier stages of colonic carcinomas.

The p21 proteins are thought to function as transducers of signals from the extracellular environment to the nucleus in a system intimately involved in the control of cellular proliferation (Hurley *et al.*, 1984; Kamata and Feramisco, 1984). Activation, even by mutation, of *ras* appears to result in the delivery of a continuous signal rather than a regulated one (Sweet *et al.*, 1984). *Ras* activation may be an early event in the development of adenomas, which are known to show a

shorter cell cycle time as well as expansion of the proliferating compartment relative to the normal mucosa (Bleiberg & Galand, 1976; Deschner & Lipkin, 1976). Indeed, cycle times in adenomas are shorter than those of carcinomas. A further carcinogenic stimulus or stimuli may be required for conversion of adenomas to invasive carcinomas, with sustained elevations of *ras* expression perhaps being no longer necessary. An analogous situation of early *ras* activation has been described in the context of chemical skin carcinogenesis in mice, where c-Ha-*ras* has been found to be activated at the stage of benign papilloma formation (Balmain *et al.*, 1984).

The findings of this study differ significantly from those described by Thor *et al.* (1984), who found *ras* p21 expression to correlate with the depth of invasion of colonic carcinoma within the bowel wall. Using different monoclonal antibodies raised to synthetic peptides reflecting part of the p21 protein structure, they found p21 expression in normal colonic mucosa and colonic adenomas to be negative or very low, whilst carcinomas expressed relatively high levels. This was interpreted as indicating *ras* activation to be a late stage in the development of colonic malignancy. The reasons for this discrepancy are not clear, but the different methods used in raising these antibodies suggest they may have very different specificities from Y13 259.

It would be of great interest to determine whether the elevated p21 expression detected in adenomas is a product of the normal cellular oncogene, or of the activated mutated gene. The monoclonal antibodies currently available are unable to distinguish between the mutated p21 protein and the normal, but analysis of restriction fragment polymorphism of DNA extracted from tumours may be a more promising approach. Work is currently proceeding in our laboratory to address this question.

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Evaluation of a monoclonal antibody to *ras* peptide, RAP-5, claimed to bind preferentially to cells of infiltrating carcinomas

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Summary RAP-5, a monoclonal antibody raised against a p21^{ras} peptide, has been claimed to show immunohistochemical localisation of cells with infiltrative properties in human tumours. We confirmed that this antibody reveals pronounced cellular heterogeneity in human colonic neoplasms but could find no obvious relationship to infiltrative activity. RAP-5 bound to many different cell types, neoplastic and normal. In order to clarify the specificities of RAP-5 we applied it to two cell lines: nontumorigenic hamster fibroblasts in which *ras* expression is barely detectable, and a vigorously tumorigenic line derived from these fibroblasts by insertion of the human mutated Ha-*ras* oncogene in a high expression vector. Another antibody to p21^{ras}, Y13-259, clearly distinguished between these cell lines both on immunoblots and immunocytochemically, but RAP-5 did not. Rather, it bound to proteins of a variety of molecular weights in both cell lines. The results show that RAP-5 is unlikely to be a useful reagent for detection of *ras* associated proteins in human tissues.

Although the mutated form of the human Harvey *ras* oncogene was the first transforming gene of cellular origin to be identified (Der *et al.*, 1982; Parada *et al.*, 1982; Santos *et al.*, 1982), the precise role of *ras* oncogenes in human malignancy is far from established. *Ras* genes code for a 21,000 dalton protein, p21^{ras}, which is located on the inner face of the plasma membrane (Shih *et al.*, 1979; Willingham *et al.*, 1980, 1983), binds GTP and possesses GTPase activity (Sweet *et al.*, 1984; Gibbs *et al.*, 1984; McGrath *et al.*, 1984). Mutations in the vicinity of codons 12 and 61 of the *ras* genes lead to products deficient in GTPase, but not GTP binding activity, and these products in particular have been associated with carcinogenesis. Thus, insertion of mutated *ras* genes into cultured cells confers upon them a transformed phenotype and tumorigenicity in animals (Reddy *et al.*, 1982; Tabin *et al.*, 1982; Taparowski *et al.*, 1982); mutant p21^{ras} introduced to cells by micro-injection can also initiate proliferation and effect transient phenotypic changes akin to transformation (Feramisco *et al.*, 1984; Stacey & Kung, 1984); and *ras* gene mutation at the critical sites has been shown to be an early event in experimental chemical carcinogenesis (Sukumar *et al.*, 1983; Balmain *et al.*, 1984). In around 15% of human solid tumours, there is evidence for the presence of mutated *ras* genes (reviewed by Weinberg, 1985).

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Amplification of *ras* genes has also been recorded in such tumours, although it is rare (Pulciani *et al.*, 1985). Hyperexpression of *ras* mRNA, and raised levels of p21^{ras} (without necessarily implicating mutation of the gene) have been reported in primary human tumours in a variety of sites (Slamon *et al.*, 1984; Spandidos and Agnantis, 1984; Spandidos *et al.*, 1985; Tanaka *et al.*, 1986; Kurzrock *et al.*, 1986). Some studies suggest that *ras* expression increases in parallel with aggressive behaviour in neoplasms of the human colon (Horan Hand *et al.*, 1984), breast (Ohuchi *et al.*, 1986) and prostate (Viola *et al.*, 1986), but the opposite result has also been reported (Spandidos & Kerr, 1984; Gallick *et al.*, 1985; Williams *et al.*, 1985; Kerr *et al.*, 1986).

Recently, a series of monoclonal antibodies termed RAP1-5 has been raised against a synthetic peptide, consisting of amino acids 10 – 17 of mutated humans Ha-*ras* p21. In immunohistochemical studies, these antibodies were claimed to show striking preferential localisation to infiltrative carcinoma cells, as compared to non-infiltrative neoplasms and normal tissues. In this paper, we examine the reactivity of RAP-5 with a range of normal and pathological human tissues, and with rodent cells lines in which widely divergent levels of expression of human Ha-*ras* p21 had been achieved through genetic manipulation (Spandidos & Wilkie, 1984). We have found these cell lines, together with comparison with another antibody (Y13-259) of proven specificity to p21^{ras}

(Furth *et al.*, 1982; Lacal & Aaronson, 1986), to be particularly helpful in assessing the important claims made for this new antibody.

Materials and methods

Fibroblast cell lines

CHL and FH05T1 were maintained *in vitro* as described previously (Spandidos & Wilkie, 1984). CHL cells were originally obtained by culture of fibroblasts from Chinese hamster lung. FH05T1 cells were derived from CHL cells by transfection with the plasmid pH05T1, which contains the mutated human Ha-*ras* (T24 bladder carcinoma cell line) oncogene adjacent to the SV40 transcriptional enhancer sequence. Cytocentrifuge preparations were made from these cells after disruption of the monolayers by gentle treatment with EDTA and trypsin.

Human colorectal tissues

Tissues were obtained within minutes of surgical removal. Some portions were immediately frozen in liquid nitrogen and stored at $-80°C$ prior to preparation of cryostat sections, whilst others were fixed in 4% neutral buffered formaldehyde at room temperature for processing in paraffin. In all, frozen material was studied from normal colonic mucosa (5 cases), colorectal adenomas (6) and adenocarcinomas (6). Material from the 6 adeno-carcinomas was also processed in paraffin. For comparison, formaldehyde fixed paraffin processed material representing *other normal and pathological tissues* was selected from departmental files: tonsil (1), intradermal naevus (1), malignant melanoma of skin (1), and bronchial carcinoid tumour (1).

Immunocytochemistry

Immunocytochemical analysis with RAP-5 and Y13-259 was carried out on 5 μm cryostat sections fixed for 15 min in either 4% neutral buffered formaldehyde or acetone, using a streptavidin-biotin immunoperoxidase method as described previously (Williams *et al.*, 1985). With RAP-5, the same techniques were also applied to dewaxed, rehydrated paraffin sections. Briefly, sections or cytocentrifuge preparations were washed in Tris buffered saline, (TBS – sodium chloride 150 mM, Tris HCl 10 mM, pH 7.6) and nonspecific binding blocked by application of 10% normal rabbit serum in TBS (NR-TBS). The primary antibody was applied for 30 min at room temperature at the optimum dilution in NR-TBS. For RAP-5 this dilution lay between 1:10,000 and 1:20,000, whilst for Y13-259 the optimum dilution for use with

tissue sections was 1:100 and with cytocentrifuge preparations 1:500. The sections were washed in TBS, and incubated for 30 min at room temperature in the second antibody. For RAP-5, this was sheep anti-mouse immunoglobulin (Amersham International) and for Y13-259 goat anti-rat immuno-globulin (Sigma), both biotinylated and diluted 1:50 in NR-TBS. After further washing in TBS the sections were incubated for 15 min with streptavidin-biotinylated horseradish peroxidase complex (Amersham) diluted 1:200 in NR-TBS. The reaction was developed after a final wash in TBS, with diaminobenzidine solution $(1 \, mg \, ml^{-1})$ (BDH) in 50 mM Tris HCl pH 7.6, containing 10 mM imidazole activated immediately prior to use with H_2O_2. The sections were briefly counterstained with haematoxylin, dehydrated and mounted.

Negative controls were included for each case, consisting of sections treated identically to the ·others but with NR-TBS replacing the primary antibody. Invariably, these gave no immuno-peroxidase reaction save over macrophages and polymorphonuclear leukocytes within the tissues.

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Immunoblots

These were prepared from lysates of CHL and FH05T1 cells. Washed cell pellets were lysed in 100 mM sodium chloride, 10 mM Tris pH 7.5, 0.1% SDS, 1% NP40 at 4°C. Insoluble residue was removed by centrifugation at $30,000 \, g$ for 30 min and the supernatants were denatured by heat im-mediately prior to electrophoresis. Approximately 20 μg of protein was loaded per track on 15% polyacrylamide gels, blotted on nitrocellulose, and detected by Indian ink (Hancock & Tsung, 1983), or immunostaining. We used essentially the same conditions for immunostaining of the nitrocellulose filter as for the cytological preparations, with the exception that the antibody dilutions used were 1:100 and 1:1000 for both Y13-259 and RAP-5. Incubation was for 2 h at room temperature.

Results

Binding of RAP-5 and Y13-259 to human tissue sections

We confirmed, in the present series of experiments, our previous results on the staining pattern of Y13-259 on acetone-fixed frozen sections of human colorectal tissues. Normal mucosa and the epithelium of most adenocarcinomas showed low levels of reactivity, whilst in general adenomas showed staining of greater intensity. We did not observe specific staining in nonepithelial cell types.

RAP-5, applied to formaldehyde-fixed frozen sections of the same and other blocks showed a different pattern: although normal epithelium tended to stain at low levels, there were no consistent differences between infiltrative and non-infiltrative neoplasms. Infiltrative carcinomas sometimes showed a moderate reaction, but sometimes were negative, whilst non-infiltrative lesions also gave positive reactions. Within individual tumours there was considerable variation in the distribution of positively staining cells, and in the intensity of the reaction (Figure 1a, b). We frequently observed moderate staining of the muscularis propria. Serosal mesothelium also consistently gave a strong reaction. In formaldehyde-fixed paraffin and frozen sections of a variety of other tissues, RAP-5 gave strongly positive reactions, notably in the cells of an intradermal naevus, malignant melanoma, and carcinoid tumour, but there was no obvious relationship with aggressive activity (Figure 2a–c). Although only single cases of these conditions were studied, the results indicate that reactivity to RAP-5 is not restricted to epithelial cells or to cells originating from any one germ layer.
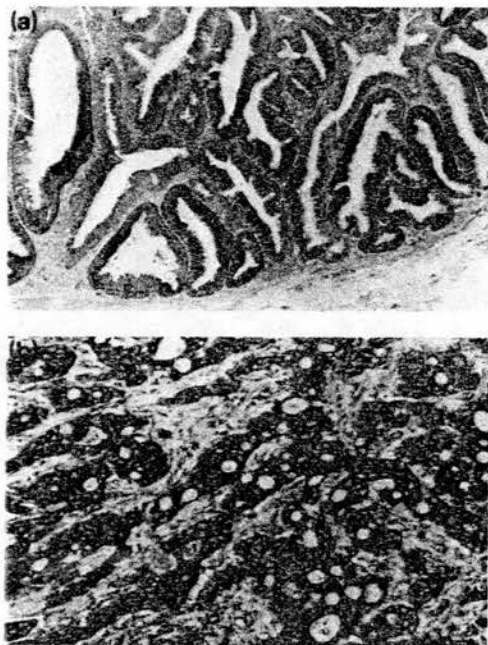

Figure 1 Immunoperoxidase detection of RAP-5 binding to formaldehyde fixed paraffin sections of human colonic adenoma (a) and infiltrative colonic carcinoma (b). Heterogeneity of cellular staining is evident in both the benign and malignant tumour. (× 50, a; × 160, b).

Binding of RAP-5 and Y13-259 to proteins in ras-expressing cell lines

As previously reported (Williams *et al.*, 1985), Y13-259 applied to acetone fixed cytocentrifuge preparations of the *ras*-transformed cell line FH05T1, yielded strong immunochemical staining over all cells (Figure 3a), whereas less than 5% of the parental, untransformed CHL fibroblasts gave positive reactions. Analysis of the antibody binding proteins on immunoblots, after SDS-polyacrylamide gel electrophoresis, confirmed that Y13-259 at both 1:100 and 1:1000 dilution detected a single protein, of apparent mol. wt 21 kd in FH05T1 cells (Figure 4a). In extracts of CHL cells the same binding protein was either undetectable or present in much ·reduced quantity. Indian ink staining of the nitrocellulose blots, or staining of unblotted polyacrylamide gels with kenacid blue showed that in terms of proteins identifiable by these means the extracts from FH05T1 and CHL cells were closely similar.

In contrast with these results, immunocytochemistry using RAP-5 as the primary antibody revealed no differences between the CHL and FH05T1 cells (Figure 3b, c). All cells of either type were negative after acetone fixation, but after fixation in formaldehyde gave strongly positive staining over a wide range of dilutions down to 1:20,000. Analysis of the antibody binding proteins was attempted on immunoblots. At dilutions of 1:1000 RAP-5 scarcely defined discrete protein bands in gel tracks loaded with extracts of either CHL or FH05T1 cells (Figure 4b). At tenfold higher concentration, a number of proteins of a wide range of molecular size appeared to bind to the antibody; the majority of these were present in similar quantity in FH05T1 and CHL extracts (Figure 4c).

Discussion

RAP-5 is secreted by a hybridoma derived from spleen cells of a mouse immunised with a *ras* octapeptide linked to thyroglobulin (Horan Hand *et al.*, 1984). The octapeptide had the amino acid sequence 10 – 17 of the mutated human (T24) Ha-*ras* p21, and selection of the hybridoma was based upon preferential binding of its immunoglobulin to the mutated as opposed to the non-mutated peptide. In practice, however, RAP-5 was found by its originators to detect epitopes present in a far higher proportion of human tumours than are associated with transforming mutations of the *ras* gene family. It was assumed that cross-reaction with non-mutated p21 was responsible. Specificity of RAP-5 for p21 *ras* was adduced from
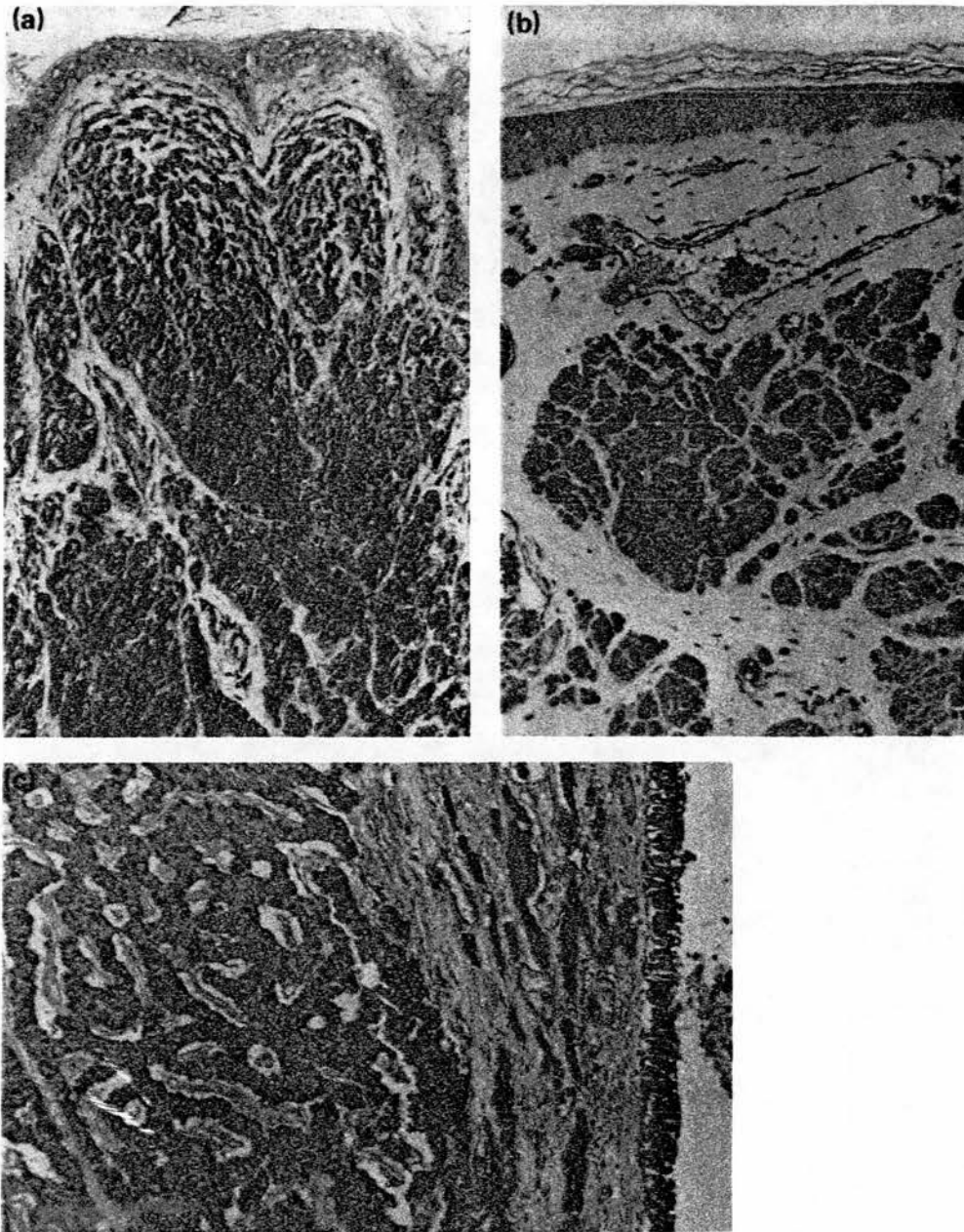
Figure 2 Immunoperoxidase detection of RAP-5 binding to formaldehyde fixed paraffin sections of human intradermal naevus (a), malignant melanoma of skin (b), and bronchial carcinoid tumour (c). The naevus and tumour cells show positive staining, with some heterogeneity, and positive cells are also identified in the overlying epidermis (a & b) or respiratory epithelium (c). (× 160).
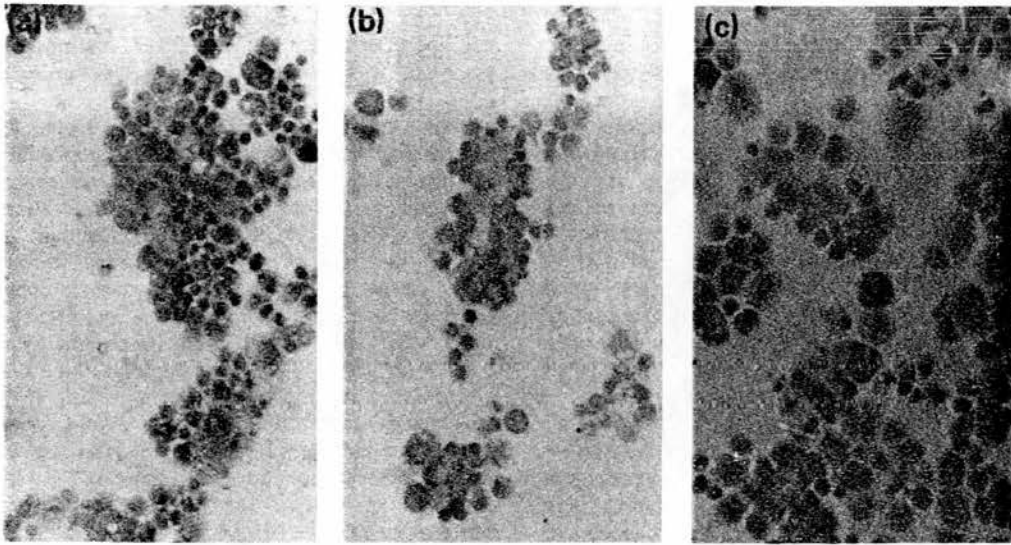
Figure 3 Positive staining of cytocentrifuged FH05T1 cells by Y13-259 (a). Similarly treated CHL cells consistently gave no staining. In contrast, both FH05T1 cells (b) and CHL cells (c) showed positive staining with RAP-5. (× 160).
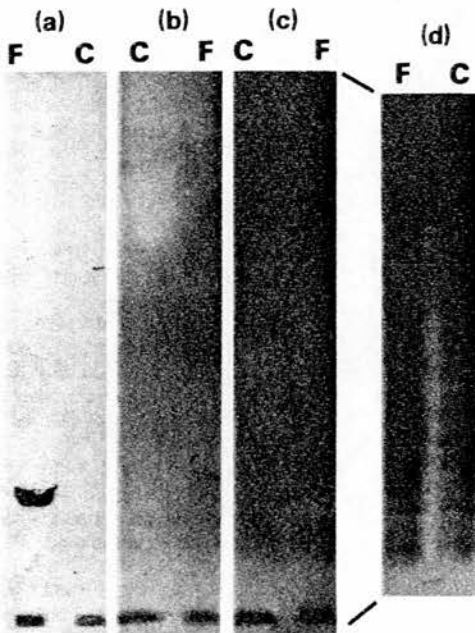

Figure 4 Electrophoresis of proteins from FH05T1 (F) and CHL cells (C). In (a), an immunoblot stained with Y13-259 at 1:100 dilution, a single band of 21 Kd appears in the FH05T1 extract, but is absent from the CHL extract. RAP-5 staining of a blot of identical extracts, at 1:1000 dilution (b) or 1:100 dilution (c) showed multiple reactive proteins in both FH05T1 and CHL cells. For comparison, similarly loaded tracks in the original polyacrylamide gel, stained with kenacid blue, are shown (d) with the position of 14 Kd and 24 Kd marker proteins (→).

competition binding studies and from immunoblots, although the concentrations of antibody used in the latter were unexpectedly high. Published data on RAP-5 do not yet include studies on a wide range of neoplastic and normal tissues. The major interest in this new antibody derived from the observation that it detected heterogeneity within human breast, colonic and prostatic neoplasms, preferentially staining areas showing infiltration of adjacent tissues, provided it was applied to sections fixed in formaldehyde (Horan Hand *et al.*, 1984; Thor *et al.*, 1984; Viola *et al.*, 1985, 1986; Ohuchi *et al.*, 1986). There were obvious implications of great fundamental and practical importance in this suggestion.

The data presented in this paper demonstrate the value of unequivocal biological test systems for such novel and potentially exciting reagents. FH05T1 cells and the parental CHL fibroblast line differ from one another in the possession of a mutated Ha-*ras* gene, in expression of the gene at the level of transcription (a feature which we confirmed in dot blots of RNA extracted from cells of similar passage history to those described here), in expression of a protein with the molecular size expected of $p21^{ras}$, in availability of this protein for detection by immunocytochemical methods, and in the ability to generate rapidly growing aggressive tumours in immune suppressed animals (Spandidos & Wilkie, 1984; Spandidos, 1985). A reagent capable of distinguishing aggressive from non-infiltrative cells on the basis of *ras* expression ought to discriminate between these two cell lines, but RAP-5 did not. Rather, under the conditions

described by its originators, it seemed to detect proteins plentiful in both cells, and also in many human cell types, both normal and neoplastic, of a variety of embryological derivations. These results do not exclude the possibility that RAP-5 may bind to a *ras* peptide, but indicate that it also recognises other widely distributed epitopes which are not related to *ras* expression. A similar conclusion has recently been reached on the basis of immunohisto-chemical studies on human breast tissue (Ghosh *et al.*, 1986). Other antibodies raised against small oncogene peptides have been shown in the past to be capable of reaction with epitopes common to many cellular proteins despite the appearances of specificity in immunoabsorption studies (Nigg *et al.*, 1982).

The role of *ras* products in tumour aggression remains undecided. In experimental animals, cells transformed by the mutated *ras* gene have been shown to be capable of both infiltration and metastasis (Spandidos & Wilkie, 1984; Muschel *et al.*, 1985; Thorgeirsson *et al.*, 1985), but *ras* expression does not always confer aggressive properties. Normal *ras* genes are expressed physio-logically in non-dividing tissues (Spandidos &

Dimitrov, 1985), and in one phaeochromocytoma cell line insertion of the products of the mutated *ras* gene led to differentiation and replication arrest (Bar-Sagi & Feramisco, 1985). Several different groups have presented evidence from animal tumours and human colonic neoplasia that activation (by mutation or hyperexpression) of the *ras* gene is a feature of early rather than late neoplasia (Sukumar *et al.*, 1983; Balmain *et al.*, 1984; Williams *et al.*, 1985; Yuspa *et al.*, 1985).

It seems probable that immunohistochemical methods will remain important in attempts to clarify the role of *ras* and other oncogenes in human neoplasia. This paper highlights the value of genetically modified cell lines in the critical evaluation of antibodies raised against oncogene proteins and peptides, and in particular casts serious doubt on the usefulness of RAP-5 in detection of human *ras*-coded, or *ras*-associated proteins.

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APPENDIX 2

Preparation of SDS-Polyacrylamide Gels

Stock solutions

A	50% w/v acrylamide in DDW		
B	1M HCl		48 ml
	TRIS		36.6 g
	DDW	to	100ml
	Adjust pH to 8.8		
C	1M HCl		48 ml
	TRIS		5.98 g
	DDW	to	100ml
	Adjust pH to 6.8		
D	TRIS		30.3 g
	Glycine		144 g
	DDW	to	1000 ml
E	1.3% w/v bisacrylàmide in DDW		

Separating gel (15%)

Mix: 15 ml soln. A
        15 ml soln. E
        0.5 ml 10% SDS
        6.25 ml soln. B
        13 ml DDW
        0.25 ml 10% (w/v) ammonium persulphate (fresh)
Degas in vacuum chamber.
Add 20 ul Temed (tetramethylene diamine - BDH Chemicals)
Pour between very clean glass plates and overlay with 1 ml.
DDW. Allow to set for 30 mins in warm room, or incubator.

Stacking gel (5%)

Mix: 2 ml soln. A
        2 ml soln. E
        0.2 ml 10% SDS
        4.8 ml soln. C
        16.8 ml DDW
        0.2 ml 10% w/v ammonium persulphate
Degas, then add 20ul Temed. Pour off DDW overlay, then
layer stacking gel on to separating gel.
Insert comb, allow to set.

APPENDIX 3

Preparation of PLPD fixative

For 400 ml:

150 ml 0.05 M phosphate buffer

50 ml 8% paraformaldehyde

0.43 g anhydrous sodium metaperiodate

2.74 g lysine HCl

5 g potassium dichromate

200 ml DDW

Phosphate Buffer

12.5 g disodium hydrogen orthophosphate anhydrous
(MW 142)

or

15.6 g disodium hydrogen orthophosphate dihydrate
(MW 178)

2 g sodium dihydrogen orthophosphate dihydrate
(MW 156)

Dissolve in 2 l of DDW

To prepare 8% paraformaldehyde

For 200 ml:

16 g paraformaldehyde powder

1 ml 5M NaOH

to 200 ml with DDW

Filter and store in the dark at 4 $^\circ$C.

Fixation

Tissues should be trimmed to 5mm thickness. Fixation is for a minimum of 18 hrs in the dark at 4 $^\circ$C. Fixed tissues must be washed in running tap water overnight to remove dichromate precipitate.