A STUDY OF METASTASIS IN COLORECTAL CARCINOMA USING DNA RECOMBINANT AND MOLECULAR HYBRIDISATION TECHNIQUES

by

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A thesis submitted in fulfilment
of the requirements for the
Degree of Doctor of Medicine at the
University of Edinburgh

April 1986

Revised October 1987

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DECLARATION

I hereby declare that the work presented in this thesis was planned, carried out and interpreted by myself other than where clearly indicated.

ACKNOWLEDGEMENTS

The work presented in this thesis was carried out at the Beatson Institute for Cancer Research, Bearsden, Glasgow whilst the author was supported by the McGhie Cancer Research Fellowship (based in the University Department of Pathology, Glasgow Royal Infirmary, under the supervision of Professor R.B. Goudie) and, subsequently, by a Cancer Research Campaign Fellowship (based in the University Department of Surgery, Glasgow Royal Infirmary under the supervision of Mr. C.S. McArdle).

My thanks are due in particular to Dr. G.D. Birnie of the Beatson Institute, Leader of group R3 in which this work was carried out, for making this project possible and also to Professor R.B. Goudie and Mr. C.S. McArdle for their advice and encouragement. I am also indebted to Mr. I.G. Finlay (University Department of Surgery, Glasgow Royal Infirmary) for stimulating discussions.

My thanks are due in addition to various members of group R3 at the Beatson Institute, especially Jennifer Burns, Anne Sproul and Dr. Mary Warnock for their practical advice and assistance without which this project would not have been possible.

Finally I am most grateful to May Habbick for her careful and conscientious preparation of this typescript.

ABBREVIATIONS

A,G,C,T,U.	Adenine, guanine, cytosine, thymine, uracil.
bp	Base pairs.
BSA	Bovine serum albumin.
dCTP	Deoxycytidine triphosphate.
DNAase	Deoxyribonuclease.
DNA	Deocyribonucleic acid
cDNA	Complementary DNA.
rDNA	DNA encoding rRNA.
DTT	Dithiothreitol.
ds	Double-stranded.
EDTA	Ethylenediaminetetra acetic acid.
GMAG	Genetic Manipulation Advisory Group.
HEPES	N-2-hydroxy ethyl piperazine N1-2-ethane sulphonic acid.
Ig	Immunoglobulin.
MOPS	Morpholinopropanesulphonic acid
poly(A)+	Polyadenylated.
poly(A)-	Non-polyadenylated.
R.I.	Refractive Index.
RNA	Ribonucleic acid.
mRNA	Messenger RNA.
rRNA	Ribosomal RNA.
snRNA	Small nuclear RNA.
RNAase	Ribonuclease.
SSC	0.15M NaCl, 0.015M Sodium
SDS	Sodium dodecyl sulphate.

ss	Single-stranded.
TCA	Trichloroacetic acid.
Tris	2-amino-2 (hydroxymethyl) propane 1:3 diol.
TEMED	NNNONO-tetramethylethylenediamine.

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ABSTRACT OF THESIS (Regulation 7.9)

It is well known that the presence of metastatic disease in colorectal carcinoma is associated with a dire prognosis. It has more recently been shown that prognosis following apparently-curative resection for colorectal carcinoma is principally determined by the presence or absence of occult metastatic disease at the time of surgery. There is evidence furthermore, based on study of the rate of growth of metastases from these tumours, to suggest that they may behave as metastasizing or non-metastasizing variants from a very early stage in their natural history. There is however no clinically applicable means currently available of accurately predicting the metastatic potential of a primary tumour. This is of considerable importance in planning and evaluating further therapeutic strategies.

A study was therefore undertaken to attempt to phenotypically characterise colorectal cancer by cloning complementary DNA (cDNA) libraries representing individual highly-and moderately-abundant mRNA species. cDNA was transcribed from polyadenylated RNA from two tumour specimens, one from a patient with disseminated disease and the other from a patient who had remained disease-free. Approximately 1,000 recombinant clones were picked for each library and were cross-screened using Grunstein-Hogness and, subsequently, dot hybridisation techniques to select the four most consistent putatively metastasis-related clones. These clones were then used as radiolabelled probes to screen mRNA from a series of samples of colorectal carcinomas (n=12), normal mucosa, adenomatous polyps and other tumour and tissue types. Steady-state transcription levels of these sequences were in general higher in the colorectal carcinomas than in normal mucosa. These RNA transcripts were also seen in several other tumour types but were scarcely detectable in normal cells such as leucocytes and cultured fibroblasts. The elevations seen in the colorectal tumours did not correlate with conventional staging criteria nor with clinical outcome over a follow up period of up to three years nor, in particular, with the development of metastatic disease. The presence of high steady-state levels of these RNA sequences appears therefore to be only tumour-associated. The lack of correlation between them and a possible metastatic phenotype suggests that if such critical sequences exist they may be expressed at low levels or that relatively small differences of expression may occur either in abundantly-expressed RNA sequences or, possibly, in a large

cont'd/....

INTRODUCTION

INTRODUCTION

1. Metastasis: General Considerations

Cancer metastasis, that is the growth of a tumour at a site separate and distinct from the primary, is one of the critical and defining characteristics of malignancy and as such is responsible for most therapeutic failures in cancer medicine. This is a fact of considerable significance given that approximately half of all malignant tumours will have already metastasized by the time of clinical presentation (Sugarbaker and Ketcham, 1977). Little however is known of its biological basis despite its being the object of much study and there is in particular no clinically-applicable means of accurately predicting the metastatic potential of a primary tumour, clearly of importance in determining subsequent therapeutic strategies.

It is recognised that the process is complex and multifactorial, involving a sequence of steps each of which is in itself necessary but not sufficient although which of these might be most critical is not clear. (For reviews see Carter, 1976; Weiss, 1977; Poste and Fidler, 1980; Folkmann, 1982). The sequence includes the initial growth and vascularisation of the primary tumour, invasion of adjacent normal tissues, (largely it is thought, as a consequence of tumour pressure, active cell movement and release of lytic enzymes) and penetration into blood and lymphatic vessels within and around the tumour, as well as penetration of body cavities. Detachment of cells from the primary tumour must then occur which may again be related to the activity of proteolytic enzymes. It has long been

known that in many tumours the cells are more readily disassociated than in normal tissue (Coman, 1953). These cells are then disseminated throughout the body although it has been inferred from clinicopathological (Willis, 1973) as well as direct experimental studies (Fidler, 1970; Butler and Gullino, 1975) that this step is highly inefficient and in one model system it has been shown that less than 1% of potentially-metastatic cells injected survived to produce metastases (Fidler, 1970). It has similarly been shown in human tumours that the presence of circulating cancer cells by no means correlates with subsequent development of metastatic disease (Salsbury, 1975). It is nonetheless generally considered that good surgical technique should minimise the vascular dissemination of tumour cells or possible local wound implantation. Factors which are thought to be important at this stage include cell size, clumping of cells (which generally produces a higher incidence of metastases in any tumour cell type (Liotta et al, 1976)) and cell-deformability, which may favour survival in passage through capillaries. It is known that the circulation is an inimical environment in any case to many normal cells e.g. fibroblasts. To survive at a distant site cells must then adhere to and penetrate the vascular (usually capillary) endothelium. For reasons which are not understood this involves arterial walls infrequently. This step apparently involves embolic trapping in fibrin and platelet thrombi, classically described in the rabbit ear-chamber by Wood (Wood, 1958), although it is not clear at what stage of arrest these coagulation phenomena occur. Tumour cells

must then adhere to a probably exposed and possibly damaged endothelial basement membrane by means of mechanical and electrostatic forces as well as, probably, tumour cell receptors for e.g. laminin and fibronectin. Extravasation of cells occurs either by a process akin to diapedesis and/or bursting through. Growth and invasion of tissue at the new site then occurs, dependent upon the acquisition of a vasculature, probably promoted by substances such as tumour angiogenesis factor (TAF) (Folkmann, 1974) and possibly upon local cellular (e.g. inflammatory cells, or osteoclasts in bone) or tissue effects (e.g. mediated by growth and/or chemotactic (Lam et al, 1981) factors). These may be critical since the presence of viable tumour cells in any organ does not guarantee metastatic tumour growth (Hewitt and Blake, 1975) and see below. The prognostic significance of the presence of tumour cells in regional lymph nodes is also unclear and may simply reflect in the early stages an active filtering role which may be later overcome when nodes may act themselves to generate metastatic disease. The therapeutic implications of such nodes similarly remains controversial (Weiss, 1977; Sugarbaker and Ketcham, 1977; Folkmann, 1982).

It is of interest that many features of the metastatic process undergone by malignant tumour cells have parallels in normal physiology e.g. the invasive behaviour of trophoblast, the extensive migrations undergone in embryogenesis by e.g. neural crest cells and the emigration of leucocytes out of the blood stream (Sherbet, 1982). It would appear that "reactivation" of the genetic loci responsible for such behaviour also occurs in malignant cells.

Both clinical (Willis, 1973) and various experimental observations have revealed a number of further unexplained and curious features of the metastatic process. These include the marked variation in metastatic potential of different tumour types (including the existence of a group of so-called "intermediate" tumours such as basal cell carcinoma or ameloblastoma which, although locally invasive, do not appear to metastasize) and differences in routes of metastasis in different tumour types. (It is well known that in general sarcomas preferentially metastasize via the blood stream whereas carcinomas do so more via lymphatics although these routes are far from mutually exclusive due to the existence of extensive lymphatico-venous anastamoses; tumours such as melanoma, neuroblastoma and teratoma appear to utilise both routes equally). There are, in addition, marked differences in organ predilection for metastases from different tumours which cannot entirely be accounted for in haemodynamic terms, although such an explanation has been put forward (Ewing, 1928), and current accounts generally conflate this view with one deriving from the hypothesis describing such events in terms of the properties of both "seeds" and "soil" (Paget, 1889). Such differences are well exemplified by the behaviour of nephroblastoma and of adrenal neuroblastoma both of which drain into the renal vein yet the preferred metastatic site for the former is lung whereas the latter more frequently metastasises to bone. The importance of haemodynamic factors is nonetheless illustrated by the fact that carcinomas of the lower third of rectum, which drain into the systemic circulation

via the inferior vena cava, metastasize more frequently to lung then to liver whereas for comparable tumours of the upper third of rectum draining into the portal venous system the converse is true (Weiss et al, 1984). Such metastatic patterns may in addition be complicated by the fact that metastases may themselves give rise to further metastatic deposits. Interestingly, changes in patterns of metastases have been observed in recent years following chemotherapy in e.g. osteosarcoma (Folkmann, 1983) and carcinoma of the breast (Amer, 1982). Clinically-evident sites of predilection for metastatic disease cannot either be accounted for in terms of type of tissue nor its embryological origin. The much more common involvement of certain sites such as liver, lung, and bone as compared to skeletal muscle or gut is poorly understood, although in experimental models in vitro organ-specific adhesion of metastatic tumour cells (Netland and Zetter, 1984) as well as the existence of a hepatocyte receptor interacting with liver-metastasizing tumour cells (Cheingsong-Popov et al, 1983) have been demonstrated.

The existence of organ-specific cultured tumour cell lines (e.g. Kim, 1979; Tao et al, 1979; Stackpole, 1981) and demonstration of such specificity even towards ectopically implanted e.g. lung in thigh (Sugarbaker et al, 1971) or parabiotic tissue (Schour and Faraci, 1974) is evidence of the importance, at least experimentally, of tumour cell properties in determining the outcome of the metastatic process. The recent clinical demonstration that the circulation of cancer cells as a consequence of peritoneo-venous shunting for malignant ascites did not

result in lung metastases is entirely consistent with such experimental observations (Tarin et al, 1984).

A further poorly-understood feature of the metastatic process is the phenomenon of "dormancy" of metastases, most notably manifest as the late (years or decades after initial tumour treatment) appearance of metastatic disease in e.g. carcinoma of the breast or malignant melanoma. Reasons suggested for this latency include a balance of cell division and cell loss or suspension of cells in a non-proliferative (G_0) state possibly due to a lack of mitogenic stimuli (Alexander, 1983). It is possible that host factors may play a controlling role in this situation (see (Woodruff, 1982) and below). The occasionally observed clinical phenomena of regression (in e.g. clear cell renal carcinoma) or of "rebound" growth of metastases following removal of the primary, suggestive of some systemic effect of a primary tumour on secondary deposits, is similarly not understood (Folkmann, 1983). Although there is circumstantial clinical and direct experimental (e.g. Eccles et al, 1980) evidence (the latter using antigenic tumours in immunosuppressed animals) implicating host immune response in the outcome of tumour metastasis, it is generally accepted, as discussed at greater length below, that there is as yet no conclusive evidence for such a response having any significant influence on the outcome of established malignant disease (Weiss, 1977; Poste and Fidler, 1980; Alexander, 1982).

Increasing awareness of the nature and complexity of the metastatic disease process has also contributed to the growing realisation that the biological nature of a tumour

probably determines cure rates more than the type and extent of local therapy and has correspondingly led to greater conservatism in the use of radical surgery (Forrest, 1976) as well as a re-assessment of the timing of and indications for adjuvant therapy (Fisher, 1984).

2. The Natural History of Colorectal Carcinoma

Colorectal carcinoma, is the second most common cancer in the western world (Doll, 1972) and accounts for about one sixth of malignant disease in the U.K. The basis of treatment is surgical resection. Estimates of the crude 5 year survival figure for patients presenting with colorectal cancer over the past 30 years have ranged from approximately 25-45% (eg. Grinnell, 1953; Donaldson and Welch, 1974 and Corman et al, 1979). A recent estimate from the U.S. National Cancer Institute Surveillance, Epidemiology and End Results Programme Update quoted a 5 year survival figure of just less than 50% (Cancer Statistics, 1984). In these series approximately 20% of patients already have disseminated disease at the time of initial presentation and a further 5-10% have extensive local disease which prevents curative resection. The 5 year survival however in the approximately 75% of patients undergoing apparently-curative resection is only of the order of 50-60%, with the majority of deaths occurring within 2-3 years of surgery (Whittaker and Goligher, 1976; Gill and Morris, 1978; Welch and Donaldson, 1978). Of these patients undergoing apparently-curative resection approximately 10-20% develop local recurrence, 20-30% develop hepatic metastases and a further 5-10% develop pulmonary metastases (Welch and Donaldson, 1978 and

Hughes et al, 1981). Of these patients with recurrent disease it is claimed that up to 20% may be cured by further local resection or resection of hepatic or pulmonary metastasis (August et al, 1984 and Hughes et al, 1981).

For those patients who have undergone apparently-curative resection there is however at present no means of predicting which patients will be cured and which will ultimately develop a fatal recurrence of disease, although many prognostic indices have been evaluated in this context.

It is generally agreed that accurate and meaningful staging of tumours is of the greatest importance in interpreting and planning therapy (Beahrs and Myers, 1983 and International Union Against Cancer (UICC) TNM classification, 1978). Current methods of tumour staging are based largely upon anatomical and morphological considerations. The most widely-used staging system in colorectal carcinoma is based on that originally proposed by Dukes (Dukes, 1932), initially in the context of rectal carcinoma, where three stages of varying prognostic significance were described based on the extent of tumour invasion through the bowel wall and spread beyond. In this system stage A describes tumour limited to within the bowel wall, stage B describes extension to extra-rectal tissues and stage C describes the presence of metastatic tumour in regional lymph nodes. Various modifications of this system have been subsequently described which to some extent have also had the effect of confusing the interpretation of the prognostic significance of this staging system (Phillips

et al, 1984). These include that by Astler and Coller (Astler and Coller, 1954) which subdivided stage B into B1 describing tumour extending into but not penetrating the muscularis propria and B2 describing penetration of the muscularis propria, as well as sub-divisions of stage C into C1 describing tumour limited to the wall but without nodal involvement and C2 where tumour has penetrated through the bowel wall in conjunction with involvement of lymph nodes. Further variations of this Astler-Coller staging have also been proposed (Gunderson and Sosin, 1974) and a further category (Dukes D) is widely used to describe the presence of metastatic disease. Although Dukes' staging has been of some use clinically it has been long recognised that this classification provides no more than a relatively crude indication of prognostic probability (Woods, 1980; Finlay and McArdle, 1982) and the clinical outcome for patients within the differing stages may vary significantly. Indeed, in the original series reported by Dukes (Dukes, 1932) the 5 year survival of patients in his group B was approximately 80%, but approximately 30% of the patients in group C also survived 5 years. The other prognostic index routinely employed is that of tumour morphology. It has long been known that in general poorly-differentiated tumours have a worse prognosis than the well-differentiated ones (Grinnell,1939). However this correlation with prognosis is also only very broad and marked inconsistencies occur between the degree of tumour differentiation and clinical outcome (Finlay and McArdle, 1982). The presence of perineural and in particular venous invasion by the primary tumour has also been shown to be

generally associated with a worsened prognosis (Talbot et al, 1980). Measurement of serum levels of carcinoembryonic antigen (CEA), despite initial enthusiasm regarding its potential as prognostic index (NIH Consensus Statement, 1981), is not widely performed since it has been clearly shown that the presence of elevated pre-operative levels do not correlate with clinical outcome (Lewi et al, 1984), and that subsequent elevations are not an invariable feature of advanced recurrent or disseminated disease (Finlay and McArdle, 1983) nor do they distinguish between these forms of recurrence (Hine and Dykes, 1984). Studies of ploidy in these tumours have shown in general an inverse relation between increasing the degrees of aneuploidy and survival although this is not absolute (Wolley et al, 1982 and Rognum et al, 1983).

A number of other features of these tumours or of the host response to them which are associated with worsened prognosis have been described although none of them are of absolute prognostic value individually. These include tumour site, with rectal carcinomas in general having a worse prognosis than colonic overall, and those of the transverse and descending colon having a worse prognosis than those of ascending colon and sigmoid colon. A worsened prognosis is also seen in those tumours with a reduced infiltrate of lymphocytes and plasma cells and with decreased sinus histiocytosis and presence of para-cortical immunoblasts in regional lymph nodes (Moertel and Thynne, 1982). Studies using a leucocyte adherence inhibition test, initially thought to be of some prognostic value, failed to distinguish clearly between prognostic categories

(Ichiki et al, 1980). Studies of the length of the mucosal transitional zone of increased intestinal crypt depth, known to be associated with colo-rectal carcinomas, showed a significant negative correlation with early Dukes' stages but were apparently unable to distinguish prognostic categories of more advanced disease (Greaves et al, 1980). Other studies of features of possible prognostic significance have included that of the demonstration of circulating tumour cells in the mesenteric venous blood draining colorectal carcinomas (Fisher and Turnbull, 1955) although some doubt has subsequently been cast on the validity of such studies due to inaccuracies in the identification of cancer cells (Weiss, 1977). This can not in any case be correlated with clinical outcome (Griffiths et al, 1973; Salisbury, 1975). Other studies of tumour characteristics have included that of the anomalous expression of HLA-DR (class II major histocompatability) antigens. In one study this could not obviously be related to histological appearance (Daar et al, 1982 and Fabre, 1982) although in another study a more homogeneous staining pattern was apparent in more poorly-differentiated tumours although there was no clear relationship with overall survival (Rognum et al, 1983). Expression of immuno-globulin secretory component (SC) is in general more uniform and widespread in lower grade and stage carcinomas although the prognostic value of reactivity is apparently limited by the substantial heterogeneity of tumours in their reactivity (Isaacson, 1982 and Arends, 1984a). It has been suggested that variation in cell kinetic parameters might be related to the behaviour of these

tumours although one study reported was unable to confirm this (Bleiberg et al, 1984). Expression of the gastro-intestinal carcinoma-associated antigen (GICA) is not apparently related to prognosis as an independent parameter (Arends, 1984b). Similarly, production of plasminogen activator (PA) in these tumours although increased relative to normal tissue (Corasanti et al, 1980), cannot be related consistently to prognosis. Study of components of epithelial basement membrane showed that no prognostic significance could be related to the presence of fibro-nectin in and around tumours but, interestingly, the amount of laminin was shown to be, in general, greater in low-grade tumours and inversely correlated with subsequent development of metastatic disease and therefore with increased survival (Forster et al, 1984).

3. <u>Metastasis in Colorectal Carcinoma</u>

Although it has long been known that the presence of metastatic disease at the time of clinical presentation in colorectal carcinoma is associated with a dire prognosis, the median survival being only of the order of 6 months (Bengtsson et al, 1981), it has been difficult to assess the presence or absence of metastatic disease at initial presentation as an independent prognostic factor since it is well known from post-mortem studies of patients dying post-operatively (Goligher, 1941) that its incidence has been considerably underdiagnosed clinically. More recent studies however using improved diagnostic techniques such as the administration of radiolabelled microspheres (Mooney et al, 1981) and computerised tomography (CT) (Finlay and McArdle, 1982) have revealed an incidence of "occult"

hepatic metastases in about 30% of patients undergoing apparently-curative resection. Follow-up of these patients showed the presence of metastatic disease at the time of clinical presentation to be in fact the most critical prognostic factor regardless of Dukes' staging and that it accounted almost entirely for the anticipated pattern of mortality (Finlay and McArdle, 1982). In that study there was a mortality of only 5% following apparently-curative resection due to local disease but, most interestingly, no further hepatic metastases were seen to develop following surgical resection. Use of sequential CT scanning furthermore has made it possible to study the rate of growth of these metastases. This has shown that they display Gompertzian growth kinetics (ie. characterised by an increasing doubling time with growth) as has previously been demonstrated for both experimental and human tumours (Steel, 1982). Extrapolation based on that data showed that the metastases had been in fact present for a mean period of some 3 years prior to surgery (Finlay et al, 1982). This would be consistent with suggestions that metastases may well develop at a time very close to the establishment of a primary tumour beyond an in-situ state (Franks, 1973; Fisher, 1984). It also raises the possibility that these tumours may behave from an early stage in their natural history as either metastasizing or non-metastasizing variants, only indirectly related to the extent of their anatomic spread.

Although satisfactory chemotherapeutic agents are not yet available for this disease, those patients with metastatic disease obviously constitute a high-risk group

who ought to be considered for further therapy such as regional administration of cytotoxic drugs (Taylor, 1981). Experimental immunotherapy of patients with advanced disease using autologous vaccines consisting of X-irradiated, viable tumour cells admixed with BCG is also being currently evaluated for these tumours (Hoover, 1984). Similarly, identification of patients who have been cured by surgery or who develop only localised recurrent disease, which is at least partially-responsive to local radio-therapy (Moertel and Thynne, 1982), would be desirable. Although the use of CT scanning would appear to be useful it is expensive, time-consuming and only approximately accurate, being associated with both false negatives and positives (Finlay and McArdle, 1982; Koehler et al, 1984). The phenotypic determinants however, or, possibly, features of a host response which might be responsible for such variation in the behaviour of tumours remain poorly-understood and there is in particular no precise means of predicting the metastatic potential of a tumour, clearly of importance in addressing these therapeutic issues.

4. Approaches to the Study of Metastasis

Despite increasing refinement and sophistication of investigative techniques the study of metastasis has advanced conceptually little beyond the hypothesis of Paget (Paget, 1889) describing the process in terms of the property of "seeds" and "soil".

Any experimental and especially quantitative study of metastasis, which is an exclusively in vivo phenomenon, is complicated by the lack of suitable models, since few tumours in experimental animals metastasize and no useful

animal system yields quantitatively predictable metastases, and amongst transplantable tumours very few metastasize. Furthermore, the applicability to the clinical situation of results derived from highly artificial in vivo-in vitro experimental systems is open to serious doubt (Weiss, 1977; Hewitt, 1978; Rockwell, 1980; Fisher, 1984). This is compounded by the fact that in normal situations such as embryogenesis, as is well recognised, local tissue environment is important in determining subsequent development (Tarin, 1976 and Sherbet, 1982), and the existence of possible "tissue phenotypes" (Pitts, 1980) may be of critical importance in the development of neoplasia.

The properties of tumour cells themselves have been the subject of extensive investigation, although the exact relevance of such observations as have been made to the processes of invasion and in particular metastasis remain in most cases, unclear (Carter, 1976; Fisher, 1984).

The morphology of the constituent cells of a malignant tumour gives, in general, as mentioned in the context of colorectal carcinoma, some indication of its metastatic propensity, with poorly-differentiated tumours being in general more likely to metastasize than those which are well-differentiated (Glucksmann, 1948) although this is far from consistent.

Non-morphological criteria of metastatic propensity remain poorly defined. Study of variation in cell kinetics between the tumours has shown correlations between the rapidity of doubling time and risk of development of metastasis (Slack and Bross, 1975). This however may simply be due to a higher growth rate in both primary and

secondary tumour since rapid growth may also be seen in benign tumours, e.g. giant fibroadenoma of the breast, which do not metastasize. In addition, certain carcinomas of e.g. thyroid, prostate and kidney which have relatively long doubling times produce metastases with a high frequency.

The cell surface of neoplastic cells has been particularly closely studied on the assumption that their behaviour may be mediated thereby through interaction with the extra-cellular environment. Changes in almost all aspects of the cell surface have been described in relation to malignant transformation (Nicolson and Poste, 1976). Attempts to relate these changes to the metastatic potential of different tumour cells however have been in general unrewarding and at times contradictory, different changes varying apparently in relation to different experimental systems. At best, quantitative changes have been observed in tumour cells of differing metastatic capability and no consistent biochemical changes have so far been identified in relation to a metastatic phenotype (Guy et al, 1979; Raz et al, 1980; Steck and Nicolson, 1983 and Steele et al, 1983). The involvement of tumour cell surface changes in the metastatic process has however been demonstrated by various approaches. These include modification of cell-surface properties by e.g. enzymatic means which has shown some alteration of experimental tumour cell metastatic capability following trypsinisation (Sargent et al, 1983). Similarly, use of the glycosylation inhibitor tunicamycin in B16 melanoma cells causes biosynthetic alterations of cell-surface glycoproteins and

causes both morphological and adhesive changes with consequent reduction in metastatic capability (Irimura et al, 1981). These changes were also shown to be correlated with the loss of a specific class of B16 sialo-galacto-protein. Further experimental evidence implicating cell surfaces in metastatic colonisation potential comes from experiments using shed plasma-membrane vesicles from a highly-metastatic B16 cell line to modify a low-metastatic cell line by surface membrane fusion which conferred an increased metastatic ability onto the originally low-metastasizing sub-line (Poste and Nicolson, 1980). Overall differences in cell surface characteristics have also been implicated relative to separability by density gradient centrifugation of cells of different lung colonisation potential (Grdina et al, 1977).

Study, mostly <u>in vitro</u>, of defined tumour cell populations of known metastatic capability have also been undertaken on the assumption that such changes may reflect phenotypic characteristics related to metastases. These have included study of cell-surface glycoproteins which are thought to play a particularly important role in cell behaviour. These have been studied by e.g. assays of radiolabelled lectin binding to specific cell-surface glycoproteins (Nicolson, 1982) and in certain model systems, notably the B16 melanoma, some changes in metastatic cell-surface proteins have been shown, e.g. in brain metastasizing melanoma sub-lines (Brunson et al, 1978).

Similarly changes in the expression of carbohydrates on cell-surface glycoproteins in cells of differing

metastatic capability have been seen e.g. in an experimental model using murine lymphoma cells (Cheingsong-Popov et al, 1983). In addition, quantitation of the neuraminidase-accessibility of cell-surface sialyl residues have shown changes apparently correlated with metastatic capability in some systems (Yogeeswaran and Salk, 1981) although this does not seem to be a general feature of other systems and this finding contradicts other studies (e.g. Raz et al, 1980).

The study of glycolipids in highly metastatic cells has revealed changes in certain species although the changes in general seem to be complex and non-specific (Nicolson, 1982). One study however, using high-resolution nuclear magnetic resonance (NMR) has claimed that in one *in vitro* model system at least a highly-metastatic cell line was associated with an increased ratio of cholesterol to phospholipids and an increased amount of plasma membrane-bound cholesterol ester. (Mountford et al, 1984).

Study of the adhesive properties of metastatic cells have also been undertaken since this may be obviously of considerable importance in determining the metastatic potential of tumour cells as well as of their organ preferences (Nicolson, 1982). In experimental studies using B16 melanoma cells, monoclonal antibodies which block adhesion of these cells to tissue culture plastic can also be used to abrogate their metastatic potential (Vollmers and Birchmeier, 1983). Other studies using shear-techniques in model systems have similarly, demonstrated differences in adhesive properties between cell lines of different metastatic potential (Elvin and Evans, 1984).

Study of cell deformability, possibly of much importance in permitting tumour cells to partake in the metastatic process, has also been studied *in vitro* using the B16 melanoma system (Raz and Ben Ze-ev, 1983). It was found in this study that growth of melanoma cell lines of low metastatic potential *in vitro* in spherical configuration on a non-adhesive substrate showed a marked increase in the metastatic capability of those cells as compared to those grown as a monolayer in a flat configuration. This was accompanied by a marked decrease in synthesis of the intermediate filament vimentin. Further study using the B16 melanoma model also demonstrated an apparent correlation of intracellular cyclic AMP (cAMP) accumulation with capacity for experimental metastasis and it may be that, at least in this system, cAMP metabolism is linked with biochemical pathways responsible for the generation of metastasis (Sheppard et al, 1984).

Study of the production of gap junctions in tumour cells has shown this to be variably disorderd (Loewenstein, 1979) although a relationship of this to the metastatic potential of tumour cells has not been demonstrated (Pauli et al, 1978).

Study of the production of a variety of, mostly degradative, enzymes in tumour cells has revealed a large number of changes many of which are doubtlessly important in metastasis. As with many other features of the metastatic process, their production may be considered individually as probably necessary but not sufficient. Thus, differences in production of these enzymes has not been convincingly correlated with metastatic potential

(Nicolson, 1982). These enzymes may be important in breaking down the extra-cellular matrix and surrounding tissue as well as perhaps activating host enzymes. High levels of proteases, especially collagenases, are usually present in invasive tumours and metastatic potential has been experimentally related to degradation of basement membrane collagen (Liotta et al, 1980) whilst its absence has been correlated with a low-metastasizing experimental tumour (Tarin et al, 1982). This enzyme may be particularly important in degrading components of epithelial and endothelial basal lamina and abnormalities of the presence of e.g. laminin have been reported to be associated with adverse prognosis in colorectal carcinoma (Forster et al, 1984). Other lysosomal proteases such as cathepsin B may also be important in this context. The serine protease plasminogen activator has also been shown to be present in general in high levels in tumour cells including human colorectal carcinoma (Corasanti et al, 1980) although apparently the levels of this enzyme in a variety of systems cannot be correlated with metastatic potential (Nicolson, 1982). Its important role in fibrinolysis however, may also implicate it in the phenomenom of platelet aggregation around tumour cells which has, in some cases at least, been shown to be associated with increased metastatic potential of tumour cells lines (Perlstein et al, 1980).

A variety of other products have been shown to be released from tumour cells which may be of importance in their successful completion of the metastatic process. These may include e.g. tumour antigens, the shedding of

which may represent a means of evading host tumour responses (Woodruff, 1982). Tumours are also known to secrete substances such as prostaglandins which may affect processes such as bone resorption, macrophage function and platelet aggregation (Nicolson, 1982). The production e.g. of prostaglandin D2 in the B16 melanoma system correlates with metastatic potential (Fitzpatrick and Stringfellow, 1979). Similarly secretion of substances such as osteoclast-activating factor may be of importance in determining the survival of metastases (Carter, 1976). The production of angiogenesis factor by both primary and secondary tumour cells is clearly of great importance in order to produce neovascularisation (Folkman, 1982). However, abnormal production of this factor is by no means exclusively associated even with malignancy and has also been shown in premalignant breast disease (Jensen, 1982).

It would thus appear that, as with many features of malignancy, phenotypic alterations important in metastasis may represent abnormalities of only a quantitative or temporal rather than absolute nature.

5. The Relation of Phenotypic Variation in Primary Tumours to Metastasis.

Evaluation of any tumour cell characteristic in relation to metastatic potential would clearly be complicated if it is heterogeneously expressed among the cell population of the primary tumour. It is generally accepted that most spontaneously-occurring human tumours are monoclonal in origin, this having been most notably demonstrated by studies of the X-linked heterozygosity of black women for the two forms of the enzyme glucose-6-

phosphate dehydrogenase, only one form of which is expressed in any somatic cell (Fialkow, 1976). This proposition is further supported by the finding of consistent karyotypic abnormalities in certain leukaemias and by the production of monotypic immunoglobulins in plasma cell myelomas. It has however long been recognised that tumours may undergo progression of various characteristics (Foulds, 1969). It has been further suggested that such progression and generation of phenotypic diversity occurs as a consequence of the well-recognised genetic instability of malignant tumour cells (Nowell, 1976). Thus, phenotypic heterogeneity of both primary tumours and their metastases has been demonstrated for a variety of characteristics (Woodruff, 1983). These include ploidy measurements in primary and metastatic tumour (Rabotti, 1959), expression of oestrogen receptors in breast cancer (Holdaway and Bowditch, 1983) cell DNA content in small-cell carcinoma of lung as measured by flow cytometry (Vindelov et al, 1980) and of a variety of biochemical markers and cell-surface antigens in the same tumour (Minna et al, 1982; Baylin et al, 1978). Quantitative study of tumour cell nuclear DNA content in different primary breast tumours and their metastases showed however similar patterns in the different sites suggesting a homogeneity of cell populations for a primary tumour and its secondaries (Auer et al, 1980). In vitro characterisation of cloned cell lines from a primary colon carcinoma has also shown variation of growth rates and of histological appearance (Brattain et al, 1981). Heterogeneity of cell-surface antigen expression has been

observed in e.g. cell lines established from various melanoma metastases where variation in pigmentation and expression of HLA-DR antigens was also observed (Albino et al, 1981) as well as in expression of the Ca antigen in various tumours (McGee et al, 1982). Variation in immunological characteristics of tumour cell sub-populations has also been observed (e.g. Miller and Heppner, 1979). Of some therapeutic importance also is the observation of heterogeneity of responsiveness to chemotherapy observed in various clinical and experimental tumours and their metastases (Donelli et al, 1977; Schlag and Schreml, 1982).

It has furthermore been suggested on the basis of experimental work using in vitro-in vivo animal models, initially with the B16 mouse melanoma tumour (Fidler and Kripke, 1977), that primary malignant tumours are also heterogeneous with respect to the metastatic potential of cell sub-populations within this tumour. This proposition was initially based on tumour cell cloning experiments from a transplanted B16 melanoma when individual clones were compared for their metastatic potential and it was found that experimental lung colonisation varied widely between cloned B16 cell populations. Using this model it was demonstrated that the metastatic heterogeneity between different cell clones was not produced by the cloning procedures and it was concluded that some cells with a higher metastatic potential existed within the parental B16 tumour (Fidler and Kripke, 1977). Similar results have been obtained by different investigators using different tumour types e.g. lymphosarcoma (Nicolson et al, 1981),

carcinoma (Talmadge et al, 1981) and sarcoma (Chambers et al, 1981). Variant metastatic cell sub-populations have thus been obtained by selection procedures either *in vivo* or *in vitro* and the implications of these findings as regards such a hypothesis are propounded by various of these authors (e.g. Poste and Fidler, 1980; Hart and Fidler, 1981). Other investigators however, using different model systems have failed to confirm these findings and have shown that random selections of cell populations from primary tumours have comparable metastatic potential and that, in particular, biopsies of metastasis showed a comparable potential, although one of the implications of the sub-population hypothesis would be that a selected metastatic population should have a higher overall metastatic capability (Tarin and Price, 1979; Giavazzi et al, 1980; Weiss et al, 1983 and Alexander, 1983). The criticism has also been voiced (e.g. Alexander, 1983) that the sub-population hypothesis as regards metastatic potential of a primary tumour is based entirely upon experiments in which *in vitro* cloning procedures were used prior to tumour cell inoculation. This procedure may well vitiate interpretation of such results since it is well known that cells in culture undergo phenotypic drift and this has also been shown in the context of metastatic properties (Neri and Nicolson, 1981; Tveit and Pihl, 1981). Interestingly, in this context it has also been shown using B16 melanoma lines that propagation in culture of mixed cell lines appears to have the effect of conferring an overall phenotypic stability on the cell populations although when cloned individually the cell

lines give rise to clones of highly variable metastatic potential (Poste et al, 1981).

So far, therefore, there appears to be no direct evidence for the applicability of any hypothesis regarding metastatic heterogeneity in spontaneously-arising human primary tumours although clearly only a relatively small proportion of the cells in any tumour will be clonogenic. In colorectal carcinoma this has been estimated to be of the order of 15% (Wright, 1984). In addition, as mentioned previously, there now appears to be good evidence that colorectal carcinoma behaves consistently overall from an early stage in its natural history as metastasising or non-metastasising variants.

6. Study of Host Response in Metastasis

Tumour metastasis can be shown at least in experimental systems to be influenced by host local tissue factors at the site of metastasis (Milas, 1984). It may be that variation in e.g. the reaction of stromal cells and the production of a tumour capsule may be of importance in determining the successful growth of metastatic deposits. It has been clearly shown that local tissue damage, inflammation, as well as hormonal microenvironment may influence the outcome of tumour metastasis. Experimental metastasis of a mammary carcinoma cell line in nude mice is for example increased by the administration of exogenous oestrogens. An increased incidence of experimental metastases has been shown at sites of tissue trauma or inflammation, following radiotherapy and hormonal treatment (Milas, 1984). It has however not been shown that variation in such host response is of importance in

determining variation in the incidence of tumour metastases in human tumours.

In the investigation of possible host factors involved in determining the outcome of the metastatic process, study has been principally directed towards possible host immune response and over recent years has produced a vast and contradictory literature the full implications of which are not yet clear (Alexander, 1982; Woodruff, 1982 and Lachmann, 1984). Much of this study was based on the, initially widely-accepted, immune-surveillance hypothesis which attributed a host resistance to tumours through the destruction of immunogenic tumours by mature immune T-lymphocytes (Burnet, 1970). This theory however has been subject to considerable criticism in recent years (Prehn, 1977; Poste and Fidler, 1980; Lachmann, 1984) and although it has long been known that many experimentally-induced tumours in animals carry tumour-specific transplantation antigens (Alexander, 1982),it has not been convincingly shown that spontaneously-arising human tumours posess any or significant immunogenicity (Prehn, 1977; Hewitt, 1978; Klein and Klein, 1977; Lachmann, 1984) and in general the attempt to define human tumour-specific antigens has not been successful (Old, 1981). Furthermore it has not been shown that patients suffering from deficiencies of either specific or non-specific immune mechanisms nor those immune-depressed or suppressed develop a greater than expected incidence of the majority of tumour types. Thus, although variation in the outcome of tumour metastases has been shown in a number of experimental animal tumour systems, where, incidentally, manipulation of

immune responses may result in either increases or decreases in the incidence of metastases (Nicolson, 1984; Alexander, 1982), it is not clear how far, if at all, such data can be extrapolated to human tumours (Alexander, 1982; Lachmann, 1984).

The failure to demonstrate such mechanisms does not of course preclude their existance (Woodruff, 1982) and it may be that this represents some immune selection or escape mechanisms possibly involving antigen "shedding" and immune complexes which may act as blocking factors. Such a phenomenon has been demonstrated in vitro where it can be shown that tumour-bearing animals lymphocytes demonstrate positive cytotoxicity which can be blocked by their serum (Lachmann, 1984). It may also be the case that in the early stages of tumour development or in weakly-antigenic tumours a weak stimulatory anti-tumour response may occur which may have the effect of stimulating the growth of tumours rather than suppressing it, a phenomenon which has been described as "sneaking through" (Prehn, 1972). Changes in cell-surface antigen expression between metastatic deposits and a primary tumour have been described in experimental systems (Schirrmacher and Bosslet, 1980) in a murine lymphoma although such antigen loss does not appear to be a common characteristic of metastatic cells (Nicolson, 1984).

More recent work has concentrated on the possible role of non-adaptive immune responses in determining the outcome of metastases. These include the role of armed or tumouricidal macrophages which are frequently found in the context of primary tumours and which are experimentally

demonstrable to have tumouricidal properties. However there appears to be no direct evidence that their presence plays a significant role in the outcome of the metastatic process (Alexander, 1976). It has also been postulated that natural killer (NK) cells may be effective against metastatic cells during their transport in the circulatory system, and in many experimental models levels of NK activity correlates inversely with subsequent formation of metastases. It has thus been shown experimentally for example that resistance to leukaemia in mice is associated with a high NK cell phenotype and that grafting of high NK bone marrow to deficient animals confers an increased resistance (Karre et al, 1980; Warner and Dennert, 1982). Similarly it has been shown that in a spontaneously-occurring mammary carcinoma of rats that tumour cells in metastatic deposits possessed a higher resistance to NK-mediated cytolytic killing (Brooks et al, 1981). Again there is no direct evidence of the involvement of these mechanisms in the development of metastases in human tumours and the evidence in favour of this view remains circumstantial (Lachmann, 1984). It is also of interest to note that deficiencies of these non-adaptive immune mechanisms are associated with problems with infections and immune-complex disease rather than with the development of tumours (Lachmann, 1984).

Study of alterations in the expression of membrane-associated antigenic determinants encoded by the major histocompatibility complex (MHC) in various experimental systems (Katzav et al, 1983 and Eisenbach et al, 1983) has however provided further suggestive evidence for the

importance of host-tumour cell interaction in determining the outcome of the metastatic process. It is well-known that these antigens play a critical role in the elicitation of immune responses against cell surface antigens of both virus-infected and some malignant cells (Stutman and Shen, 1978) and it has thus been postulated that variable expression of MHC components expressed on neoplastic cells may determine host-tumour immune relationships and thereby affect the capability of tumour cells to evade immune destruction and subsequently generate metastases. How far the phenotypic shifts seen in the expression of the murine H2 locus are paralleled by events in spontaneous human tumourigenesis, particularly in the context of metastasis, are however not clear, although, as mentioned in the context of colorectal carcinoma, anomalous expression of HLA-DR antigens of the MHC class II has been observed in various primary tumours.

There appears therefore as yet to be no conclusive evidence that any host immune response, still poorly understood, plays a critical role in controlling the development of human tumours or more particularly that of their metastases.

7. Predictive Assays for Metastasis

There is no clinically-useful biological assay system currently available for predicting the metastatic potential of a primary tumour. *In vitro*-*in vivo* models have been developed for the study of tumour invasion using techniques such as inoculation adjacent to rat femurs (Pauli et al, 1980) or invasion of tumour cells into precultured fragments of embryonic chick cardiac muscle (Mareel, 1984)

which may be able to define features of tumour cell invasiveness which are important in the formation of metastasis. Use of a clonal assay using a bi-layer Agar system has also been used to successfully culture clonogenic tumour cells including metastatic melanoma (Meyskens et al, 1981). A test based on subjective "epigenetic grading" of the cellular responses of relatively undifferentiated chick embryo blastoderms following implantation of tumour cells was able to distinguish experimentally metastasizing and non-metastasizing (essentially benign) forms of a hamster lymphosarcoma (Sherbet et al, 1980). A similar model for the assay of metastasis using a chick chorio-allantoic membrane in which an increased incidence of metastasis generally correlates with tumour size on the membrane has also been reported (Ossowski and Reich, 1980). Preliminary data have also been obtained using a variety of human tumour biopsies, including colorectal carcinoma, for injection into athymic nude mice (Hart, 1984). Variable factors such as site of injection and the hormonal status of the recipient mice appear to have significant effects on the metastatic behaviour of the tumour although it appears that the most important factor is the intrinsic character of the tumour cells themselves. It is however not yet clear whether such a test can be used to predict the behaviour of primary human tumours. Evaluation of such biological assays appears to be generally very subjective, the results are highly empirical and they do not so far appear to have applicability as useful, routine prognostic tests.

8. Molecular Approaches to the Study of Cell Phenotype: General Considerations

Different cell types are distinguished by both qualitative and quantitative differences in their protein content. These arise as a result of variation in the transcription, processing, stability and translation of cellular messenger RNA (mRNA), and steady-state levels of mRNA constitute the principal determinant of phenotypic differences between cells (for reviews see Lewin, 1980; Perry, 1981; Brown, 1981; Darnell, 1982; Alberts et al, 1983 and Nevins, 1983). It has been estimated that in typical eukaryotic cells, with the exception of neural cells, there exist some 10,000-20,000 individual mRNA species each present at markedly-different copy numbers. These are generally divided into three categories corresponding to those of high-, moderate- and low-abundance (Lewin, 1980; Williams, 1981; Alberts et al, 1983). The low-abundance mRNAs comprise the vast majority with approximately 10,000 different species which are present at only 5-15 molecules per cell. A further 500-1,000 species are present at a moderate abundance of several hundred copies per cell and relatively few species, approximately 5-10, are present at a high-abundance of several thousand copies per cell. It is clear that a typical eukaryotic cell contains therefore several hundred thousand RNA molecules representing the 10,000-20,000 different RNA species present.

Comparison of total mRNA populations by hybridisation analysis has shown clear, although often subtle, differences overall in different normal cell types

(Young et al, 1976; Hastie and Held, 1978), in cells at different stages of development e.g. during myogenesis (Affara et al, 1977) and in normal compared to regenerating rat liver (Wilkes et al, 1979) as well as in virally-transformed cells (Williams et al, 1977), in normal compared to neoplastic rat liver (Jacobs and Birnie, 1980) and between normal and neoplastic rat mammary tissue (Supowit and Rosen, 1980). Although overall differences in the steady-state transcription levels of mRNA populations may be detected by hybridisation analysis such an approach is relatively crude since possibly-significant differences in the abundances of individual mRNAs may be obscured. However recent developments in molecular cloning techniques have made possible the study of, at least moderately-and highly-abundant, individual mRNAs. (See below).

Variations in the steady-state levels of different mRNA species are held to be the overall result of variation in the series of steps (described below) which constitute RNA biosynthesis, the exact contribution of each of which is not yet fully understood but of which transcriptional control (see Tobin, 1979) is thought to be by far the most important although the importance of the role played by variation in e.g. message stability and translation is also becoming increasingly apparent.

9. RNA Biosynthesis

(For reviews see Lewin, 1980; Perry, 1981; Brown, 1981; Darnell, 1982; Alberts et al, 1983 and Nevins, 1983). Transcription of eukaryotic RNA from its DNA templates catalysed by three distinct RNA polymerase enzymes (I-III) which are involved in transcription of ribosomal (pol I),

messenger (pol II) and of 5S ribosomal and transfer (pol III) RNA respectively. Control of transcription as judged by the rate of synthesis of nuclear RNA appears to be the most important level of control. Although the details of these mechanisms are not yet fully understood it is thought that site-specific binding proteins may either alter chromatin structure or facilitate or prevent the attachment of RNA polymerases to specific sites of the DNA. Generation of a primary transcript occurs from a region of the genome described as a transcriptional unit which contains the appropriate signals for initiation and termination of transcription. These units may be described as simple or complex depending on whether they have the potential to generate single or multiple proteins. The transcription start site is located around a codon of AUG and is at the sequence specifying the 5' terminus which ultimately generates the amino (NH2) terminus of the protein. So far, however, recognisable sequences representing a termination signal have not been defined. Initiation of RNA synthesis by RNA polymerase requires also the presence of further as yet poorly-characterised factors as defined by in vitro assay systems.(Alberts et al, 1983 and Nevins, 1983). Transcription of mRNA from various genes depends also on the, markedly-differing, efficiency with which differing RNA polymerase II start sites function.

Further elements acting on gene transcription include a promotor region approximately 25-30 base pairs upstream from the transcriptional start site, including the sequence TATA known as the Goldberg-Hogness Box, and whose function

appears to be related to the accurate positioning of the start of transcription. This sequence appears to be analogous to the prokaryotic promotor sequence known as the Pribnow Box, which is required to initiate transcription, although deletion of the TATA sequence does not abolish transcription of eukaryotic genes (Rosenberg and Court, 1979). Further sequences known as enhancers have also been described. These are usually present far upstream (200-300 nucleotides) from the site of transcription and are able to confer high levels of expression on genes transcribed at low levels and apparently act in a tissue-specific fashion. They are able to act on heterologous genes in cis and independently of position or orientation in in vitro systems. They have been shown to be present in nucleosome-free regions and it is thought that such a chromatin structure, which is hypersensitive to the action of nucleases, may also be associated with enhancer activity. Such elements were first and most fully characterised in viruses, notably Simian virus 40 (SV40), and were shown in this context to be capable of acting in a wide variety of tissues and hosts. The enhancer elements present in retroviruses play in addition a critical role in oncogenesis. It has more recently been suggested however that enhancer and the promotor elements overlap in fact, both physically and functionally, and that these regulatory elements are comprised of a modular arrangement of short sequence motifs each with a specific function in conferring inducibility, tissue-specificity or a general enhancement of transcription. These appear to be binding sites for nuclear proteins whose mechanism of action on chromatin is

not yet clear (Serfling et al, 1985; Reudelhüber, 1984). These would include positive and negative regulatory elements as have been described e.g. in the context of responsiveness to hormones, heavy metal ions, heat shock or, negatively, as in the T antigen of the SV40 early transcript. Such transcriptional control has been demonstrated to be tissue-specific e.g. for liver-related mRNA (Derman et al, 1981) and also e.g. for the expression of ovalbumin RNA in chicken oviduct and non-target tissues prior to hormonal stimulation where scarcely detectable levels of transcription are observed, in contrast to the thousands of cytoplasmic copies generated following hormonal induction (Tsai et al, 1979). Variation in transcription has also been demonstrated to be developmentally regulated in e.g. Dictyostelium (Blumberg and Lodish, 1980) and for myoglobin (Affara et al, 1977). It should be noted that the existence of such control does not of course preclude regulation at subsequent levels of processing. Variation of the actual start site has also been shown to be involved in differential tissue expression e.g. in transcription of pancreatic and salivary mouse alpha amylases (Hagenbuchle et al, 1981).

A specific transcription termination site has not yet been identified although termination occurs downstream of the 3' polyadenylation site and it is possible that sequences beyond this participate in generating an RNA cleavage site. Control of transcription at this point could be important in transcriptional units encoding multiple proteins as in e.g. the adenovirus late t transcripts, immunoglobulin heavy μ chains and calcitonin

mRNA (Nevins, 1983). The frequently observed phenomenon of premature termination of transcription, whose significance is not yet understood, could also conceivably play a regulatory role in transcription. At the 3' end of the transcript in approximately one quarter of primary transcripts a poly A tail of some 100-200 residues of adenylic acid are added. This is catalysed by a further poly A polymerase enzyme and occurs approximately 30 base pairs downstream of a region which includes an AAUAAA sequence whose exact significance is not understood but may be spatially related to transcript cleavage and subsequent poly A polymerisation. (Fitzgerald and Shenk, 1981). The function of this poly A tract is not yet entirely clear but is thought to be possibly related to processing and export of the transcript and cytoplasmic stability of mRNA. This represents a further possible regulatory step in transcription and differential poly A site selection appears to occur for e.g. adenovirus major late proteins (Nevins, 1983). Further modifications of the RNA pol II primary transcript include the internal methylation of approximately 1/400 adenylate residues at the N^6 position and in the 2'O ribose position of the penultimate nucleotide throughout the RNA (Banerjee, 1980). The significance of these modifications is not yet known. In addition (probably before transcription is complete) a 7mG cap at the 5' end of the transcript is also added whose function is thought to lie probably in subsequent initiation of translation through interaction with specific proteins and binding to ribosomes. These modifications are also possibly protective against exonucleolytic

degradation. Primary transcripts (known as heterogeneous nuclear RNA-hnRNA - due to their variety of sizes) form nuclear complexes with several proteins to form ribonucleoprotein packages. This is thought to be important in subsequent processing and transport. Only a small fraction of these short-lived and unstable hnRNAs survive to become stable cytoplasmic mRNA. The latter represents only approximately 3% of the steady-state total cell RNA although hnRNA accounts for more than a half of RNA transcription. The remaining approximately 97% is composed of ribosomal RNA (Alberts et al, 1983).

Before mRNA is exported from the nucleus to the cytoplasm, splicing of transcripts occurs which removes non-coding intervening sequences or introns which are a marked feature of eukaryotic genes in contrast to to those of prokaryotes (Cech, 1983). This results in the conversion of very long hnRNA to much shorter mRNA molecules of some 500-3,000 nucleotides in length. Most eukaryotic genes (with exceptions such as the histones and alpha interferon) contain several and in some instances (e.g. the alpha chain of procollagen) up to 50 such introns. Their excision involves complexing to small nuclear ribonucleoprotein particles (snRNP) which are a minor component of ribonucleoproteins. These are characterised by the presence of many small and ubiquitous nuclear RNAs such as the Ul RNA (so-called due to the abundant presence of uracyl nucleotides) which has been shown to bind to 5' splice sites. Splicing is thought to be accomplished by the enzymatic cleavage and ligation reaction which removes the intron sequences by the

production of a so-called lariat formation, whose three dimensional conformation is probably of great importance, and which leaves the coding sequences intact. In some cases e.g. ribosomal and mitochondrial RNA it has been shown that the excision and ligation process is catalysed by the intron itself (Cech, 1983). Further snRNP molecules may be involved in the direction of processing and in guiding RNA molecules. Much of these intron sequences is thought to represent random evolutionary material, although certain highly conserved donor and acceptor splice sites must also be present (Breathnach and Chambon, 1981). It is also thought to be possible that the structure of the introns themselves may be important in the generation of different transcripts. One consequence of the presence of multiple introns is that differential splicing patterns may generate different protein products from the same primary RNA transcripts. How this is controlled is not yet clear. This has been demonstrated for adenovirus P16 major late proteins where, in addition, differing 3' ends may be generated by differential cleavage and adding of A at different points (Ziff, 1980). Such mechanisms are also involved in the generation of membrane and secreted forms of immunoglobulin μ heavy chains during the process of lymphocyte differentiation (Early et al, 1980). Transport of mRNA then occurs into the cytoplasm although only for fully-processed molecules.

Regulation of gene expression may also occur due to variation in the stability and half-life of cytoplasmic mRNA molecules both for different specific mRNAs in the same cell as well as variation in the half-life of the

same mRNA species in a given cell type under different circumstances. This has been shown for casein mRNA in mammary glands whose mRNA copy number increases enormously in rsponse to prolactin treatment and which is associated with an approximately 20 fold increase in the half-life of the mRNA (Guyette et al, 1979). In addition, specific destruction of certain mRNA species appears to occur in e.g. developing erythroblasts where globin is conserved preferentially when non-globin RNAs appear to be destroyed (Bastos et al, 1978). Similarly, during the development of the slime-mould Dictyostelium, a new set of RNA species is formed following cell aggregation but which is specifically destroyed if its developmental cycle is interrupted following aggregation (Chung et al, 1981). A further extreme example of mRNA stability is the well-known "masking" of mRNA in unfertilised sea-urchin eggs. This RNA is capable of involvement in rapid protein synthesis however following fertilisation (Brandhoest, 1976).

Thus, although steady-state cellular mRNA levels reflect the outcome of various regulatory steps in RNA biosynthesis and as such constitute the principal determinant of cell phenotype, it is clear that variation in those preceding steps as well, in particular, as in subsequent translation may also have considerable phenotypic significance.

10. Approaches to the Study of Individual mRNA Species: Cloning of Complementary (c)DNA Libraries

Study of the molecular determinants of variation in cell phenotype have been greatly advanced in recent years by the use of techniques making possible the cloning of

individual mRNA species into complementary (c)DNA libraries (Old and Primrose, 1981; Williams, 1981; Maniatis et al, 1982). This has been made possible in large part by the use of DNA recombinant and molecular cloning technology based on the isolation of viral and bacterial enzymes used for reverse transcription, ligation and cleavage of DNA molecules and the use of both plasmid and bacteriophage as vectors for recombinant molecules (Paul, 1982). Cloning and characterisation of the approximately 10,000-20,000 individual mRNAs transcribed actively in a typical eukaryotic cell clearly offers a greatly refined approach to the characterisation of cellular phenotype. Such a library can be much more easily screened than a comparable genomic one which would contain approximately 100,000 to 1,000,000 fragments of clonable size, and in addition yields far fewer false positives since every cDNA clone contains an mRNA sequence (Williams, 1981). The number of clones required to produce with a given probability a certain cDNA clone can be calculated according to the formula of Clarke and Charbon (Williams, 1981), and predicts that to clone the majority of the abundant and moderately-abundant mRNA species a library of some 1,000 clones would be required. In such libraries a cDNA clone is a bacterial cell transformed by a plasmid containing a DNA copy of an RNA molecule. These are created after the synthesis of double-stranded DNA copies from polyadenylated (poly A) RNA which are subsequently ligated into a restriction enzyme cleavage-site within the plasmid molecule. The plasmids used in cloning are genetically-engineered derivatives of naturally occurring drug-

resistance plasmids which contain sufficient genetic information to ensure their own replication and to include proteins which confer resistance to one or more antibiotics. These properties are made use of in subsequent screening procedures. Screening of such libraries using radiolabelled reverse-transcribed cDNA probes containing the sequences of interest may be performed on bacterial colonies lysed *in situ* initially or in secondary procedures using bulk preparations of plasmid DNA. Such screening procedures permit detection of sequences expressed at down to, at best, 0.1% of the mRNA population (Williams, 1981). Detection of low-abundance sequences however is very much more complicated due to very low "signal to noise" ratios at these levels.

Isolation and characterisation of an individual cDNA clone permits determination of its nucleotide sequence and may be used to study its corresponding genomic clone if available.

The use of such techniques has made it possible to isolate cDNA clones representing mRNA sequences which are differentially expressed in differing normal human tissues (Crampton et al, 1980 and Affara et al, 1983), in cells at different stages of development (Williams and Lloyd, 1979), in virally-transformed cells *in vitro* (Scott et al, 1983) in normal and neoplastic (experimentally-induced) mouse colon (Augenlicht and Kobrin, 1982) and in normal and neoplastic rat liver (Yamamoto et al, 1983). Such techniques have also been used in a molecular approach to the classification of human leukaemias by study of differences in abundance of individual mRNA sequences as

defined by hybridisation with selected cDNA clones (Wiedemann et al, 1983; Birnie et al, 1983).

11. The Molecular Pathology of Cancer: General Considerations

It has long been assumed that somatic genetic mutation and/or transposition and subsequent abnormalities of gene expression play an important role in carcinogenesis (Cairns, 1981 and Klein, 1981). Evidence for this has included the fact that various forms of radiation and most known carcinogens or their active metabolites are mutagens (Poirier and DeSerres, 1979) as well as the demonstration of tumour-specific chromosomal abnormalities, most notably the so-called Philadelphia chromosome in chronic myeloid leukaemia (Weiss and Marshall, 1984; Klein and Klein, 1984). Chromosomal abnormalities such as trisomy 7 and 12q- have also been reported at the premalignant stage in colorectal cancer (Becher et al, 1983). These non-random cytogenetic abnormalities have been further implicated in carcinogenesis by their location, in many cases, at or near chromosomal regions bearing cellular oncogenes (see below and Yunis, 1983). Thus, in chronic myeloid leukaemia the c- **abl** oncogene is translocated from chromosome 9 to 22 (de Klein et al, 1982) and in Burkitt's lymphoma the c- **myc** oncogene is translocated from chromosome 8 to 14 and juxtaposed to an active immunoglobulin locus (Dalla-Favera et al, 1982). The documentation of heritable (LeBeau and Rowley, 1984) and constitutive (Yunis and Soreng, 1984) chromosomal "fragile" sites in cancer and their association in some cases with such chromosomal rearrangements is of further interest in this context. Such sites are evident

as non-stained gaps, usually on both chromatids, seen in metaphase chromosome preparations. Their structure is not understood but they are thought to represent chromosomal segments which do not undergo normal compaction during mitosis (LeBeau and Rowley, 1984). It may well be that these "fragile" sites act as predisposing factors for chromosomal rearrangements in cancer and that they bear genes of importance in carcinogenesis. Further cytogenetic abnormalities apparent as deletions and seen in association with certain tumours e.g. with a hereditary component such as Wilms' tumour (Koufos et al, 1984) and retinoblastoma (Murphree and Benedict, 1984) have furthermore been adduced as evidence for the existence of a group of tumour-suppressor genes or "anti-oncogenes" whose function must be lost in the process of carcinogenesis. Thus in the case of retinoblastoma both alleles of the rb-1 gene on chromosome 13 are lost in the tumour cells (Murphree and Benedict, 1984). This evidence is consistent with earlier reports of the suppression of tumourigenic behaviour in hybrids derived from the fusion of malignant with normal cells (e.g. Harris et al, 1969; Stanbridge et al, 1982).

Further evidence for a somatic mutation theory is derived from the high incidence of cancer in patients suffering from conditions such as xeroderma pigmentosum and ataxia telangiectasia which are characterised by defects in DNA repair mechanisms (Paterson, 1976).

Evidence does also exist that cancer may not always involve irreversible mutation but rather epigenetic control mechanisms, as in the well-documented case of apparent normalisation of teratocarcinoma stem cells in chimaeric

mice following their inoculation into blastocysts (Mintz and Illmensee 1975 and Pierce et al, 1979). The increasing weight of evidence however implicates genetic abnormalities in the development of most tumours. Much of this has again been provided through the recent application of DNA recombinant and cloning techniques to isolate and characterise individual genes involved in oncogenesis.

12. Oncogenes

The documentation in recent years of the activity of a group of cellular oncogenes, first identified as homologues of known oncogenic retroviruses (see Table 1, after Paul, 1984), has been contributing greatly to the understanding of cancer at the molecular level (Bishop, 1983; Paul, 1984; Weiss and Marshall, 1984). The concept of oncogenes was first proposed in the context of animal tumour viruses when it was suggested that such retroviral oncogenes might reside dormant in the germ lines of all species (Huebner and Todaro, 1969). It has however been consequently shown that retroviral oncogenes have been derived by a process of recombination and mutation from normal cellular proto-oncogenes which appear to be highly conserved in evolution.

The use of cloned retroviral oncogene probes has shown several such oncogenes to be the transforming genes in biological transfection assays using tumour DNA to transform NIH 3T3 fibroblasts in culture, and abnormal oncogene activity at either a qualitative (e.g. point mutations as in the case of **ras**) or quantitative level (e.g. increased expression of a normal gene product by mechanisms such as gene amplification or chromosomal translocation as in the case of **myc**) has now been

demonstrated in a variety of culture-derived and primary human tumours (Cooper, 1984). Activated oncogenes have also been detected in tumours induced experimentally by agents such as radiation (Guerrero et al, 1984) or chemical carcinogens (Sukumar et al, 1984; Balmain and Pragnell, 1983) confirming that their activation in carcinogenesis is consistent with the pre-existing evidence from classical experimental and epidemiological studies. Although activated cellular oncogenes related to known retroviral oncogenes were initially defined in terms of their ability to transform 3T3 fibroblasts in culture, by no means all of the cellular homologues of the retroviral oncogenes are active in this assay which appears preferentially to detect **ras** genes. In addition several further oncogenes have been detected using this assay which do not appear to be related or only distantly to any known viral oncogene (e.g. B- **lym** and N- **ras**). Precise definition of what actually constitutes an oncogene has become therefore somewhat unclear in order to accomodate the still-growing list (currently about 40) and has been generally extended to include both the above categories as well as cellular proto-oncogenes abnormally expressed in primary tumours. Transcription of a number of cellular proto-oncogenes has been similarly demonstrated in a variety of tissue types and at various developmental stages (Gonda et al, 1982; Muller et al, 1982), although in the majority of cases very little is yet known of their exact physiological roles. It is generally thought however that they are concerned with the regulation of cell growth and development (Heldin and Westermark, 1984) and it has been hypothesised that

abnormalities of such growth-regulatory mechanisms may be critically concerned with the generation of malignancy (Sporn and Roberts, 1985).

On the basis of what is known of the biochemical properties and cellular-localisation of the various oncogene products they are generally assigned to four broad groups (Hunter, 1984).

(i) Phosphokinases, associated with the cell membrane. This group includes **src** , **yes** , **fps** / **fes** , **fgr** , **ros** , **abl** and **erb** -b, which have a specific tyrosine phosphokinase activity, as well as a smaller related group including **fms** , **mos** , and **raf** / **mil** . All of these have in common a region of homology with the catalytic unit of cAMP-dependent kinase. The **erb** -b gene product has been shown to have extensive homology with a truncated form of the epidermal growth factor (EGF) receptor. In addition to possessing tyrosine phophokinase activity the **src** and **ros** products have been shown to have the ability to phosphorylate phosphatidyl inositol, a precursor of diacyl-glycerol and triphospho-inositide, both of which can act as second messengers.

(ii) GTP Binding Proteins. This group is represented by the **ras** family of proteins which are also associated with the cell membrane. Along with the **src** product these are probably the best characterised of the oncogene products. These proteins have both GTP binding and GTP-ase activities. The latter is markedly reduced in the mutated oncogenic version (Sweet et al, 1984). They show significant homology

to the G binding proteins (Hurley et al, 1984) and more recent evidence based on study of mutant **ras** genes in yeast suggest that **ras** p21 is involved in modulating adenylate cyclase activity (Toda et al, 1985) and thus in regulating cellular cAMP levels and may thus function as a second messenger of transducer between receptors and adenylate cyclase.

This gene family has been shown to be dispersed on various chromosomes with Ha- **ras** 1 and 2 localised on chromosomes 11 and X respectively and Ki- **ras** 1 and 2 on 6 and 12 respectively (O'Brien et al, 1983).

(iii) Growth Factors. The **sis** oncogene has extensive homology with the beta chain of platelet-derived growth factors (PDGF). This appears to be the only growth factor encoded by an oncogene itself.

(iv) Nuclear Proteins. This group includes **myc** , **myb** , **fos** , B- **lym** and p53. These display only distant similarities and less significant evolutionary conservation. Expression of e.g. **myc** and **fos** appears to follow mitogenic stimulation of cells by exogenous growth factors and may well play a role in the regulation of cell entry from G0 to G1.

An alternative division of oncogenes into two "complementation" groups has also been used (Land et al, 1983) to distinguish them on the basis of their effects on cell transformation in vitro. It has been shown that one group including **ras** , **src** and polyoma middle T, is

responsible for anchorage independence in culture and production of tumour growth factors (TGFs), whereas members of a second group, including **myc** , **myb** , p53 and adenovirus ElA, confer sensitivity to the effects of the first group and are responsible for conferring immortalisation on cells in culture. This boundary does not appear to be completely hard and fast however since transformation of primary rat embryonic cell lines can be achieved by a mutated **ras** gene alone in conjunction with transcriptional enhancers (Spandidos and Wilkie, 1984).

The idea of complementary oncogenes is nonetheless consistent with the well-established notion of carcinogenesis being a multi-step process, variously estimated to involve from 2-7 steps (Klein and Klein, 1984; Paul, 1984), as is the fact that in many tumours several oncogenes appear to be activated (e.g. B- **lym** or **ras** and **myc** in Burkitt's lymphoma) although what contribution exactly each one may play and at what stage is not understood.

Such classifications are still also consistent with a modified version of an autocrine hypothesis of cancer, which as originally propounded (Sporn and Todaro, 1980) suggested that generation of a malignant phenotype might occur by endogenous production of growth factors acting on receptors of their producer cell. It would now appear that cancer cells may also become independent of growth factors by abnormalities in post-receptor controlling events. Thus, although it is known that many tumour cells, both spontaneously-occurring and virally-induced, produce a variety of growth factors, including tumour growth factors (TGF alpha and beta) and PDGF, the growth factor autonomy

of cancer cells may be more critically related to alterations of a receptor or post-receptor signalling pathway, which may also have theeffect of altering cell sensitivity to exogenous growth factors (Sporn and Roberts , 1985). The demonstration, furthermore of growth inhibitory effects of TGF beta (Roberts et al, 1985) suggests that abnormalities of such "negative" growth factors may also be critical in malignancy.

Although activation of various oncogenes has been demonstrated in a wide range of tumour types and at different stages of tumourigenesis, the implications of such activity are not yet fully understood. Nor is it clear how far such activity may be responsible for variation in the malignant phenotype. It appears that in any one tumour type different oncogenes or versions of an oncogene may be active and there appears to be no absolute tissue specificity of oncogene activation (**ras** family oncogene activation has been described in e.g. lymphoma, melanoma, carcinoma and sarcoma (Balmain, 1985)), although some pattern of preferential tissue involvement may yet emerge. In addition only about 10-15% of tumours can be shown to contain an activated oncogene using current transfection assays. This figure may reflect variation in the nature of oncogene mutations which in the case of **ras** most commonly involves "hot spots" around codons 12 and 61 (Balmain, 1985), although in a series of urothelial tumours recently reported (Fujita et al, 1984) neither the presence of a transforming **ras** oncogene in a small percentage (10%) of cases nor the presence or absence of a documented mutation in these appeared to be related to

tumour stage. Indeed activation of **ras** oncogenes has been reported at the premalignant stage in the mouse skin carcinogenesis model (Balmain et al, 1984) as well as in a late and highly-aggressive variant arising from a mouse lymphoma (Vousden and Marshall, 1984).

Of the 10-15% of tumour DNAs active in the 3T3 transfection assay the majority appear to contain activated members of the **ras** family of oncogenes. Activated Ki- **ras** genes have been demonstrated in colorectal carcinomas (Pulcini et al, 1982 and McCoy et al, 1983) due in one case to a codon 12 mutation in a Ki- **ras** 2 gene resulting in a coding substitution of valine for glycine at this position (Capon et al, 1983).

Oncogene amplification however has been shown to be correlated in the case of N- **myc** to advancing tumour stage in neuroblastoma (Brodeur et al, 1984) and in the case of c- **myc** to a more aggressive tumour variant in small-cell carcinoma of the lung (Little et al, 1983). Variably elevated levels of steady-state transcripts of several oncogenes have now been reported in a wide range of tumours (Slamon et al, 1984) but the possible clinical significance of this is not yet clear.

13. Other Tumour-Associated Genetic Abnormalities

Further understanding of the molecular pathology of cancer will also depend on the characterisation of other genes inappropriately activated or inactivated in malignancy by use for example of further biological assay systems and by techniques such as the cloning of complementary (c)DNA libraries. As mentioned previously this approach has already been used to isolate sequences

from virally-transformed cells in vitro (Scott et al, 1983) and the isolation of sequences expressed in different classes of leukaemia (Birnie et al, 1983). Whether such genes are activated in conjunction with or as a consequence of other oncogene activation is not yet clear.

Other apparently non-specific genetic abnormalities occurring in malignancy include gene amplification, i.e. increased copy number of genes (Schimke, 1984; Stark and Wahl, 1984), associated cytogenetically with chromosomal double-minutes (DM) and homogeneously-staining regions (HSR). These have been demonstrated in primary colorectal cancer (Reichmann et al, 1980) and an overall increase in the incidence of such double-minutes in metastatic relative to primary breast tumours was reported in one study although this was not an invariable finding (Gebhart et al, 1984). Amplification of various oncogenes has also been reported in different tumour types as previously mentioned, including amplification of c- **myc** in a colonic carcinoma cell line (of neural endocrine characteristics) although the significance of this is not clear (Alitalo et al, 1983).

Hypomethylation as a general feature of genomic DNA in cancer has been reported in a variety of tumour types (Diala et al, 1983) and this phenomenon has also been observed in specimens of colorectal carcinoma as well as in premalignant polyps (Goelz et al, 1985). The implications of this in terms of phenotypic variation is again not clear.

The demonstration of overall degeneration of DNA sequence organisation in a series of gut cancers using a

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complex mixture of many human foetal liver DNA fragments as probe (Humphries, 1981) appears similarly to be a gross finding which could not easily be related to subtle differences related to variation in a malignant phenotype.

The existence of an, as yet uncharacterised, multigene family, apparently activated in a range of different tumours (Haninia et al, 1983), although possibly of fundamental importance in carcinogenesis, has not yet been evaluated with reference to variation in a malignant phenotype.

A further approach to the definition of variation amongst tumours may lie in the study of genomic restriction fragment length polymorphisms (RFLPs) generated by the use of restriction endonuclease enzymes, which may arise due to single base changes in particular genes or as variable length polymorphisms due to differences in the copy number of tandem repeat sequences in non-coding flanking regions (Wainscoat and Thein, 1985). These techniques, although hitherto most useful in the study of disease linkages, in prenatal diagnosis and in study of somatic homozygosity in loci associated with familial cancers (Wainscoat and Thein, 1985) have also been used to demonstrate oncogene activation (of e.g. Ki- **ras** codon 12 using the Sac I enzyme (Santos et al, 1984)) and, conversely, to demonstrate the absence of such mutations in Gardner's Syndrome with its predisposition to colorectal cancer (Barker et al, 1983). Similarly, study of the inheritance of different variable length polymorphisms associated with the Ha- **ras** oncogene in the normal (white) cells of cancer patients showed the presence of abnormal allelic

restriction fragments (Krontiris et al. 1985). Such studies, however, are still at an early stage and whether they will ultimately contribute to the characterisation of the genetic determinants of variation in a malignant phenotype remains speculative.

14. Plan of Investigation

In an attempt to define a clinically-useful marker for metastasis in colorectal cancer as well as to provide a possible means of studying the biology of metastasis and perhaps ultimately contribute to new therapeutic approaches to it, it was decided to make use of novel techniques which have recently become available and which make it possible to clone individual RNA sequences into cDNA libraries and so study cell phenotype at this level. This approach affords the particular advantage of isolating and studying previously-unknown gene sequences. The strategy employed was to use two different tumours, one of which had already metastasised (a Dukes D) and one which had not done so at the time of surgery, nor did so on follow-up of more than three years (a Dukes B), as the source of material for construction of cDNA libraries. It was thus hoped, by cross-screening these libraries, to identify cloned sequences related to such differences in behaviour and to correlate transcription of these with metastatic capacity in a series of tumours.

Since (Ki) **ras** oncogenes have been shown to be activated in colorectal cancer it was also decided to study the significance of variation in the level of **ras** family oncogene RNA transcripts in relation to variation in the clinical behaviour of the same tumours.

Table I **Viral oncogenes. Some are related, e.g. v-fps and v-fes appear to be the same oncogene from different species.**

Virus	Species of Origin	v-onc	Type of Tumour	Proto-oncogene
Rous sarcoma	Fowl	v-src	Sarcoma	Yes
Fujinama sarcoma	Fowl	v-fps	Sarcoma	Yes
Yamaguahi sarcoma	Fowl	v-yes	Sarcoma	Yes
Rochester-2 sarcoma	Fowl	v-ros	Sarcoma	Yes
Myelocytomatosis MC29	Fowl	v-myc	Carcinoma, sarcoma, leukaemia.	Yes
Avian erythroblastosis	Fowl	v-erbB	Leukaemia, sarcoma	Yes
Myeloblastosis	Fowl	v-myb	Leukaemia	Yes
Avian SKV770	Fowl	v-ski		Yes
Reticulo-endotheliosis	Turkey	v-rel	Leukaemia	Yes
Moloney murine sarcoma	Mouse	v-mos	Sarcoma	Yes
Harvey murine sarcoma	Rat	v-H-ras	Sarcoma	Yes
Kirsten murine sarcoma	Rat	v-Ki-ras	Sarcoma	Yes
3611 murine sarcoma	Mouse	v-rab	Sarcoma	Yes
Abelson murine leukaemia	Mouse	v-abl	Leukaemia	Yes
FBJ murine osteosarcoma	Mouse	v-fos	Sarcoma	Yes
Feline sarcoma	Cat	v-fes	Sarcoma	Yes
Gardner-Pasheed feline sarcoma	Cat	v-fgr	Sarcoma	Yes
McDonough feline sarcoma	Cat	v-fms	Sarcoma	Yes
Simian sarcoma	Monkey	v-sis	Sarcoma	Yes

A. MATERIALS

1. Chemicals

Actinomycin D	Sigma Chemical Company
Agarose	BRL Inc.
Ampicillin	Sigma Chemical Company
ATP	Sigma Chemical Company
Bacto agar	Difco Laboratories
Bacto tryptone	Difco Laboratories
Bacto yeast extract	Difco Laboratories
Bovine serum albumin	Sigma Chemical Company
Bromophenol blue	BDH Chemicals
Caesium chloride (99.99% pure)	BRL Inc.
Calf Thymus DNA	Sigma Chemical Company
Chloramphenicol	Sigma Chemical Company
Chelex 100	Bio Rad Laboratories
Collodion bags	Sartorious
Deoxynucleotidetriphosphates	Boehringer Corporation
Dextran sulphate	Sigma Chemical Company
Diethylpyrocarbonate	Sigma Chemical Company
Dithiothreitol	Sigma Chemical Company
Ethidium bromide	Sigma Chemical Company
Ficoll 400	Pharmacia
Formaldehyde	BDH Chemicals Ltd.
Formamide	Fluka Chemische Fabrik

Guanidinium thiocyanate	Fluka Chemische Fabrik
HEPES	Sigma Chemical Company
Kodak X-Omat R X-Ray Film	Kodak (U.K.) Ltd.
β-mercaptoethanol	Sigma Chemical Company
MI 96 scintillant	Packard Instruments
Nitrocellulose-0.10μM membrane	Sartorious
Oligo (dT)3 cellulose	BRL Inc.
Polyvinylpyrolidone	Sigma Chemical Company
Poly(A)	Sigma Chemical Company
Poly(C)	Sigma Chemical Company
Repelcote	Hopkins and Williams
Salmon sperm DNA	Sigma Chemical Company
Sephadex G50 and G100	Pharmacia
Sodium bicarbonate	Flow Laboratories
Sodium dodecyl sulphate	BDH Chemicals
Sodium lauryl sarcosine	Sigma Chemical Company
Sodium morpholinopropane-sulphonic acid	Sigma Chemical Company
Sucrose	Schwartz/Mann
TEMED	BDH Chemicals
Tris-HCl	Sigma Chemical Company
Triton X-100	Sigma Chemical Company
Trizma base	Sigma Chemical Company
Urea	Pierce Chemical Company
Vanadyl-Ribonucleoside Complex	BRL Inc.
X-Gal	BRL Inc.

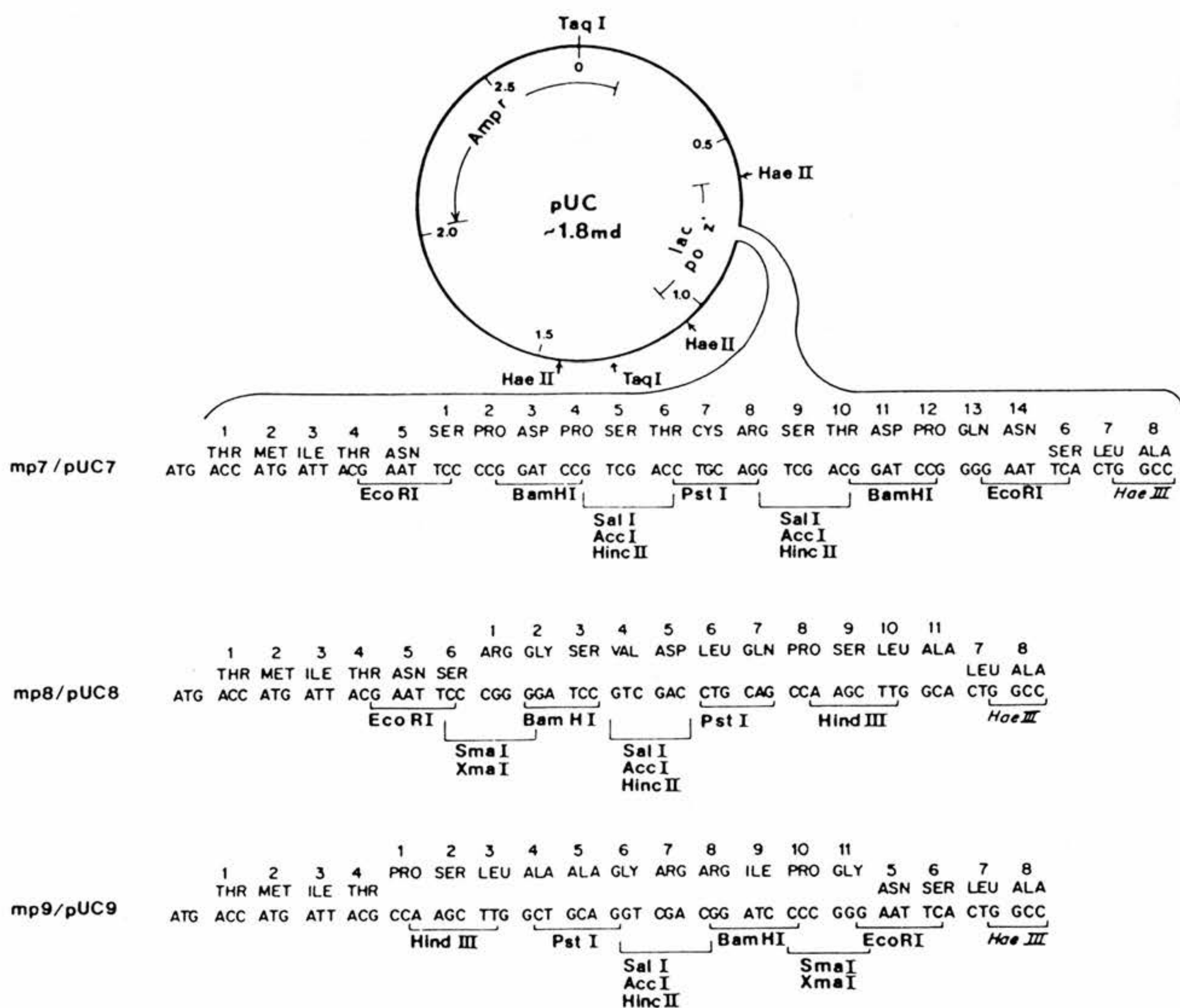
2. Enzymes

Avian myeloblastosis virus Reverse transcriptase	Division of Cancer and Prevention, National Cancer Inst.
Calf-intestinal alkaline phosphatase	BRL Inc.
E.coli DNA polymerase I	Boehringer Mannheim Corporation.
E.coli DNA polymerase I (Klenow fragment)	Boehringer Mannheim Corporation.
Lysozyme	Sigma Chemical Company
SI nuclease	BRL Inc.
T4 DNA ligase	New England Biolabs Inc.

3. Bacterial and Plasmid Strains used for Cloning

The plasmid vector used for cloning purposes was the M13mp7-derived pUC8 plasmid which contains a multiple cloning site and the bacterial host strain which was used for transformation was the E.coli K-12 strain JM83 (Vieira and Messing, 1982). Details of the plasmid cloning site are shown in the genetic map below.

Genetic manipulations were carried out under GMAG category 2 containment conditions.



Genetic map of pUC plasmids (from Vieira and Messing, 1982)

B. METHODS

1. Collection of Specimens

Colectomy specimens were opened in operating theatre and blocks of tumour tissue of approximately 1g in weight, subsequently confirmed by histology, were dissected out and snap frozen immediately in liquid nitrogen and stored at -70°C. Specimens of adjacent, histologically normal, colorectal mucosa were similarly dissected off and stored.

2. Treatment of Glassware and Culture Media

Glassware for use with solutions of RNA were siliconised with "Repelcote" and sterilised in 0.1% diethylpyrocarbonate. After each of these procedures glassware was baked overnight at 80°C. All solutions employed in bacterial culture were sterilised by autoclaving at 14.5psi (120°C) for 20 minutes.

3. Preparation of Total Cellular RNA

Specimens were pulverised under liquid nitrogen in a procedure devised to handle solid tumour specimens by several blows with a mallet and grinding with a mortar and pestle. Powdered tumour was then suspended in a solution of 5M guanidinium thiocyanate, 50mM Tris-HCl, 50mM EDTA, and 5% β mercaptoethanol (pH 7.0) as described by Chirgwin (Chirgwin et al, 1979). Long DNA strands were sheared by a 15 second burst of sonication sonication. Sodium lauryl sarcosine from a 20% (w/v) stock solution was then added up to 2% (w/v). The solution was warmed at 65°C for 3 minutes until clear and then cooled on ice. The solution was then overlaid onto a 2.5ml cushion of 5.7M CsCl (which had previously

been passed through chelex to remove heavy metal ions), 60mM EDTA pH 7.0 (R.I. 1.3994) in 14ml polypropylene tubes. The tubes were balanced and centrifuged at 35,000rpm at 15°C for 20 hours on a B60 6x14 swing-out rotor. The supernatant was then aspirated and the tubes drained and wiped clean. The RNA pellets were dissolved in sterile water and precipitated twice from 0.2M sodium acetate (pH 6.8) and 2.5 volumes of absolute alcohol at -20°C overnight. Concentration of RNA was measured using a Unicam SP500 spectrophotometer at A260. An O.D. 260nm. (optical densitometry) measurement of 1 is equivalent to 40µg.ml of RNA.

4. Isolation of Polyadenylated RNA by Oligo (dT) Cellulose Chromatography

Poly(A)+ RNA was isolated from total cellular RNA as originally described by Aviv and Leder (Aviv and Leder, 1972) by the use of oligo(dT) -cellulose to bind the 3' poly(A) "tail" present in a a small percentage of RNAs. A 2ml column of swollen oligo (dT)-cellulose (T3) was prepared and poured and then washed thoroughly with at least 10 bed volumes of 0.1M NaOH. This was then washed thoroughly with more than 20 bed volumes of binding buffer (0.5M NaCl, 10mM Tris-HCl, 0.1% (w/v) Sodium lauryl sarcosine, 1mM EDTA pH 7.5). Total RNA was dissolved in sterile water and an equal volume of 2x binding buffer at a concentration of, optimally, 100µg/ml to minimise aggregation and non-specific binding. The RNA was passed over the column twice, which was then washed with 10 bed volumes, of 1x binding buffer and the O.D. of 1ml. fractions was read until the

O.D. = 0. The poly(A)$^+$ bound to the column was then eluted with sterile water and its concentration measured by O.D. before being pooled and the whole procedure repeated. The final eluate was precipitated with 1/10th volume 2M sodium acetate (pH 6.8) and 2.5. volumes of absolute alcohol, and recovered by centrifugation in a Sorvall HB4 rotor at 10,000rpm at -10°C for 20 minutes.

5. Synthesis of First Strand of Complementary (c) DNA

This was carried out by oligo (dT)-primed reverse transcription using a method similar to that described by Getz (Getz et al, 1975) to a specific activity of 500cpm/ng. This was achieved by using a 1:20 dilution of low activity 20 Ci/mmol ^{3}H dCTP to prevent excess breakdown. The following reagents were used in a 1ml reaction mix containing 10μg of poly(A)$^+$ RNA: a triphosphate mix containing dATP, dGTP, and dCTP to a final concentration of 1mM and ^{3}H dCTP to a final concentration of 0.05mM all of which was lyophilised and then added to 50mM Tris-HCl, (pH 8.2), 10mM magnesium acetate 10mM D.T.T., 40mM NaCl, 100μg actinomycin D and 60 units/μg of RNA AMV reverse transcriptase. The RNA and oligo dT and H_2O were mixed first and heated to 60° for 1 minute, then left to anneal for 30 minutes at room temperature. The other reagents were then added, the reverse transcriptase last of all, and the mix was incubated at 37°C for 1.5 hours. The reaction was terminated by adding EDTA to a final concentration of 25mM. The mix was then eluted with 50mM Tris pH7, 50mM NaCl over a Sephadex G-50 column together with 20μg of

yeast tRNA to separate unincorporated ^{3}H dCTP. The solution was made 0.1M NaOH and left at 37°C for 1 hour to hydrolyse the RNA template. It was then neutralised with acetic acid using a drop of phenol-red indicator until its colour turned salmon pink. The yield of (c)DNA was calculated by counting 5µl fractions mixed and vortexed with 500µl H_2O and 5ml of Packard M196 scintillant fluid. The yield in ng of ss (single-stranded) cDNA = $\frac{\text{cpmx200/ml.}}{\text{500cpm/ng}}$

An aliquot of 1µl was reserved to be used as a control in the S1 assay. The (c)DNA was then precipitated by adding 1/10th vol. 2M sodium acetate (pH 6.8) and absolute ethanol to 2.5 volumes and leaving overnight at -20°C. This was again pelleted by centrifugation at 20,000rpm,-10°C for 20 minutes.

6. Synthesis of Second Strand of Complementary (c)DNA

This was performed by a modification of the method described by Affara et al (Affara et al, 1981). The single-stranded cDNA was incubated in a 500µl reaction mix containing 30mM Tris-HCl, 5mM $MgCl_2$, 0.5mM β-mercaptoethanol pH 7.5 and 1.5mM dCTP, dGTP, dATP, dTTP and 200 units of DNA polymerase ("Klenow" fragment) at room temperature for 5 hours. The mix was extracted in phenol: chloroform twice and the aqueous ethanol layer precipitated at -20°C and dissolved in 10mM Tris-HCl, 1mM EDTA pH8.

7. Trial S1 Nuclease Digestion

Prior to bulk S1 digestions a trial was performed to ensure that an acceptable degree of double-stranding was

present. 1μl aliquots of both single-stranded cDNA and double-stranded cDNA were incubated in a 350μl reaction mix containing 30mM sodium acetate, 50mM NaCl, 1mM $ZnSo_4$, 5% glycerol, pH 4.6 with 6 units of S1 nuclease (Vogt, 1973) at 37°C for 1.5hours. The mixes were then chilled on ice. A 100μl aliquot was taken from each and counted after mixing with 0.5ml H_20 and 5ml M196 scintillant fluid. A 200μl aliquot was then mixed with 50μl of DNA/BSA carrier (100μg/ml E.Coli DNA and 1μg/μl BSA) and 50μl of ice-cold 2M perchloric acid. After 20 minutes on ice the mix was centrifuged at 1,000rpm or 3 minutes at 0°C. 150μl of each supernatant was removed and counted. These represent the acid-soluble DNA. The acid-soluble fraction of total cDNA represents the fraction which is single-stranded.

8. Bulk S1 Nuclease Digestion of ds cDNA

Cleavage of hairpin loops in the synthesised double stranded cDNA was performed using a modification of the method described by Fantoni (Fantoni et al., 1979). The double-stranded cDNA was dissolved in 500μl of 10mM Tris-HCl pH 8.0, 1mM EDTA, 200μl of 3x S1 Buffer (90mM sodium acetate, 150mM NaCl, 3mM ZnS04 45% glycerol pH 4.6) and 30μl (150 units) of S1 nuclease. The mix was incubated for 3 hours at 37°C. The reaction was stopped with 10μg of 0.25M EDTA and tRNA carrier was added. The mix was extracted twice in phenol: chloroform and chloroform extracted in ether which was blown off finally with gaseous N_2. The DNA was recovered by precipitation with 2.5 volumes of absolute alcohol in 0.2M sodium acetate pH 6.8.

9. Repair Reaction to Generate Blunt Ends

This was performed in a mix of 100μl double-stranded cDNA in 2x buffer (60mM Tris pH 7.5, 10mM $MgCl_2$, 1mM β mercaptoethanol), 80μl of 15mM stock triphosphates (dGTP, dATP, dCTP, dTTP) and 20μl of DNA polymerase I (1 unit/μl). This was incubated at 37°C for 1 hour. The reaction was stopped by the addition of sodium acetate to 200mM and then extracted with an equal volume of chloroform. 10μg of yeast tRNA carrier were then added and the double-stranded cDNA was precipitated in 2.5 volumes of absolute alcohol and 0.2M sodium acetate pH 6.8. The pellet was then dissolved in 200μl of 10mM Tris-HCl pH 7.5, 1mM EDTA.

10. Ligation of Double-Stranded cDNA into SmaI Cut pUC 8

"Blunt end" ligation of double-stranded cDNA into Sma I cut and phosphatased pUC8 plasmid vector (kindly provided by Dr. M. Ramsden of the Beatson Institute) was performed by a modification of the method of Cochet (Cochet et al, 1979) and Fantoni (Fantoni et al, 1979). The following reaction mix was employed: 5ng double-stranded cDNA (10μl), 50ng pUC8 (1.6μl), 1μl 20x Ligase Buffer (0.6m Tris pH 8.0, 100mM $MgCl_2$, 200mM DTT, 25mM EDTA), 1.5μl 15mM ATP, 1μl T4 Ligase (1000 units/ml) and 5μl H_2O. This was incubated for 3 hours at room temperature.

11. Preparation of Competent Cells

The JM83 strain of <u>E.Coli</u> was used for transformation and cells were prepared by a modification of the method of Dagert and Ehrlich (Dagert and Ehrlich, 1979). An inoculum of <u>E.Coli</u> JM83 was incubated overnight at

37°C in 4ml of L-broth (10g. Bactotryptone, 5g yeast extract, 10g NaCl in 1l, of distilled water). This was then added to 100ml of L-broth in a 500ml Erlenmeyer flask and incubated in an orbital shaker at 37°C until the O.D. 600nm, was approximately 3.0. This was then decanted into sterilised plastic gasketed Sorvall bottles and chilled on ice for 10 minutes. They were then centrifuged for 5 minutes at 4.500rpm at 4°C in a Sorvall GS-3 rotor. The pellets were then suspended in 20ml of ice-cold 0.1M filter-sterilised calcium chloride and kept on ice for 20 minutes. They were then centrifuged again at 4.5K, 4°C for 5 minutes and the pellet re-suspended in 2ml of 0.1M $CaCl_2$ and stored at 4°C overnight.

12. Transformation of Competent Cells with pUC8 Recombinant Plasmid

The 20µl ligation mix previously described was added to 80µl of transformation buffer (50mM MOPS pH 7.8, 75mM $CaCl_2$, 1mM EDTA, 100mM KCl, 5% v/v glycerol) and mixed with a 100µl aliquot of competent cells. This mix was left on ice for 20 minutes and then at 37°C for 15 minutes, before being inoculated into 1.8ml of pre-warmed L-broth. This was then incubated for 2 hours at 37°C to permit the establishment of plasmid-conferred antibiotic resistance. 100µl aliquots were then plated out onto 5cm-1.5% L-agar-containing Petri dishes (7.5g. Bacto-agar in 500ml of L-broth and overlayed with 10ml 1.5% w/v agar containing 20µg/ml of "X-Gal" (2% w/v solution of phenylmethyl sulphuryl fluoride) and 100µg/ml of ampicillin). The plates were incubated for

18 hours at 37°C. The transformation efficiency was determined by calculating the number or colonies grown per μg of uncut plasmid used in a control experiment to generate transformants. Negative controls were also undertaken using cut plasmid without additional ligated double-stranded cDNA.

13. Identification and Harvesting of Transformed Cells

The pUC8 plasmid (Vieira and Messing, 1982) is a Rec A+ M13 derivative of approximately 1.8md containing both an ampicillin and β-Galactosidase gene. The latter contains the palindromic SmaI site CC"CGG which is
G"GCC
rendered inactive by insertional mutagenesis if foreign cDNA is ligated into it. These features make it possible to identify cDNA containing recombinants when grown in transformed E.Coli on ampicillin and "X-Gal" containing medium. The latter is in consequence no longer metabolised by β-Galactosidase to produce the characteristic blue colony colour but appears instead white. The latter colonies growing on ampicillin containing medium should therefore contain cDNA-plasmid recombinants. Such colonies were picked-out using sterile tooth picks and grown in 96 well microlitre plates in 100μl of L-broth containing 5%(v/v) glycerol and 100μg/ml of ampicillin. These plates containing colonies of transformed cells collectively constitute a cDNA "library".

14. Screening of cDNA Libraries using the Grunstein - Hogness Technique: Preparation of Filters

The microtitre plates containing colonies of transformed

cells were replica-plated onto autoclaved Millipore nitrocellulose filters (0.45µm pore) using transfer plates and grown on 9cm L-agar Petri dishes supplemented with 100µg/ml ampicillin, for 18 hours at 37°C. The filters were then removed and the bacterial colonies lysed in-situ and the DNA immobilised by the method of Thayer (Thayer, 1979). The filters were placed on sheets of Whatmann 3MM paper in a plastic tray saturated sequentially with lysis and neutralising solutions. Between each application the filters were blotted for one minute on dry Whatmann 3MM paper. The solutions used were as follows:-

1) 10mM Tris-HCl pH 8.0, 4% (w/v) sucrose, 10mg/ml lysozyme applied twice for 5 minutes.
2) 0.2% Triton-X-100, 0.5M NaOH twice for 5 minutes.
3) 1.5M NaCl, 1.0M Tris-HCl pH 7.6 twice for 3 minutes.
5) 2x SSC once for 1 minute. The filters were applied colony side uppermost. They were air-dried and baked for 2 hours at 80°C.

Prehybridisation was performed by immersing the filters overnight at 65°C in heat-sealed plastic bags containing 20mls of the following solution:-

5xSSC, 10x Denhardt's solution (0.02% bovine serum albumin, 0.02% poly vinyl-pyrollidone, 0.02% Ficoll 400), 0.1%SDS, 10mM NaP4 pH 7.4, 10µg/ml heat denatured poly A, 10µg/ml heat denatured poly C, and 100µg/ml heat denatured Salmon Sperm DNA.

15. Preparation of 32P-dCTP cDNA Probe

This was done by a modification of the method of Rothenburg and Baltimore (Rothenburg and Baltimore,

1976) and probe was labelled to a specific activity of $5x10^7$ counts/minute/µg. A typical reaction mixture of 50µl contained 1µg of poly $(A)^+$ RNA, 100µCi 32P-dCTP (400Ci mmol), actinomycin D 70µg/ml, 200µm dATP, dGTP, dTTP, oligo dT 500µg/ml, RNAsin 20 units, and AMV reverse transcriptase 24 inits reverse transcriptase buffer (50mM Tris-HCl pH 8.3, 8mM $MgCl_2$, 50mM KCl, 25mM Dithiothreitol). This mixture was incubated for 1.5 hours at 37°C. NaOH was then added to a final concentration of 300mM for a further 20 minutes at 37°C to remove the template RNA. 3µl of phenol red indicator was then added and the solution neutralised by adding 2M sodium acetate (pH 4.8) until the solution turned salmon pink. Unincorporated nucleotides were removed by passing the mixture over a Sephadex G-100 column pre-treated with 50µg of yeast tRNA in 0.1xSSC (0.15M NaCl, 0.015M Na citrate). One ml fractions were collected and 5µl aliquots counted on a scintillation counter after mixing with 0.5ml H_2O and 5ml of Packard M196 scintillant fluid. The pooled ^{32}P dCTP-labelled fractions were then denatured by boiling for 5 minutes followed by immediate cooling on ice prior to being added to pre-hybridisation buffer.

16. Grunstein-Hogness Hybridisation

^{32}P-dCTP radiolabelled cDNA probe was added to plastic bags containing filters and approximately 10ml pre-prehybridisation buffer at a concentration of $5x10^6$cpm/10ml and the bags were thermally sealed. These were then incubated at 65°C for 18 hours. The filters were then washed as follows:-

In 3xSSC, 0.1% SDS at 65°C with agitation for 30 minutes, once in 1xSSC, 0.1% SDS for 30 minutes, and three times in 0.1xSSC, 0.1% SDS at 65°C for 30 minutes. The filters were then air-dried on Whatmann's 3MM paper before being sealed in plastic bags. They were then exposed to Kodak X-omat film with Ilford Fast Tungstate intensifying screens in a Harmer Cassette at -70°C for between 1 to 7 days.

17. Small-Scale Isolation of Plasmid DNA

This was carried out essentially as described by Birnboim and Doly (Birnboim and Doly, 1979). 5µl inocula of bacterial clones of interest from the libraries were grown up overnight in a 3ml culture in L-broth at 37°C. These were then divided and centrifuged in microfuge tubes in an Eppendorf microfuge (8,000g.) for 15 seconds. The pellet was resuspended in 200µl of lysis buffer (25mM Tris-HCl pH8, 10mM EDTA pH8, 50mM sucrose, and 2mg/ml lysozyme) and incubated on ice for 30 minutes. 200µl of alkaline/SDS solution (0.2N NaOH, 1% SDS) were then added and the mixture was left on ice for 5 minutes. 300µl of 3M potassium acetate (pH4.8) was then added and gently mixed by inversion. This mixture was then kept for 60 minutes on ice and the DNA clot was then spun down in a microfuge for 3 minutes. The supernatant was removed and divided into two microfuge tubes. 1ml of cold ethanol was added to each and precipitation achieved at -20°C for 30 minutes. This was again microfuged for 2 minutes and the supernatant discarded. The pellet was then resuspended in 200µl of a Tris-acetate solution (0.1M

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sodium acetate pH8, 500mM Tris HCl pH8).
A further precipitation in 2 volumes of absolute alcohol was performed at -20°C for 10 minutes and the precipitate spun down for 2 minutes in a microfuge. The pellet was then taken up in 40μl of RNase (100μg/ml) and incubated at 37°C for 1 hour. This was then alkali treated in 0.05N NaOH and incubated at 37°C for 30 minutes before being boiled for 10 minutes. It was thereafter cooled on ice prior to use for dotting.

18. DNA Dot Hybridisation

This was performed essentially as described by Thomas (Thomas, 1980). 4μl aliquots of plasmid DNA were dotted onto sheets of Sartorious nitrocellulose paper which had previously been immersed in 20x SSC for 5 minutes. These were then air-dried and baked for 2 hours at 80°C prior to prehybridisation. This was performed as for the Grunstein-Hogness technique for 18 hours at 65°C in a shaking water bath. ^{32}P cDNA radiolabelled probe was similarly added and allowed to hybridise for 18 hours at 65°C. Washing of filters and autoradiography were performed according to the same methods.

19. Bulk Isolation of Recombinant pUC8 Plasmids

This was again performed essentially as described by Birnboim and Doly (1979). Inocula of 5μl of selected bacterial clones from the cDNA libraries were grown up for approximately 16 hours in 3mls of ampicillin-containing L-broth (100μg/ml) and then added to 0.5l of L-broth. The cells were then harvested after overnight culture by centrifugation in a Sorvall (GS-3) rotor for 5 minutes at 5,000rpm. The pellet was then suspended

and lysed in 25ml of freshly prepared lysozyme (2mg/ml) in 25mM Tris-HCl, 10mM EDTA, 25% (w/v) sucrose, pH8.0 and left on ice for 30 minutes as described originally by Clewell and Helinski (1970). 50ml of 0.2N NaOH, 1% (w/v) SDS were added mixed by inversion and left on ice for 5 minutes. 37.5ml of sodium acetate (pH 4.8) were then added, and the solution gently mixed. After a further 1 hour on ice the mixture was centrifuged in a Sorvall GS-3 rotor for 10 minutes at 8.500rpm at 0°C. The supernatant was removed and DNA precipitated with 0.6 volumes of isopropyl-alcohol at -20°C for 1 hour. The DNA was pelleted by centrifugation for 20 minutes at 10,000rpm at -20°C and resuspended in 5mls 0.1xSSC. 6g of CsCl were added and vortexed to dissolve it. Ethidium bromide 0.25ml (10mg/ml) was then added and the overall R.I (refractive index) was adjusted to 1.3940. Each solution was then added to 10ml polycarbonate centrifuge tubes (M.S.E.) which were topped up with liquid paraffin and finely balanced. These buoyant density gradients (Birnie, 1978) were then centrifuged at 40,000rpm at 20°C for 40 hours using an M.S.E. 10x10ml titanium fixed-angle rotor. The lower of two bands which appear in the tubes (and which are easily visible in U.V. light) contains supercoiled plasmid DNA and the upper band contains both chromosomal and open circular plasmid DNA. Supercoiled DNA was thus recovered using a fixed, repelcoted Pasteur pipette. The ethidium bromide was removed by several extractions with CsCl saturated isopropanolol. The remaining aquequs layer was then

extensively dialysed in Collodion bags (Sartorious) against 10mM Tris-HCl, 1mM EDTA pH 7.6, to remove the CsCl. The plasmid DNA was then precipitated in 200mM sodium acetate (pH 6.8) and 2.5 volumes of absolute alcohol overnight and pelleted by centrifugation in a Sorvall GS-3 for 20 minutes at 10,000rpm at -20°C. After washing the pellet in 70% alcohol it was suspended in 1ml 10mM Tris-HCl, 1mM EDTA and its concentration determined by measuring its optical density at A_{260nm} when 1 O.D. unit = 50µg DNA.

20. Quantitative Dot Hybridisation

This was performed as described previously (Thomas, 1980) using fixed 100ng amounts of recombinant plasmid DNA per dot and ^{32}P-dCTPcDNA probes as described previously.

21. Preparation of Nick-Translated ^{32}P-Labelled Recombinant Plasmid Probes

Labelled plasmid probes were prepared (to a specific activity of $1x10^{6}$cpm/ml using a commercially available kit (Amersham International Inc.). 0.5µg of plasmid DNA was incubated with 20µl of nucleotide buffer solution (containing 100µm dATP, dGTP, dTTP) in a concentrated nick-translation buffer (containing Tris-HCl pH 7.8, $MgCl_2$ and mercaptoethanol) and 20µl of an enzyme solution (containing 5 units of DNA polymerase I and 100pg DNA-ase I in a buffer solution containing Tris-HCl pH 7.5, $MgCl_2$, glycerol and BSA) in a total reaction mix of 50µl at 15°C for 3 hours. Unincorporated material was removed by passage over a Sephadex G100 column in 0.1x SSC. The labelled plasmid

was boiled for 5 minutes and cooled on ice before addition to hybridisation buffer.

22. RNA Doubling-Dilution Dot Hybridisation

This was performed essentially as described by Birnie (Birnie et al, 1983). From a stock solution of RNA at a concentration of 500µg/ml twelve doubling dilutions were made by transferring a volume from each tube equal to an aliquot of sterile water previously added to it. The tubes were heated for 15 minutes at 65°C to denature the RNA and then chilled on ice. 4µl of each dilution was then dotted onto a Sartorious nitrocellulose membrane which had been previously soaked for 5 minutes in 20x SSC. This was air-dried and the RNA immobilised by baking at 80°C for 2 hours. The filter was prehybridised for at least 4 hours in 10mls of prehybridisation buffer (containing 50% formamide, 5x Denhardt's solution, - 50mM sodium phosphate pH 6.8, 200µg/ml of sonicated, denatured salmon sperm DNA, 10µg/ml poly(A) and 10µg/ml poly(C)) at 42°C. Hybridisation was performed in 10ml of buffer (containing 50% formamide 5X SSC, 1X Denhardt's solution, 10% Dextran sulphate) containing labelled, denatured probe labelled to a specific activity of $1x10^6$cpm per ml at 42°C for 18 hours. The filters were then washed in 3x SSC, 0.1% SDS at room temperature twice for 20 minutes and in 0.5x SSc, 0.1% SDS at 65°C for 20 minutes and repeated as necessary until background radioactivity was minimal. The filters were air-dried and autoradiographed as before, using Kodak X-omat film in conjunction with a Dupont Cronex intensifying screen at -70°C for 1-5 days. The end point of the RNA

titration was taken as the lowest dilution giving a signal above background on the film.

23.Dot Hybridisation with **ras** -family Oncogene Probes

Dot hybridisation with single 10μg aliquots of total cellular RNA was also performed as previously described (Spandidos and Kerr, 1984) using ^{32}P-labelled nick-translated Hi Hi3 (Ellis et al, 1980) and BS9 (Ellis et al, 1981) recombinant probes containing the viral Kirsten and Harvey **ras** DNA sequences respectively.

24.Quantitative and Qualitative Controls of RNA Preparations

This was performed using the pL335 (Dalla Favera et al, 1982), pAM91 (Minty et al, 1982) and pHR28 (prepared by Anne Sproul of the Beatson Institute) probes containing the human cellular **sis** , mouse actin and human 28S ribosomal DNA sequences respectively to check the quantity of RNA present in a specimen and was undertaken in conjunction with Dr. D.A. Spandidos (Beatson Institute, Glasgow) as previously described (Spandidos and Kerr, 1984). The quality of the RNA preparations was similarly checked by formaldehyde-agarose gel electrophoresis, followed by ethidium bromide staining, blotting onto nitro-cellulose and hybridisation to DNA probes. For blotting, lyphophilysed RNA was taken up in 50% formamide, 2.2M formaldehyde and 1 x MOPS buffer, heated at 55°C for 15 minutes, made 10% glycerol, 0.1% dye and layered on to a gel. The gel containing 1% agarose, 1 x MOPS buffer, and 2.2M formaldehyde was made as follows:-

the agarose was melted in H_2O, cooled to 60°C and the

10 x MOPS buffer and 13.2M formaldehyde were added. The gel was run for 3 - 4 hours at 3V/cm until the marker dye had migrated 8-10cm. It was then soaked in 10 volumes of 1 x MOPS buffer (two changes of 15 min each) and subsequently in 20 x SSC (two changes of 30 min each). RNA was blotted onto nitrocellulose in 20 x SCC for 4-6 hours. The nitrocellulose filter was washed in 3 x SSC, air-dried and baked for 3-4 hours in an 80°C oven.

25. Histopathological Evaluation of Tumour Specimens

Histopathological reports were based on standard 5µm paraffin-embedded sections stained with haematoxylin and eosin. These were checked in conjunction with Dr. F.D. Lee (Department of Pathology, Glasgow Royal Infirmary).

26. Clinical Follow-Up

Patients in this study were follow up routinely as out-patients in the Department of Surgery, Glasgow Royal Infirmary at three-monthly intervals with biannual ultrasonography and further radiological investigations as required.

RESULTS

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ADDENDUM

5. The Sephadex G-50 column was prepared by swelling 2g of G-50 overnight in 100ml of distilled water; this was then poured on top of 1ml chelating resin in a Bio-Rad 1c x 20c column.

Neutralisation with acetic acid was performed simply drop-wise until the colour of the solution turned salmon pink.

6. Precipitation of double-stranded cDNA was as described in Section 5, by addition of 1/10th vol. 2M sodium acetate (pH 6.8) and absolute ethanol to 2.5 volumes and leaving overnight at -20°C. The mix was also extracted with chloroform following phenol extraction.

9. DNA polymerase I ("Klenow" fragment) was used in the repair reaction.

RESULTS

1. Collection of Tumours and Preparation of RNA

A series of specimens comprising 24 colorectal carcinomas along with, in most cases, adjacent histologically-normal mucosa and, in some cases, adenomatous polyps found incidentally was collected. The yield of total cellular RNA from approximately 1g of tumour was typically between 500-1,000μg depending on the cellularity of the specimen. Subsequent oligo-(dt)-cellulose chromatography then yielded typically 10-20μg of poly(A)+RNA. Northern blot analysis of these samples as described previously (Spandidos and Kerr, 1984) showed a moderate amount of degradation of RNA as would be expected from primary tumour samples. Clearly identifiable and discrete bands were, however, easily discernable when hybridised with e.g. the **ras** oncogene probes. Similarly, the dot hybridisation signals obtained from these samples (Spandidos and Kerr, 1984) using the actin (pAM91) and ribosomal (pHR28) probes as positive controls (these are uniformly highly expressed in these cell types) showed uniformly high intensities. The **sis** oncogene probe (pL335) conversely, generated scarcely-detectable signals thus serving as a negative control.

2. Construction of cDNA Libraries

Double-stranded (ds) cDNA was made by a series of enzymic reactions as outlined in Figure 1. From an initial mix containing 10μg of poly(A)+ RNA a final yield of approximately 100ng of blunt-ended ds-cDNA was generated.

This relatively low yield (compared to more recent approaches) was due to the fact that each step (although this number was minimised as far as possible) involved substantial losses of cDNA. The efficiency of the initial reverse transcriptase-catalysed cDNA synthesis was calculated to be approximately 15%. Trial S1-nuclease digestion showed that approximately 60% of the cDNA was double-stranded following the second strand synthesis. Thus 40% was subsequently lost during the bulk S1 digestion performed to generate "blunt ends" by cleaving the hairpin loops. Size fractionation of the cDNAs was not performed, in order to randomly generate a full range of sequences of differing size-lengths for cloning. (Augenlicht and Kobrin, 1982).

The procedure for cloning recombinant pUC8 plasmid vector following ligation of cDNA into the Sma 1 restriction nuclease site is outlined diagramatically in Figure 2. The volume and concentration of the ligation mix were the optimum as previously determined by control experiments to test the efficiency of blunt-end ligation using this vector (performed by Dr M. Ramsden, Beatson Institute, Glasgow). The transformation efficiency obtained when plasmid was transfected into the JM83 strain of E.coli was approximately 1×10^4 colonies/μg, compared to a transformation rate of approximately 1×10^6 colonies per μg for uncut plasmid. Approximately 800 antibiotic-resistant transformants were picked for each of the 2 libraries and were grown

individually in wells in microtitre dishes.

3. Screening of the cDNA Libraries

This was performed initially using the semi-quantitative Grunstein-Hogness technique as outlined in Figure 3. By this means a total of 96 clones were identified representing mRNA species which appeared to differ significantly in abundance when screening of the two libraries was performed using homologous (same tumour) and heterologous (different tumour) probes, the majority of which (75%) were present in the library from the primary tumour which had metastasized and which were absent in the library from the primary tumour which had not. Examples of such clones are illustrated in Figure 4 which shows the results of screening of the replica of one of the microtitre dishes from the metastatic library. The other 25%, conversely, were apparently highly-abundant in the tumour which had not metastasised relative to the one which had. Due to the relative crudity of the Grunstein-Hogness technique, a further round of screening was carried out using more quantitative (but labour-intensive) dot hybridisation techniques using mini-preparations of the 96 selected recombinant clones. These were also screened using homologous and heterologous probes in order to select those sequences which differed most consistently in transcriptional levels between the two tumours. These blots were not retained. On this basis 4 recombinant clones only were selected for bulk preparation of quantifiable amounts of plasmid DNA for use as probes to

screen a series of tumour and other tissue types. These putative metastasis-related clones (PMRC) selected from the original 96, were numbers PMRC1, PMRC7, PMRC35 and PMRC89 and were all selected from the "metastatic" tumour library.

4. Screening of a Series of Tumours and Normal Tissue Samples with ^{32}p-labelled Recombinant DNA Probes (PMRC1,7,35 & 89).

This was performed by RNA doubling-dilution dot hybridisation, the results of which are shown in Figures 5-8, and which represent the best obtained after several rounds of screening; these although individually of variable quality technically were, in general, consistent. This technique allows obvious and sensitive (although subjective) visual comparison to be made between specimens, the range of intensities is exponenial, and also permits numerical quantitation of reactivity and hence abundance of a particular RNA species in a given specimen. ("Cut off" points were also independently assessed by another group member). Quantitation is based on a value of 1 arbitrarily ascribed to the first dot and, subsequently, values of 2,4,8 etc. for subsequent dilutions. The arbitrary hybridisation "cut-off" point is in fact somewhat easier to interpret on the original autoradiographs when trans-illuminated than in the Figures shown.

The complete list of values obtained for a series of specimens including colorectal carcinomas, adenomas, normal

mucosa and other tumour and cultured tissue types is shown in Table 2. It is evident that the range of values obtained extends up to one hundred-fold, and, in a few cases (e.g. specimens 2,10,19), several hundred-fold. Abundance of these RNAs is generally (e.g. PMRC 1) much higher in carcinomas than in normal mucosa where this was available for comparison, as in specimens 20/21 and 22/23, where the difference is approximately ten-fold. Relatively low steady-state transcript levels of these sequences, comparable to that seen in normal mucosa, were observed in the two adenomatous polyps studied.

The numbers (n) of different tissue types falling into different arbitrary categories of RNA abundance for e.g. PMRC1-related sequences shows this overall pattern clearly when tabulated as follows:-

Abundance of PMRC1-related RNA	Colorectal Carcinoma (n)	Adenomatous Polyp (n)	Mucosa (n)
1024 - 128	7	0	0
64 - 32	11	1	3
32 1	0	1	2

Low levels only were seen in the specimens of carcinoma of breast (specimen 29), melanoma (specimen 30) and HL60 promyelocytic leukaemia cell line (specimen 31). Very low steady-state transcript levels only were seen in the specimens of normal tissue (cultured fibroblast specimen 26,

or in white cells specimen 28). (RNA from HL60 leukaemia and from white cells was kindly provided by Jennifer Burns of the Beatson Institute).

Although in general higher values were seen using e.g. PMRC1, it is not possible to compare directly the levels of RNA transcripts seen with the different probes in this series since the reactivity seen depends also on variable factors such as degree of ^{32}P radiolabelling, which in turn depends also on the length of cDNA insert in the respective probes. Similarly, because of the variability in using the same probe on different occasions, use of a constant internal control (e.g. a cell line such as HL60 or preferably of a colorectal carcinoma) would be necessary for any extended series.

Due to limitations of re-using nitrocellulose filters, it was not possible to perform direct quantitative comparisons using other selected "random" probes, as would have been ideal. However, aliquots of the same specimens were separately quantitated by densitometric analysis as previously described (Spandidos and Kerr 1984) and this, together with the generally-consistent pattern of results between probes and amongst specimens, supports the overall accuracy of the original quantitations based on optical densitometry. (See Methods, Section 3).

Thus, transcript levels of these sequences appear to be higher, though variably so, in the specimens of colorectal carcinomas relative to othere tumours or normal tissue

types. The values observed in the samples of mucosa were, however, in several cases also higher than in other tumour or tissue types, suggesting that their transcription may be tissue (i.e. colorectum)-related. It should be noted that where different biopsies of the same tumour were studied as an incidental look at possible tumour cell heterogeneity, broadly similar ranges of value (within 1-2 dilutions) were in fact obtained (e.g. tumours 2/22. 16/17. 19/20). However, some discrepancies did also occur e.g. samples of mucosae 15/21 in general and 16/17 probed with PMRC89.

A few further anomalies were also apparent in interpretation of the hybridisation signals in e.g. Figure 5 where no signal is seen for dilution 1 in samples 12 and 13. This may be due to this aliquot having been accidentally omitted or been subject to pressure by adhesive tape (as appears to have occurred over e.g. specimens 16-18 for dilution 7). In e.g. Figure 8, the signal seen over dilution 3 of sample 4 may have been due to filter irregularity causing probe stain, or to inadvertent deposition of RNA from another aliquot.

Even if these results are excluded from consideration, the overall lack of correlation between abundance of these RNA species to tumour metastatic potential observed in the series of carcinomas (specimens 1-12, Table 3 (a-d) remains clear. (See Section 5 and scattergram in Figure 10.).

5. Correlation of Levels of Steady-State Transcripts of RNA Corresponding to PMRC1,7,35 and 89 with Conventional Staging Criteria and Clinical Outcome in a Series of Colorectal Carconimas

The relation of values obtained for each PMRC with conventional staging criteria and clinical outcome is shown in Table 3 (a-d) and Figure 10. Follow-up on all survivors exceeds 2 years which, although relatively short, is the period during which patients who have undergone apparently-curative resection would be expected to develop disseminated disease if they are going to do so. It is in any case clear from the range of values obtained in specimens of tumours which have already metastasised that they do not appear to be related consistently to metastatic tumour behaviour.

Similarly, the range of values overlaps all Dukes' stages and histological types. They do not, in addition, appear to be related to tumour site. Although the variably-elevated levels of transcripts of these abundant RNA species appear to be cancer-related in colorectal tumours, the degree of elevation of transcript levels for the four sequences does not appear to be related either to conventional staging criteria nor to their clinical behaviour with particular reference to the development of metastatic disease.

6. Correlation of Levels of Steady-State RNA Transcripts of **Ras** Family-Oncogenes with Conventional Staging Criteria and Clinical Outcome in a Series of Colorectal Carcinomas

Specimens of RNA prepared from the same series of tumours was also probed with the recombinant HiHi3 (Ellis et al, 1980) and BS9 (Ellis et al, 1981) probes which contain the viral Kirsten and Harvey **ras** DNA sequences respectively. Variably-elevated steady-state transcript levels of these oncogenes have already been reported by our group (Spandidos and Kerr, 1984), and the level of elevation, relative to an arbitrary value of 1 ascribed to normal mucosa, quantitated by densitometric scanning of single dot hybridisations. The relation of these values to clinical outcome is shown in Table 4 and to Dukes' staging and tumour histology in Figure 9. Although the elevation of steady-state levels of transcripts range from 2-20 fold these appear to overlap in all histological types and Dukes' stages. Similarly, the values obtained do not appear to related to the clinical behaviour of these tumours with particular reference to the development of metastatic disease, on follow-up over a period of more than 2 years. This is evident even from the range of values seen within the group of tumours which have already metastasized although, as discussed above, those which have not metastasized within 2 years would be unlikely to do so.

Thus, although variably elevated steady-state levels of **ras** -related RNA transcripts are seen in these tumours they do not appear to be related to either conventional staging criteria nor to clinical behaviour with particular reference to the development of metastatic disease.

Table 2.

Tumour and tissue specificity of PMRCS 1,7,35,89 hybridised with doubling-dilutions of total cellular RNA.

Specimen Number

(Patient/Specimen)	Probes			
	PMRC1	PMRC7	PMRC35	PMRC89
1. (Ca-PK)	128	128	32	64
2. (Ca-EL)	512	512	256	256
3. (Ca-JF)	64	16	16	8
4. (Ca-HRo)	64	32	16	8
5. (Ca-HM)	64	8	8	-
6. (Ca-HRe)	32	16	8	8
7. (Ca-WP)	64	16	8	4
8. (Ca-MM)	64	512	128	256
9. (Ca-BH)	32	8	2	16
10. (Ca-ASh)	1024	256	128	128
11. (Ca-JG)	64	32	8	16
12. (Ca-DJ)	128	8	8	8
13. (Ca-GC)(A)+	128	64	16	16
14. (Polyp-IM)	1	128	64	64
15. (Mucosa-GC)	1	8	4	8
16. (Ca-MM2)	64	128	64	256
17. (Ca-MM3)	64	32	16	32
18. (Mucosa-MM)	64	4	4	2
19. (Ca-GC2)	256	4	16	8
20. (Ca-GC3)	256	4	16	16
21. (Mucosa-GC)	32	2	4	1
22. (Ca-EL2)	256	256	64	512
23. (Mucosa-EL)	64	8	8	4
24. (Polyp-MB)	32	4	-	1
25. (Mucosa-MB)	16	4	1	4
26. (Fibroblast*)	4	1	-	-
27. (Fibroblast-PC*)	8	1	-	-
28. (Leucocytes)	16	2	2	1
29. (Ca-Breast)	16	4	4	4
30. (Melanoma)	16	16	4	2
31. (Leukaemia-HL60*)	64	16	16	2

Abbreviations - 1,2,3. - refers to different biopsies.
* - cell line.
(A)+ - adjusted value for sample of (A)+ RNA.
PC - Polyposis coli.

Table 3a.

Relation of steady-state transcription levels of RNAs from a series of colorectal carcinomas as measured by hybridisation with PMRC 1 to age, sex, tumour histology, Dukes' staging and clinical outcome.

Patient	Age	Sex	Histology (Differentiation)	Dukes' Stage and Site	Follow-up (in months)	Status	PMRC 1
1. (PK)	52	M	Moderate	C (Sigmoid)	27	N.D.	128
2. (EL)	72	M	Moderate	B (Caecum)	24	N.D.	512
3. (JF)	79	M	Moderate	B (Rectum)	-	O+(post-op)	64
4. (HRo)	54	F	Moderate	B (Sigmoid)	(20)	N.D.+	64
5. (HM)	51	F	Poor	D (Rectum)	(8)	D.D.+	64
6. (HRe)	72	M	Poor	O (Rectum)	(5)	N.D.+	32
7. (WP)	75	M	Moderate	B (Caecum)	36	N.D.	64
8. (MM)	54	F	Moderate	C (Caecum)	(6)	D.D.+	64
9. (BH)	78	F	Moderate	B (Rectum)	-	N.D.+*(post-op)	32
10. (ASh)	67	M	Good	B (Trans.Colon)	(30)	N.D.+	1024
11. (JG)	54	M	Moderate	O (Sigmoid)	(12)	D.D.+	64
12. (DJ)	60	M	Poor	D (o)	(5)	D.D.+	128

1. Status of patients at follow-up or death was assessed and abbreviated as follows:-

N.D. - No evidence of disease.
D.D. - Disseminated.
L.D. - Local.
\+ - Dead.
0 - Not known.
* - Post-mortem assessment.

Table 3b

Relation of steady-state transcription levels of RNAs from a series of colorectal carcinomas as measured by hybridisation with PMRC 7 to age, sex, tumour histology, Dukes' staging and clinical outcome.

Patient	Age	Sex	Histology (Differentiation)	Dukes' Stage and Site	Follow-up (in months)	Status	PMRC 7
1. (PK)	52	M	Moderate	C (Sigmoid)	27	N.D.	128
2. (EL)	72	M	Moderate	B (Caecum)	24	N.D.	512
3. (JF)	79	M	Moderate	B (Rectum)	-	O+(post-op)	16
4. (HRo)	54	F	Moderate	B (Sigmoid)	(20)	N.D.+	32
5. (HM)	51	F	Poor	D (Rectum)	(8)	D.D.+	8
6. (HRe)	72	M	Poor	O (Rectum)	(5)	N.D.+	16
7. (WP)	75	M	Moderate	B (Caecum)	36	N.D.	16
8. (MM)	54	F	Moderate	C (Caecum)	(6)	D.D.+	512
9. (BH)	78	F	Moderate	B (Rectum)	-	N.D.+*(post-op)	8
10. (ASh)	67	M	Good	B (Trans.Colon)	(30)	N.D.+	256
11. (JG)	54	M	Moderate	O (Sigmoid)	(12)	D.D.+	32
12. (DJ)	60	M	Poor	D (o)	(5)	D.D.+	8

1. Status of patients at follow-up or death was assessed and abbreviated as follows:-

N.D. - No evidence of disease.
D.D. - Disseminated.
L.D. - Local.
\+ - Dead.
0 - Not known.
* - Post-mortem assessment.

Table 3c.

Relation of steady-state transcription levels of RNAs from a series of colorectal carcinomas as measured by hybridisation with PMRC 35 to age, sex, tumour histology, Dukes' staging and clinical outcome.

Patient	Age	Sex	Histology (Differentiation)	Dukes' Stage and Site	Follow-up (in months)	Status	PMRC 35
1. (PK)	52	M	Moderate	C (Sigmoid)	27	N.D.	32
2. (EL)	72	M	Moderate	B (Caecum)	24	N.D.	256
3. (JF)	79	M	Moderate	B (Rectum)	-	O+(post-op)	16
4. (HRo)	54	F	Moderate	B (Sigmoid)	(20)	N.D.+	16
5. (HM)	51	F	Poor	D (Rectum)	(8)	D.D.+	8
6. (HRe)	72	M	Poor	O (Rectum)	(5)	N.D.+	8
7. (WP)	75	M	Moderate	B (Caecum)	36	N.D.	8
8. (MM)	54	F	Moderate	C (Caecum)	(6)	D.D.+	128
9. (BH)	78	F	Moderate	B (Rectum)	-	N.D.+*(post-op)	2
10. (ASh)	67	M	Good	B (Trans.Colon)	(30)	N.D.+	128
11. (JG)	54	M	Moderate	O (Sigmoid)	(12)	D.D.+	8
12. (DJ)	60	M	Poor	D (o)	(5)	D.D.+	8

1. Status of patients at follow-up or death was assessed and abbreviated as follows:-

N.D. - No evidence of disease.
D.D. - Disseminated.
L.D. - Local.
\+ - Dead.
O - Not known.
* - Post-mortem assessment.

Table 3d.

Relation of steady-state transcription levels of RNAs from a series of colorectal carcinomas as measured by hybridisation with PMRC 89 to age, sex, tumour histology, Dukes' staging and clinical outcome.

Patient	Age	Sex	Histology (Differentiation)	Dukes' Stage and Site	Follow-up (in months)	Status	PMRC 89
1. (PK)	52	M	Moderate	C (Sigmoid)	27	N.D.	64
2. (EL)	72	M	Moderate	B (Caecum)	24	N.D.	256
3. (JF)	79	M	Moderate	B (Rectum)	-	O+(post-op)	8
4. (HRo)	54	F	Moderate	B (Sigmoid)	(20)	N.D.+	8
5. (HM)	51	F	Poor	D (Rectum)	(8)	D.D.+	-
6. (HRe)	72	M	Poor	O (Rectum)	(5)	N.D.+	8
7. (WP)	75	M	Moderate	B (Caecum)	36	N.D.	4
8. (MM)	54	F	Moderate	C (Caecum)	(6)	D.D.+	256
9. (BH)	78	F	Moderate	B (Rectum)	-	N.D.+*(post-op)	16
10. (ASh)	67	M	Good	B (Trans.Colon)	(30)	N.D.+	128
11. (JG)	54	M	Moderate	O (Sigmoid)	(12)	D.D.+	16
12. (DJ)	60	M	Poor	D (o)	(5)	D.D.+	8

1. Status of patients at follow-up or death was assessed and abbreviated as follows:-

N.D. - No evidence of disease.
D.D. - Disseminated.
L.D. - Local.
+ - Dead.
O - Not known.
* - Post-mortem assessment.

Table 4.

Relation of Ki and Ha-ras oncogene-related steady-state RNA transcription levels to age, sex and clinical outcome in a series of twelve colorectal carcinomas.

Patient	Age	Sex	Follow-up in months	Status	Ki-ras	Ha-ras
1.(PK)	52	M	27	N.D.	6.3	4.5
2.(IM)	83	F	(9)	N.D.+	3.5	14.0
3.(MH)	62	F	(19)	D.D.+	19.0	9.0
4.(ASm)	61	F	33	L.D.	7.0	7.6
5.(DJ)	60	M	(5)	D.D.+	3.5	1.5
6.(MM)	54	F	(6)	D.D.+	6.5	1.5
7.(HP)	81	F	(4)	D.D.+	7.0	1.5
8.(EL)	72	M	24	N.D.	9.0	1.5
9.(WP)	75	M	36	N.D.	5.0	1.5
10.(BH)	78	F	(-)	N.D.+*(post-op)	4.4	1.5
11.(JF)	79	M	(-)	O+	6.5	11.0
12.(HM)	51	F	(8)	D.D.+ (post-op)	1.9	14.0

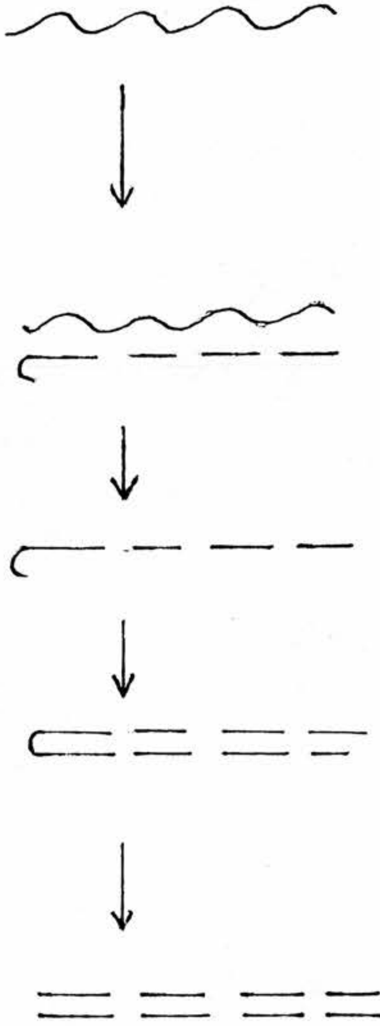
1. Status of patients at follow-up or death was assessed and abbreviated as follows:-

N.D. - No evidence of disease.
D.D. - Disseminated disease.
L.D. - Local disease.
\+ - Dead.
O - Not known.
* - Post-mortem assessment.

Figure 1.

Outline of protocol for Enzymic Synthesis of cDNAs.

RNA from "non-metastatic" tumour. RNA from "metastatic" tumour.



Poly(A)+ RNA

oligo $(dt)_{17}$ -primer Reverse transcriptase

Poly(A)+ RNA
ss cDNA

Boil

DNA Polymerase
("Klenow" Fragment)

S_1 Nuclease
+DNA Polymerase

dscDNAs

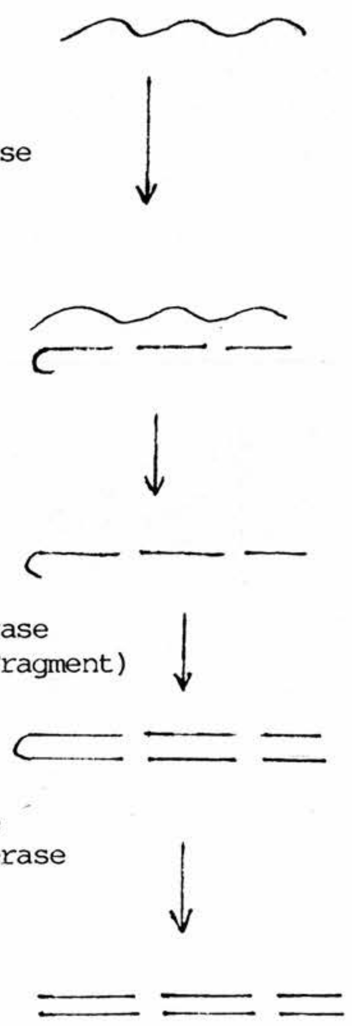


Figure 2.

Outline of transfection and cloning protocol.

CLONING

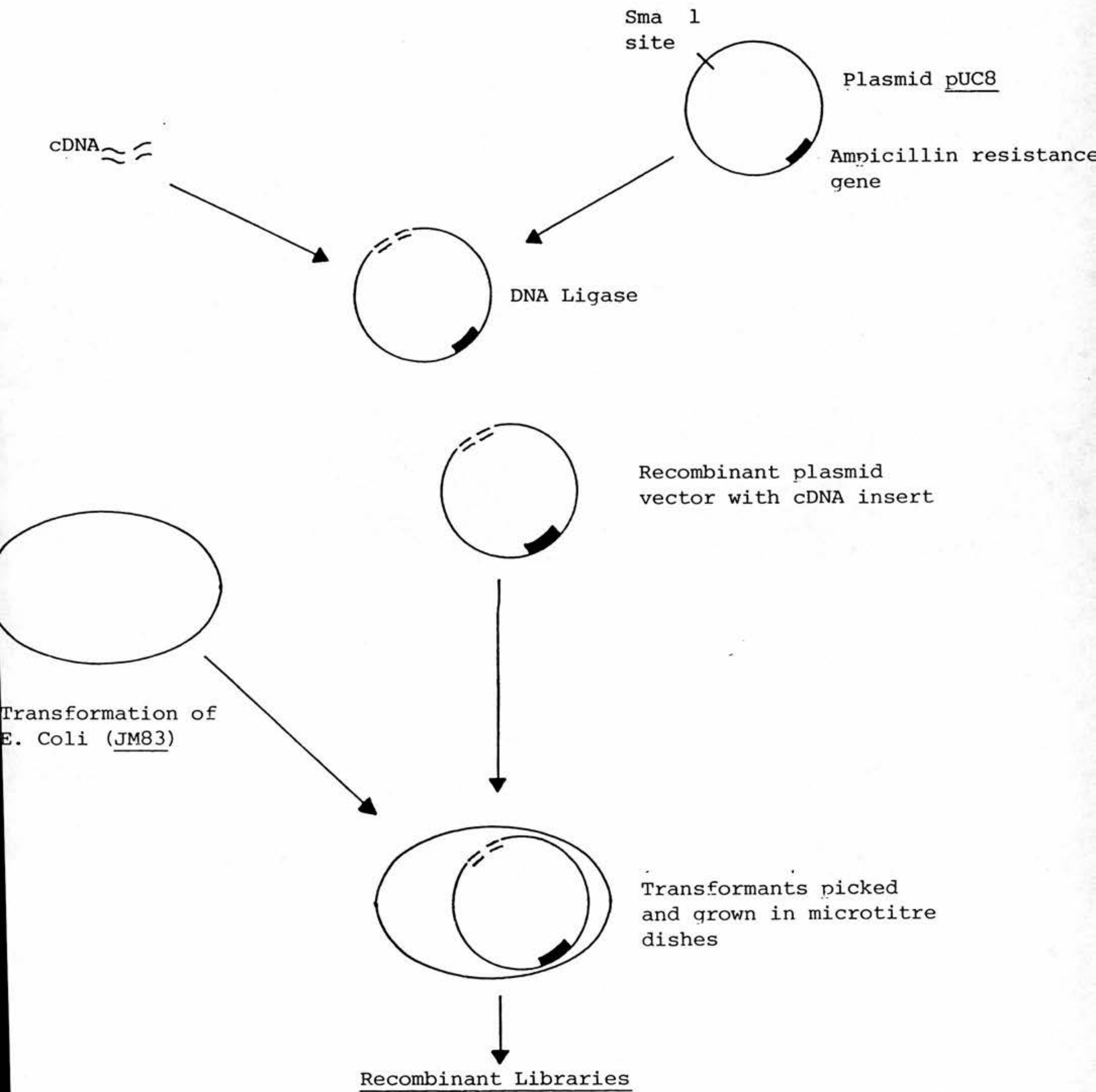


Figure 3.

Outline of protocol for screening a cDNA library using the Grustein-Hogness technique.

SCREENING BY COLONY HYBRIDIZATION

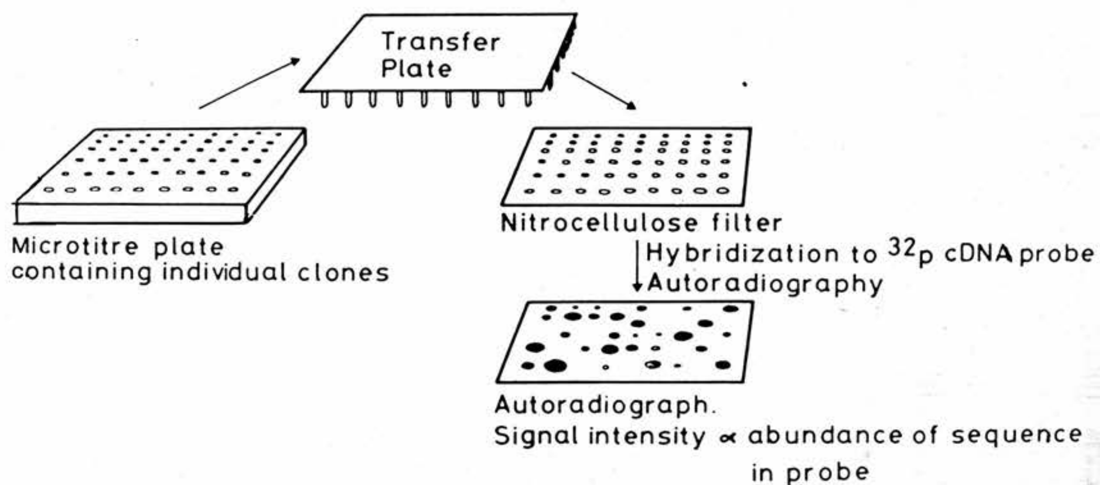
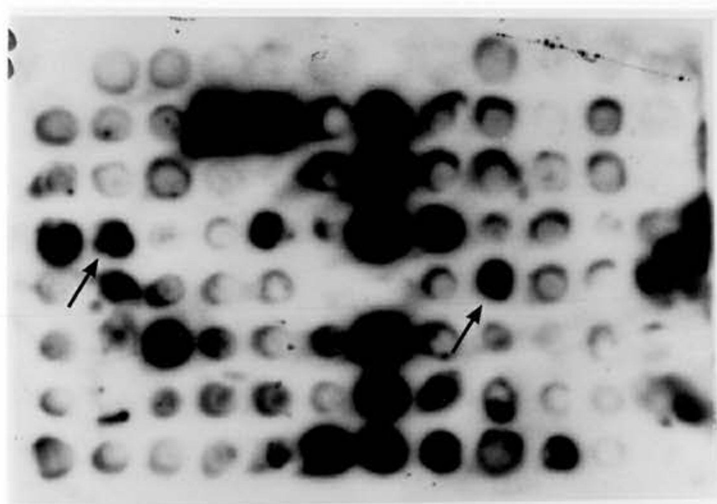


Figure 4.

Example of a plate from the "metastatic" tumour cDNA library screened with probe made from its own RNA (above) and probe made from "non-metastatic" tumour RNA (below) in homologous and heterologous screening respectively. The arrows show examples of clones which were picked due to their differing abundance as shown by a markedly-differing hybridisation signal.

Since the replica filters were screened with probes used on different occasions and labelled to differing extents, it is the relative overall pattern of intensity which is compared. A control is not included since the only relevant one would be RNA homologous to the cDNA used as probe, which the various clones already collectively represent.



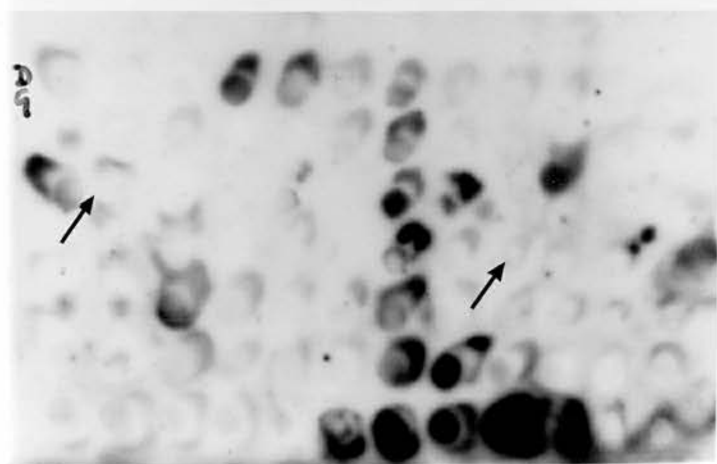
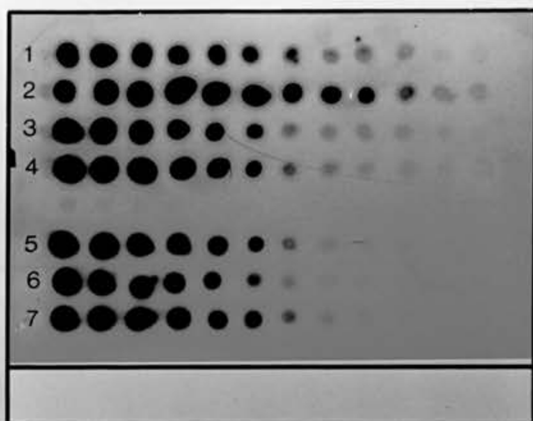
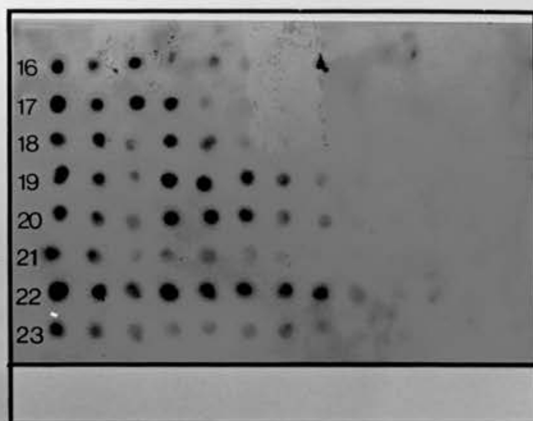
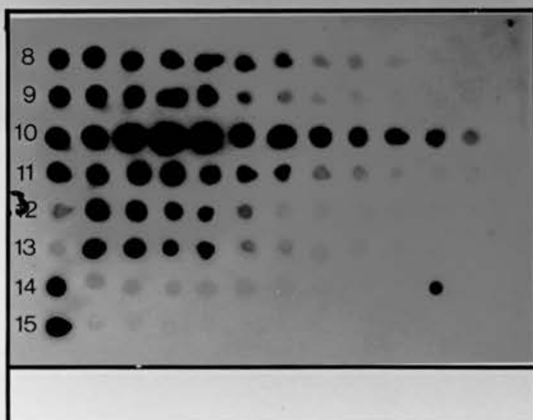


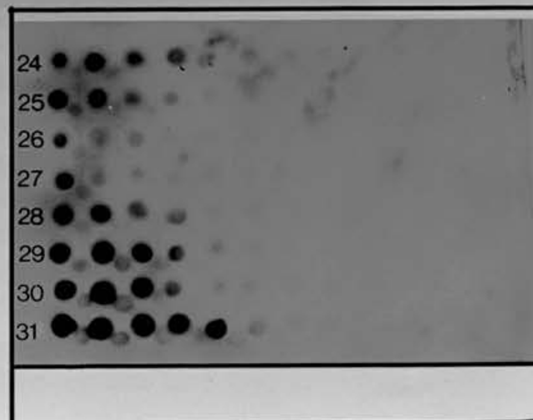
Figure 5.

Results of doubling-dilution RNA dot hybridisation of specimens 1-31 with PMRCI.





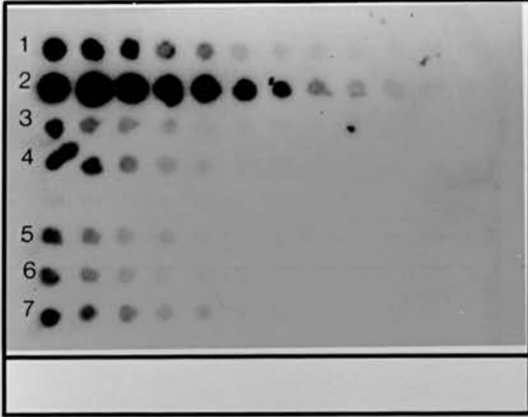




PMRC 1

Figure 6.

Results of doubling-dilution RNA dot hybridsation of specimens 1-15 with PMRC7. (The blot showing specimens 16-31 was accidentally discarded).


1
2
3
4
5
6
7

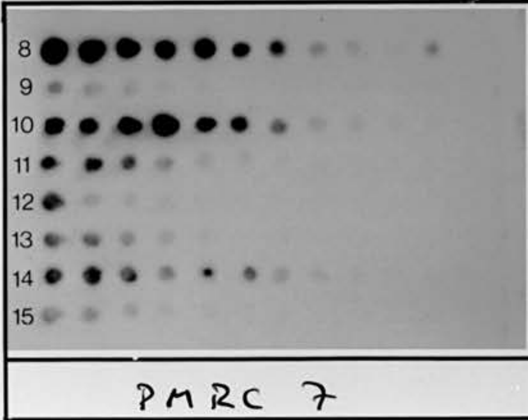
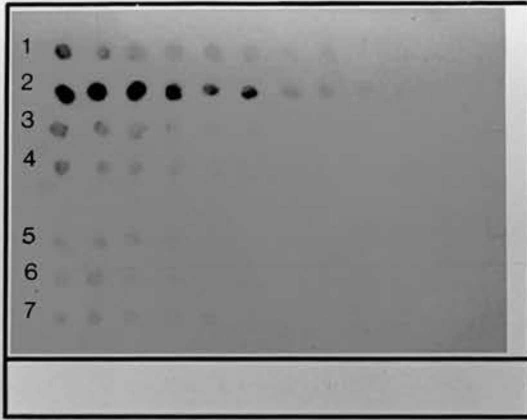
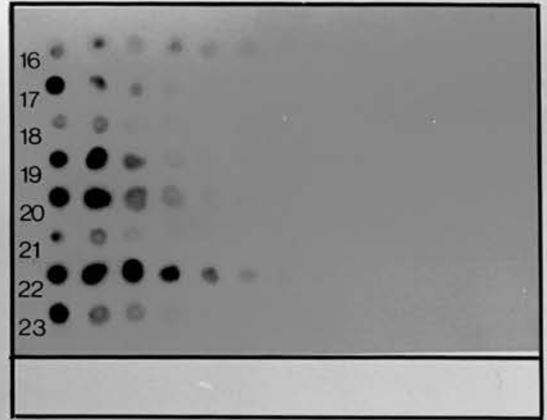
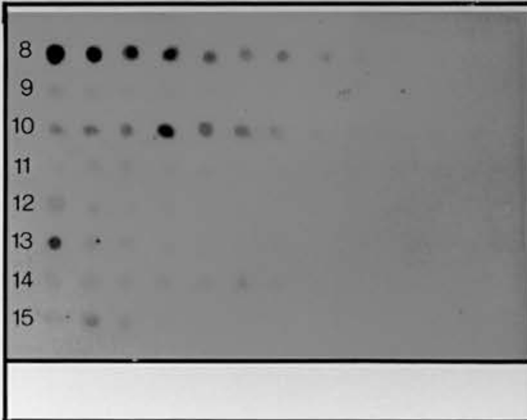

8
9
10
11
12
13
14
15
PMRC 7

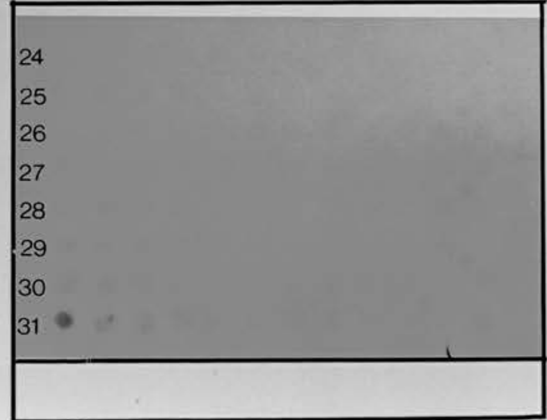
Figure 7.

Results of doubling-dilution RNA dot hybridsation of specimens 1-31 with PRMC35.





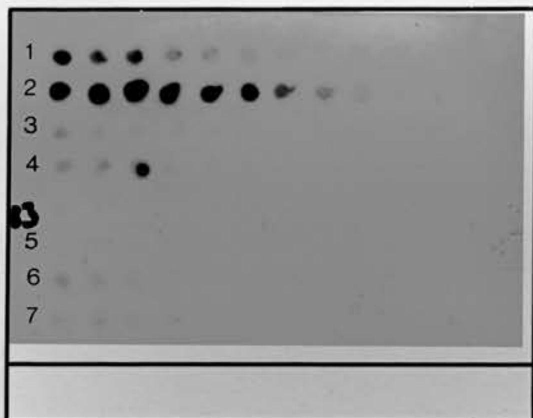


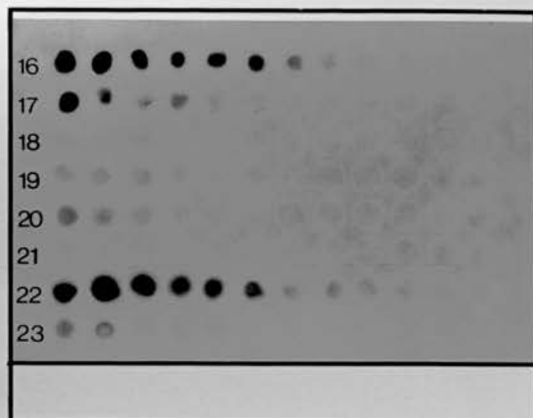


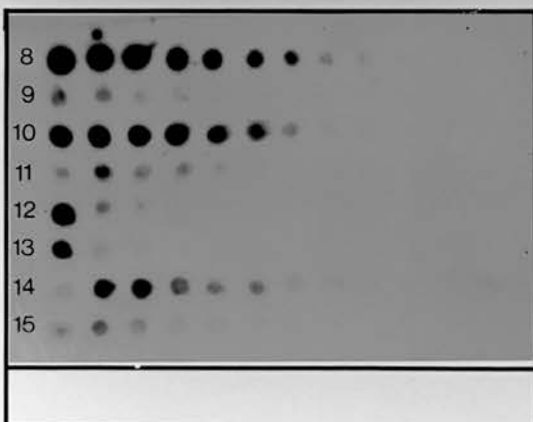
PMRC 35

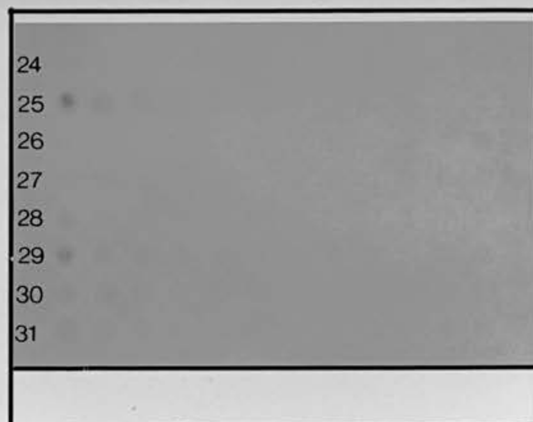
Figure 8.

Results of doubling-dilution RNA dot hybridisation of specimens 1-31 with PMRC89.





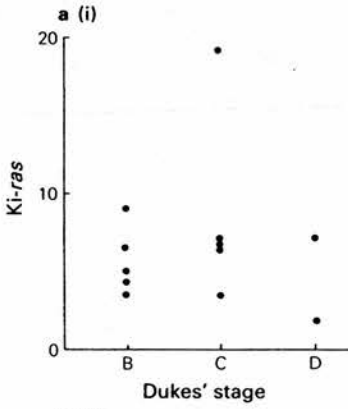


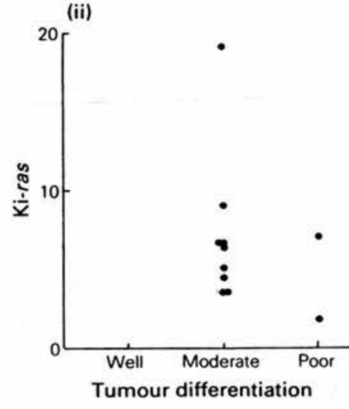


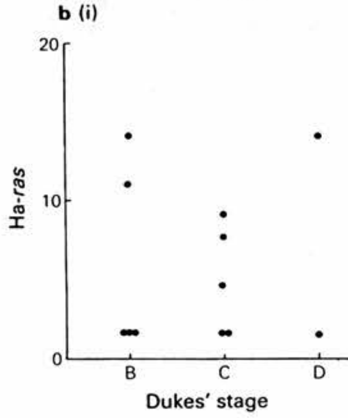
PMRC 89

Figure 9.

Relation of (a) Ki- **ras** and (b) Ha- **ras** related oncogene expression to Dukes' stage (i) and degree of tumour differentiation (ii). In one case histology was not available.


a (i)
Ki-ras
20
10
0
B
C
D
Dukes' stage


(ii)
Ki-ras
20
10
0
Well
Moderate
Poor
Tumour differentiation


b (i)
Ha-ras
20
10
0
B
C
D
Dukes' stage

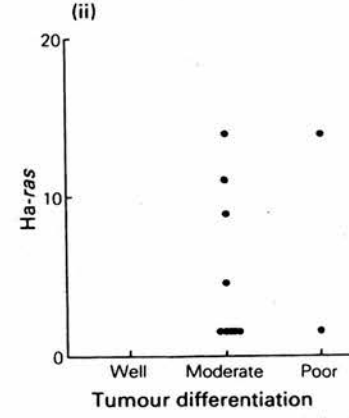
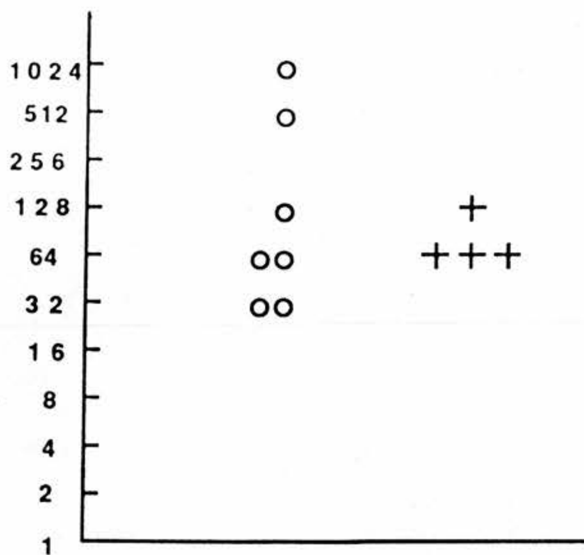
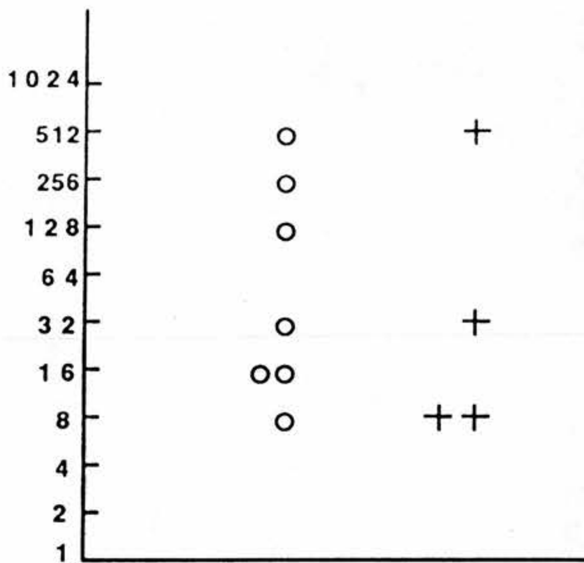

(ii)
Ha-ras
20
10
0
Well
Moderate
Poor
Tumour differentiation

Figure 10

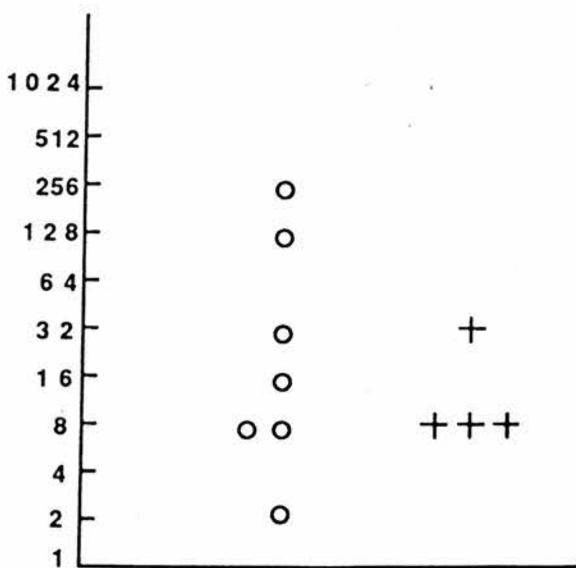
Relation of steady-state transcription levels of
(a) PMRC-1, (b) PMRC-7, (c) PMRC-35 and (d) PMRC-89
to metastasising (+) and non-metastasising (o) tumour
behaviour.



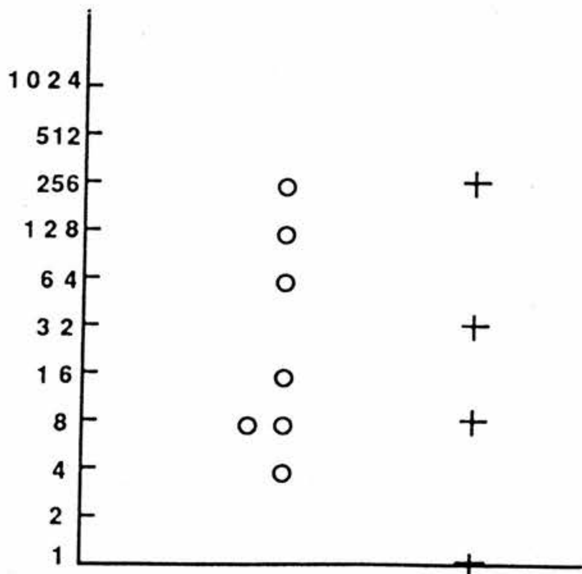
a)



b)



c)



d)

DISCUSSION

DISCUSSION

1. General Considerations

The aim of this study was to identify possibly metastasis-related differences between two primary colorectal carcinomas, which represented clinically tumours one of which had already metastasised and one which had not done so after a follow up period of, now, over 3 years. The strategy employed was to construct cDNA libraries from the moderately-and highly-abundant mRNA species from each tumour and to identify differences between them by screening these libraries using radiolabelled probe made from their RNAs in homologous (same tumour) and heterologous (different tumour) screening. cDNA clones most consistently representing differences between the two were selected and used to determine the steady-state transcription levels of these RNA sequences in a series of tumours as well as in various normal tissues and specimens of different tumour types. The values obtained were then correlated in a series of 12 carcinomas with conventional staging criteria and clinical outcome with particular reference to the development of metastatic disease. It was hoped that the transcription of such sequences might consistently relate to differing behavioural characteristics of these tumours and thus provide a means of studying the molecular pathology of metastasis as well as being of clinical usefulness as a predictive test.

A similar evaluation of the clinical significance of the variable elevation in **ras** family oncogene RNA steady-state transcripts which had been observed in these tumour specimens relative to normal mucosa in conjunction

with Dr. D.A. Spandidos (Beatson Institute, Glasgow), was also undertaken.

As shown in the previous section the four putative metastasis-related recombinant clones isolated from the cDNA libraries appeared to represent mRNA species present in general at elevated levels in a series of carcinomas of the colorectum as compared to normal mucosa and to other tumour and tissue types. Elevated steady-state transcription levels of these sequences did not however appear to be consistently related in the series of carcinomas to either conventional staging criteria (Dukes'stage or degree of tumour differentiation) nor to the clinical behaviour of the tumours with particular reference to the development of metastatic disease. These results suggest that if differing steady-state transcript levels of critical mRNA species do occur in a metastatic phenotype then it may be in RNA species of very low abundance or may be by relatively small differences in transcript levels in abundunt RNA sequences or possibly by a "battery" of changes, perhaps of a subtle nature, in a large number of RNA species. Differences in the former would not be amenable to study in such a cDNA library in which they would not be expected to be either cloned or detectable. Alternatively it is possible that such sequences may be abundantly-present in only a subpopulation of a primary tumour, or perhaps expressed only transiently.

The lack of correlation of degree of elevation of **ras** oncogene transcription with conventional staging criteria or clinical outcome similarly suggests that expression of these sequences is not directly related to clinically-

apparent phenotypic differences in these tumours although abnormal expression of these oncogenes may be, as previously suggested (Spandidos and Kerr, 1984), critical in the generation of malignancy.

In the interpretation of these results and consideration of further strategies to study the molecular pathology of metastasis, various points arise both in consideration of the techniques and strategy employed in the present study as well as of a more general nature.

2. Collection of Tumours and Preparation of RNA

Biopsies of tumour specimens were of a random nature, although in several cases multiple biopsies were taken of a single tumour and the expression of the PMRCs was generally equivalent (see Results, Section 4). The validity of this observation would appear to be confirmed by the generally comparable readings seen in e.g. different samples of normal mucosa. If the hypothesis relating to cell heterogeneity with respect to metastatic potential in primary tumours were indeed true in this tumour type, then such random sampling would not be expected to identify the presence of sequences consistently related to such a cell phenotype, although this hypothesis is currently held by several authors to be extremely contentious (Weiss et al. 1983; Tarin & Price 1979; Alexander 1983 and Giavazzi et al. 1980) (see Introduction, Section 5). Nor is there any direct evidence so far of its applicability to primary human tumours. There is, however, good evidence that these colorectal tumours consistently behave clinically as

metastasising or non-metastasising variants (see Introduction, Section 3). Random sampling of primary tumours, however, unless dealing with fairly gross differences in gene expression within populations of tumour cells, undoubtedly remains the major conceptual restriction to this approach and it is possibly surprising that bigger differences in transcriptional levels of the cloned sequences between biopsies of the same tumours were not seen. Such differences might occur due to e.g. differences in stromal cell admixture, varying tumour cell kinetics or degree of necrosis.

The samples of mucosa, although histologically normal and taken from sites several centimetres distant from tumour, might well have displayed certain cancer-associated "transitional" changes as has been previously well-documented, although the exact significance of such changes is not yet clear (see Williams 1985). It seems unlikely, however, that such minor phenotypic changes would constitute a significant problem in cross-screening RNAs for frankly cancer-/or metastasis-associated sequences. This possibility could in any case be checked if so desired by screening with probe made from RNA from non tumour-bearing mucosa, although such a specimen was not available for this study.

Yields of poly (A)+ RNA per weight of tumour were in general much lower than per comparable weight of tissue culture cells as described in previous studies due not only to lower cell content but also to difficulties in rapidly mincing tissue for treatment with guanidinium thiocyanate (Methods, Section 3) to

prevent excess degradaion of RNA by endogenous nucleases. The difficulty in obtaining intact RNAs from primary tumours is well-recognised (Slamon et al. 1984). This problem was eventually circumvented using the procedure described of pulverising tumour under liquid nitrogen (see Methods, Section 3). Nonetheless, substantial quantities (approximately 1g) of tumour material were required to extract sufficient polyadenylated RNA to clone a cDNA library using the proocol described. This precluded any consideration at the time of using smaller biopsy specimens of either tumour or of metastatic tumour deposits. Recent developments in the technology of synthesising cDNA and its subsequent cloning have, however, made it possible to generate similar-sized libraries using approximately one-tenth as much poly $(A)^+$ RNA (see Gubler & Hoffman, 1983). Such newer methods circumvent the relative inefficiency of the procedures described in this study by greatly reducing the number of steps taken in undertaking the various enzymic reactions.

3. Construction and Screening of cDNA Libraries

The cloning strategy employed to attempt to identify phenotypic differences between 2 primary colorectal tumours, one of which had metastasised and the other of which had not, is outlined in the Results, Section 2 and Figures 1 & 2. Such an approach has been made possible by the recent developments in methods of transfection of *E.coli* with recombinant cDNA plasmids and of methods for screening the transformed colonies generated, each of which contains a cloned DNA copy

representing one of the large number of diverse mRNA species. This depends on the use of a variety of enzymes to synthesise cDNA and to ligate them into a plasmid cloning site generated by use of restriction endonuclease enzymes and the identification of bacterial colonies subsequently transformed by these plasmids which confer on their host particular phenotypic characteristics (in this case ampicillin antibiotic resistance and the inability to produce beta galactosidase). Various procedures may be used for cloning and in this instance "blunt-end" ligation into the Sma I site (rather than the possibly more efficient procedure of using linkers) was used, since it affords the considerable advantage of easy excision of cloned inserts (for e.g. subsequent sequencing) using the flanking EcoR1 and BamH1 restriction sites (see Materials Section 3). Use of mutant, scarcely-viable bacterial host strains (in this case the E.coli JM83 strain of the K12 mutant) ensures compliance with the strict safety regulations surrounding such work. The large number of colonies (800 in this study) generated by this approach, collectively termed libraries, represents a random selection of the RNA species present in the population from which they were cloned. The 800 clones picked represented the maximum number which could easily be screened simultaneously using the methods described. Although many of the recombinants will contain only partial

copies of the RNAs this is immaterial to the purposes of identification and quantitation of the levels of transcription of these sequences. Since as full a cross-section as possible of RNA species was desired no selection procedure, e.g. size fractionation of the cDNAs, was carried out.

Although this strategy represents a very rewarding, if complex, means of studying differences in the abundant RNAs between different cell phenotypes, there are limitations imposed both in terms of the number and nature of individual RNA species which may be studied. Thus, although a library of the size described above would be expected to contain the majority of the highly-/and moderately-abundant sequences (some several hundred) the remainder of the cell RNAs (some 10,000 or so, see Introduction), transcribed at only a few copies per cell, (i.e. 0.1% of the population of RNAs) would have only a small fraction of its total represented. The size of library required to represent a whole range of cell RNAs may be calculated by the formula of Clark and Charbon. (See Williams, 1981 and Maniatis et al, 1982). Thus in the case of a cultured fibroblast for example, it has been calculated that N = 37,000 (P = 0.99) (where N = number of clones and P = probability). For a P value of 0.90 however, the number would be reduced drastically to about 7,000. It is clear therefore that to generate libraries representing all of a cellular RNA population, libraries of much greater size would be required. However, more severe limitations are imposed using these procedures on the identification of sequences of interest using primary screening procedures

such as the Grunstein-Hogness technique (see Methods, Section 14). Due to the very low copy number of such sequences present also in the radiolabelled probe used, such a low "signal to noise" ratio is obtained as to render impossible the detection of such sequences when exposed autoradiographically. The Grunstein-Hogness technique is furthermore only semiquantitative and rather unreliable since the dot intensity obtained in screening depends on a variety of factors including the extent of bacterial colony growth, degree of lysis, length of cDNA insert and degree of radiolabelling of the probe used. It has been calculated that the limit of sensitivity of this technique is of the order only of a 5 fold difference (Williams, 1981) which in many biological situations would represent a gross comparison. Indeed, of the 96 clones picked and grown in "minipreps" for subsequent secondary screening using quantitative dot hybridisation on the basis of the initial Grunstein-Hogness screening, the majority did not consistently differ on further cross-screening (see Results, Section 3). It is correspondingly likely that many clones representing sequences which did differ to a biologically significant extent in their levels of steady-state transcription would not have been identified using these screening procedures.

One extremely labour-intensive strategy which might be used to deal with these problems would be to make plasmid preparations of entire libraries for use in quantitative dot

blots. A more sophisticated alternative would be the isolation of sequences of interest by the use of subtractive hybridisation procedures on the two RNA populations of interest (See e.g. Scott et al. 1983 and p117.)

Total plasmid recombinant probes were used for screening the series of specimens, since it was felt that the theoretical problem that variation in levels of bacterial contamination, together with variation in levels of transcription of homologous DNAs in that population, might contribute to significant masking of the predominant (tumour) RNA species was extremely remote.

This study has therefore addressed itself to a study of mRNA sequences differing greatly in steady-state transcript levels in the different tumours. This limitation has been a major obstacle to the study of RNA transcription in a variety of other biological systems in which this may differ by only small amounts in different cell types or at different times. Nonetheless, a considerable amount of useful information may be expected to be gained using these techniques despite these limitations (see Introduction, Section 10).

4. Significance of Transcription of RNA Sequences Related to PMRCs1,7,35 and 89

Although the results of the dot hybridisations shown in Figures 1 - 4 and referred to in Tables 2 & 3 are of a relatively preliminary and technically imperfect nature, it is nonetheless clear that, taking into account the comments made regarding quantitation of specimens and a few individual

anomalies apparent in Figures 1 - 4 (see Results Section 4), PMRC1,7,35 and 89-related RNAs were not apparently confined to those primary tumours which metastasised. These sequences were in general more highly abundant in carcinomas than in corresponding premalignant or normal colorectal tissue or other tumour and tissue types. The significance of this is not clear. It seems unlikely that the differences observed between the colorectal carcinomas and mucosa can be attributed to differences in cell growth kinetics. Recent studies using stathmokinetic techniques (Pritchett et al. 1982) have suggested that cell birth-rate was only very slightly increased in the tumours, whilst the cell turnover-time was actually longer (Bleiberg and Galand, 1976). The finding of such tumour-associated variation in transcription of numbers of RNA sequences is, however, certainly consistent with other studies using similar approaches in e.g. experimental mouse colonic carcinoma (Augenlicht & Kobrin, 1982) and human leukaemias (Weidemann et al. 1983). These sequences may encode proteins which may be critical to the malignant phenotype or may simply represent genetic loci randomly and apparently inappropriately activated in malignancy (e.g. in this tumour type embryonic genes such as carcinoembryonic antigen). If so desired, further study of such sequences in clones of interest would be made possible by bulk isolation of recombinant plasmid DNA to study the size of

the transcripts by Northern Blotting techniques (Maniatis, 1982) to identify genomic clones where available (Maniatis et al, 1982) and for use as radiolabelled probes for in in situ hybridisation to permit both cellular and chromosomal localisation of these genes and the site of their transcription in vivo (Hafen et al, 1983; Cox et al, 1984). The nucleotide sequence of these clones may be determined (Sanger et al, 1977) and used to determine the primary protein structure encoded and thus its identification if the protein is already known. Such studies are not planned in the meantime since these recombinant clones are not apparently related to the object of the study, (i.e. characterisation of a possible metastatic phenotype), although they are clearly of some interest as cancer-associated sequences.

5. Significance of Transcription of **Ras** -Family Oncogenes

Although some elevation in steady-state transcription levels of **ras** -related oncogene RNA was observed in all these tumours the degree of elevation did not appear to be related to clinically-apparent phenotypic variation. As discussed above in the context of the PMRCs study of random biopsies would mean that if critical differences in tumour cell sub-populations did exist then these would not be detected. In the context of **ras** oncogene expression in these tumours we (Kerr et al, 1985) and others (Williams et al, 1985) have however demonstrated widespread positive staining of **ras** oncogene p21 by immunocytochemical means. These findings are consistent with a more recently published study showing similar elevations of **ras** oncogene p21 protein levels in these tumours which could not be

correlated exactly with conventional staging criteria. Study of metastatic tumour deposits however, showed, interestingly, that they contained relatively low levels of p21 (Gallick et al, 1985). A previous immunocytochemical study of p21 expression had shown variable reactivity in formalin-fixed paraffin-embedded sections of tumours and had suggested that this was related to more aggressive behaviour (Thor et al, 1984). The reasons for the discrepancy in reactivity seen in these studies is discussed elsewhere (Kerr et al, 1985). Our own findings have since been confirmed by subsequent reports in breast tumours (Ghosh et al, 1986), using the anti-p21 antibody described by Thor et al.

For reasons discussed in the preceding section it does not seem likely that the differences observed were in any way related to differences in cell-growth kinetics between the carcinomas and normal mucosa.

Although activated **ras** oncogenes have been demonstrated in a wide variety of human tumours (Balmain, 1985) it is not yet clear in general at what stage this occurs. **Ras** activation has been shown experimentally in mouse skin papillomas as well as carcinomas (Balmain et al, 1984) and in a more aggressive late tumour variant of a mouse lymphoma (Vousden and Marshall, 1984). Variation in the levels of transcription of various oncogenes, including the **ras** family, has now been shown in a wide range of tumours e.g. Slamon et al, 1984; Tatosyan et al, 1985) although it is not yet clear in the majority of cases what significance this might have in the context of the clinical behaviour of these tumours. A recent study of the

significance of elevated c- **myc** transcript levels in colorectal tumours has similarly failed to demonstrate any correlation with conventional staging criteria (Rothberg et al, 1985). In a previously cited study, however, it was noted, interestingly, that several specimens of metastatic tumour (although not in colorectal carcinoma) showed elevated levels of c- **sis** transcription (Tatosyan et al, 1985). A study of **ras** transcription in (B16) experimental murine melanoma cell lines of differing metastatic potential also failed to demonstrate differences in levels of **ras** oncogene transcription (Kris et al, 1985). It has, however, been shown that amplification of the N- **myc** oncogene is related to tumour stage in neuroblastoma (Brodeur et al, 1984) and a study of c- **myc** expression in a series of human leukaemias showed increased levels of transcripts to be apparently related to a more immature phenotype (Birnie et al, 1986). In a related study, we (Spandidos and Kerr, 1984) have shown elevated steady-state levels of transcripts of **ras** family oncogenes in pre-malignant as well as in malignant tumours of the colorectum as compared to normal mucosa and have hypothesised that activation of these genes at a premalignant stage might be critical in the process of carcinogenesis but not in itself sufficient. The results of the present study of the clinical significance of this variation would certainly not be inconsistent with such a hypothesis. It may therefore be that activation of a variety of other gene loci, possibly associated with the well-recognised genomic instability in cancer, (Nowell, 1976) are more directly involved in the generation of a frankly-malignant phenotype

and its variants. Since Ha- **ras** activation has now also been described in colorectal carcinoma (Greenhalgh and Kinsella, 1985) it may also be that variation in the type of **ras** oncogene activated where this occurs, or variation in the nature of the mutations around codons 12 and 61 (see Introduction, Section 12) may be significant in this context. In a series of urothelial tumours recently reported however (Fujita et al, 1984), neither the presence of atransforming **ras** oncogene in DNA in a small percentage of cases nor the absence or presence of a documented mutation in them appeared to be related to tumour stage. The presently-existing evidence would therefore seem to suggest that activation of **ras** oncogenes may be a relatively early event in colorectal carcinogenesis and that expression of high levels of **ras** p21 certainly does not appear to be necessary for a metastatic phenotype.

6. Future Investigations

The most obvious next step is to proceed to the cloning of a cDNA library from a metastatic tumour deposit itself, and a preliminary report by our group of the results of such an approach has already been made (Elvin et al, 1986). Developments in the techniques of cDNA synthesis in a greatly-reduced number of steps have made it possible to work with very much smaller amounts of polyadenylated RNA. This strategy in addition obviates the problem raised by the possibility that cells of metastatic potential may exist as only a subpopulation of a primary tumour. Identification of cDNA clones possibly associated with a metastatic phenotype would in addition make it possible to address the question of metastatic tumour cell

heterogeneity in primary tumours by using probes prepared from such clones for in situ hybridisation of tissue sections (see Hafen et al & Cox et al, 1984). It does remain possible of course that such changes observed in a metastatic tumour deposit might be induced by local tissue factors or be a result of fusion with host cells as has been described in experimental systems at least (Kerbel et al, 1983), although so far there is no evidence for this being an occurrence in human cancer. In addition, recent advances in cloning technology are making it possible to study biologically-significant RNA populations possibly present at only low abundance. Cloning vectors have been described which permit the direct selection of such cDNA clones. This approach uses single-stranded DNA packaged into phage particles which provides the larger amount of cDNA required to drive the hybridisation reaction (Kowalski et al, 1985). An alternative approach has been described to detect such low-abundance mRNAs using a probe from which common mRNAs from differing cell populations are removed by the use of plasmid DNA coupled to cellulose to provide a reusable preparative hybridisation probe capable of removing cell sequences present in common (Scott et al, 1983). This approach requires however the use of large amounts of RNA and it is not entirely clear how accurate or reproducable this technique will be. Such an approach to low-copy number sequences has, however, been used to clone the T cell receptor (Hedrick et al, 1985), although in that case a partial probe was already available.

The latter method in particular, dealing as it would with very large numbers of low-abundance RNAs, would be extremely labour-intensive. However, if no obvious differences between the transcription of the highly-abundant RNA species in metastases and non-metastatic tumour are apparent then it would obviously be necessary to consider such alternative strategies.

Approaches other than the use of cDNA libraries may well also prove to be rewarding. These would include consideration of the use of biological transfection assays analagous to those used to detect activated oncogenes in human tumour DNA (see Introduction, Section 12). Preliminary attempts at such an assay using mouse epidermal cells in culture transfected by an activated Ha-ras oncogene have already been undertaken using DNA from a series of metastatic tumour specimens in conjunction with Dr. A. Balmain (Beatson Institute, Glasgow). This cell line is locally invasive when injected subcutaneously into syngeneic mice but does not metastasise. It is hoped that transfection of tumour DNA from metastases from these and other tumours might confer metastatic capability on these cells following transfection. However, assay of their growth and behaviour in mice has so far proved unrewarding. Such approaches will undoubtedly depend on the use of very carefully-defined model systems. One report of the preliminary characterisation of a gene isolated by such an approach from a cervical carcinoma cell-line and apparently capable of conferring a metastatic phenotype has already been made (Bernstein and Weinberg 1985).

Both of the approaches discussed above attempt to define activation of genes, possibly previously uncharacterised, in the process of metastasis either by empirical correlation based on clinical behaviour or by biological assays.

A wide range of cloned gene probes is, however, now becoming available and it is possible that a judicious selection from these in study of metastatic tumour cells would reveal useful information in relation to variation in the behaviour of malignant cells. These include most obviously the list of oncogenes (see Introduction, Section 12) and it is to be anticipated that a more full documentation of their role in both carcinogenesis and variation in the clinical behaviour of tumours will generate information of biological interest and clinical usefulness, as discussed in the preceeding section. Study of the role of growth factors and their receptors in tumours, overlapping as it does with the role of oncogenes, will also undoubtedly yield information of considerable usefulness. It has for example recently been shown that the level of expression of epidermal growth factor (EGF) receptors in a series of primary breast carcinomas is related to their aggression (Sainsbury, 1985).

Study of the role of murine H-2 (MHC) determinants by transfection of H-2 genes has shown in addition this to be associated with the abrogation, in vitro at least, of the metastatic properties of a tumour cell-line (Wallich et al, 1985).

Such studies will proceed not only by evaluation of levels of transcription of these genes, but also of their

expression at the protein level by methods such as immuno-cytochemistry where suitable antibodies are available. Clearly the list of gene products of possible relevance extends considerably and includes for example various structural and immunological determinants of cell behaviour and if such studies are not to become unmanageable in size would clearly require to be rigorously selective.

7. Conclusions

If, as seems reasonable to assume, the ability of a tumour cell to metastasize is related to its phenotypic characteristics then it may be expected that study of those determinants at the level of gene transcription by the methods described above will ultimately yield information of considerable usefulness. Although there are, as discussed above, limitations, rapidly diminishing, to the techniques employed it appears from the initial part of this study that variation in the metastatic behaviour of a series of colorectal carcinomas can not be correlated with single major differences in gene expression as determined using the series of (PMRC) probes isolated and observed in random biopsies.

Similarly, variation in steady-state levels of **ras** family oncogene RNA transcripts in these biopsies do not appear to be related to variation in the clinical behaviour of these tumours, although **ras** oncogene activation has been implicated in the genesis of colorectal carcinoma.

Nonetheless, it is to be anticipated that further such studies of gene expression in metastatic tumours using similar techniques and refinements of them to study changes

in transcription of, possibly large, numbers of low-abundance RNA species will contribute valuable insights into the molecular pathology of metastasis.

This is particularly the case since such study may describe expression of previously uncharacterised genes and represents a significant advantage of the use of such techniques.

As well as being of biological interest study of such gene transcripts and their nucleotide sequences as well as their encoded peptide gene products would permit for example production of highly-specific immune reagents to them (Vitteta et al, 1983). These could have considerable diagnostic value in, for example, predictive clinical tests or in study of tumour progression and of the possible existence of metastatic cell sub-populations, as well as, possibly, therapeutic potential.

The importance of new approaches to these problems continues to be stressed in recent literature concerning e.g. tumour staging in general (Henson, 1985), as well as in the context of adjuvant local (Taylor et al, 1985) and systemic (Gastro-Intestinal Study Group 1985) therapy for colorectal cancer, in which the overall incidence and significance of "occult" metastatic disease has been confirmed by more recent studies (Leveson et al, 1985).

It is also to be anticipated that the numerous on-going studies, such as that described above, of the clinical significance of the activation and expression of cellular oncogenes and related genes such as those encoding growth factors and their receptors will contribute information of both diagnostic and possibly therapeutic value.

It is thus to be hoped that such approaches will shortly be contributing to the beginnings of an understanding of the molecular pathology of the still very poorly understood but enormously-important phenomenon of cancer metastasis.

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Nature, 287: 491-499.

APPENDIX

Isolation of putative metastasis-related cDNA clones from colorectal cancer

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We have shown that prognosis following "curative" resection for colorectal cancer is principally determined by the presence or absence of occult disseminated disease at surgery (1). Studies of rates of growth of metastases suggest that the capacity to metastasise may be due to inherent differences between tumours (2). This study aims to utilise novel technology to characterise colorectal cancer by cloning complementary DNA (cDNA) libraries representing their abundantly expressed mRNAs.

cDNAs have been transcribed from the polyadenylated RNA prepared from two colorectal cancers, one from a patient with disseminated disease and the other from a patient who remains apparently disease-free. These were ligated into the plasmid pUC8 which was used to transform E coli JM 83. Approximately 1000 recombinant clones were picked from each transformation and homologous and heterologous screeni was performed using radio-labelled probes.

Eighty four clones which showed differences between the two libraries have bee identified. These clones are now being used to screen a series of tumours to ascertain whether these findings may be consistently correlated with clinical behaviour.

These cloned probes may have considerable potential in defining clinical behaviour and in predicting the presence of occult disseminated disease in patients with colorectal cancer.

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British Journal of Surgery (1983), 70 : 486.
(Surgical Research Society Abstract)

CLINICOPATHOLOGICAL SIGNIFICANCE AND CELLULAR DISTRIBUTION OF ras FAMILY ONCOGENE EXPRESSION IN NORMAL AND NEOPLASTIC COLORECTUM

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We have previously demonstrated a variably elevated expression of ras family oncogenes in a series of polyps and carcinomata (n = 12) of the colorectum and have now correlated this with conventional staging criteria (Dukes' staging and tumour differentiation) and clinical outcome with particular reference to the development of metastatic disease. No relationship was evident between these parameters suggesting that although abnormal expression of ras oncogenes may be critical in the development of malignancy, variations in the level of their expression do not appear to be related to clinically evident phenotypic differences. Study of the distribution of the ras encoded p21 protein using the monoclonal antibody Y13-259 shows this protein to be apparently homogeneously distributed in all cells in both adenomatous polyps and carcinomata and throughout the normal foetal and adult colorectal mucosal population. This suggests that the presence of this protein is a feature of normal cellular metabolism in certain cell types and is not restricted to those involved in cell proliferation. It thus appears that neither cells at different stages of carcinogenesis nor variants of a malignant phenotype can be identified by these means.

Journal of Pathology (1985), 145 : 98.
(Pathological Society Abstract)

Br. J. Cancer (1984), 49, 681–688

Elevated expression of the human *ras* oncogene family in premalignant and malignant tumours of the colorectum

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Summary Study of expression of *ras*-related oncogenes in human premalignant polyps and malignant tumours of the colorectum, as well as in normal colorectal mucosa, shows a significant elevation in the premalignant and malignant tissues as compared to their respective colorectal mucosa. These results suggest that activation of the *ras* oncogene family occurs in the development of colorectal tumours and that elevated expression at a premalignant stage may well be critical in the process of carcinogenesis but not in itself sufficient.

Analysis of colorectal cancer at the molecular level has been stimulated by the finding that activated cellular oncogenes capable of transforming NIH3T3 mouse cells are present in adenocarcinomata of the colon (Pulciani *et al.*, 1982) and in established cell lines derived from colorectal adenocarcinomata (McCoy *et al.*, 1983). Although expression of cellular oncogenes analogous to retroviral *onc* genes has been studied in fresh and culture derived human haematopoietic neoplastic cell types at various stages of differentiation and a variety of human cell lines (Westin *et al.*, 1982a, b; Eva *et al.*, 1982), similar studies on human solid tissues have not been reported. In this study we quantified the RNA transcripts from the Ki-*ras*, Ha-*ras* and *sis* human oncogene families from a series of premalignant adenomatous polyps and malignant tumours of the colorectum, normal colorectal mucosae and various established cell lines. We wished in particular to determine whether there are significant variations in the level of expression of these genes in tumours of the colorectum and in their postulated premalignant state as compared to normal colorectal mucosa. Adenomatous polyps of the large bowel are now generally thought to represent premalignant lesions with a potential for malignant change over a period of 10–15 years varying from 5% for the predominantly tubular variety to nearer 40% for the predominantly villous (Morson, 1974). Interestingly, one of the specimens was reported as being a metaplastic polyp, which is not generally thought to be premalignant, although this is disputed (Jass, 1983; Rognum & Brandtzaeg, 1983). Our study included representatives of all the major histological types. None of them came from patients known to be suffering from any familial syndrome.

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Received 26 January 1984; accepted 13 March 1984.

Materials and methods

Tissue specimens were collected and stored in liquid nitrogen. These were subsequently pulverized under liquid nitrogen and RNA and DNA was extracted as previously described (Spandidos & Paul, 1982). Briefly, the tissue or cells were homogenized in guanidine-HCl buffer (8.0 M guanidine HCl, 20 mM sodium acetate, 50 mM EDTA, 5% β-mercaptoethanol, pH 7.0). Cell lysates were made 2% with SDS and heated at 65°C for 2 min. After vortexing, 5 ml of cell lysate were placed on a 3 ml cushion of CsCl solution (5.7 M CsCl, 50 mM EDTA pH 8.0) and centrifuged for 48 h at 40K rpm at 15°C in a 10×10Ti rotor. The RNA pellet was resuspended in 2.0 M $LiCl_2$, 4.0 M urea and left at 4°C overnight. RNA was pelleted at 10K rpm for 15 min in a Sorvall centrifuge, resuspended in $0.1 \times$ MOPS buffer ($1 \times$ MOPS = 20 mM NaMOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0) and dialyzed in the same buffer for 2 h before lyophilization. Before each experiment the quality of RNA preparations was examined by formaldehyde-agarose gel electrophoresis, followed by ethidium bromide staining, transfer to nitrocellulose and hybridization to DNA probes (see below). Ten μg of total cell RNA was spotted per dot as described (Spandidos *et al.*, 1981). Hybridizations were performed in $5 \times$ SSC, 50% formamide for 24 h at 42°C with 10 ng ml^{-1} probe as described (Wahl *et al.*, 1979) using $2 \times$ Denhardt's solution (Denhardt, 1966). ^{32}P-labelled DNA probes with specific activities of $2–3 \times 10^8$ cpm μg^{-1} DNA were made by nick-translation (Rigby *et al.*, 1977). The nitrocellulose sheets were washed in $0.5 \times$ SSC at 60°C and exposed to hypersensitized X-ray films at −70°C (Lasky & Mills, 1977). The filters were hybridized sequentially with ^{32}P-labelled nick-translated HiHi3 (Ellis *et al.*, 1981), BS9 (Ellis *et al.*, 1980), pL335 (Dalla Favera *et al.*, 1981), pHR28 (Sproul & Birnie, unpublished results) or pAM91 (Minty *et al.*, 1982) recombinant probes carrying the viral Kirsten *ras* (v-Ki-*ras*), viral Harvey *ras* (v-

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Ha-*ras*), human cellular *sis* (H-c-*sis*) and human 28S ribosomal and mouse actin DNA sequences respectively. Probes were removed by washing the nitrocellulose at 65°C with double distilled H_2O for 2 h. Approximately 100 pg of each insert oncogene DNA were spotted as a positive control. Fractionation of RNA in formaldehyde containing agarose gels and blotting on to nitrocellulose have been described elsewhere (Spandidos & Paul, 1982).

Results

RNA spot hybridization analysis

The relative levels of human Ki-*ras*, Ha-*ras* and *sis* transcripts in total cell RNA made from premalignant and malignant tissue, normal colonic mucosa and cell lines were determined using an RNA spot hybridization assay (Spandidos *et al*., 1981). Quantification of the intensities of the autoradiographic spots was carried out using densitometric scanning as previously described (Spandidos *et al*., 1981). Probe excess was confirmed by obtaining a linear autoradiographic response to serial dilutions of the various RNAs (data not shown). Results of the RNA spot hybridization analysis are shown in Figure 1 and Table I. These show firstly that transcripts from the human Ki-*ras* and Ha-*ras* related oncogenes could easily be detected in most premalignant, malignant tissues and cell line RNAs but are barely detectable in normal tissue. The human *sis* oncogene is expressed at very low levels in all types of tissue examined. Secondly, the amount of human Ki-*ras* and Ha-*ras* specific RNAs varied in different cells and tissues whereas little variation was observed in *sis* RNA levels. In particular, in the first three patients where samples were available from all three types of tissue, Ki-*ras* RNA levels in premalignant and malignant tissues varied between 9.5–20 and 3.5–19 × higher respectively than the levels seen in normal colorectal mucosa. A slightly different picture was seen when expression of the Ha-*ras* oncogene family

Table I Expression of human c-Ki-*ras* and c-Ha-*ras* oncogenes in solid tumours, normal tissue and cell lines studied by RNA spot hybridization analysis.[a]

Patient no. and cell line[b]	*Tissue histology*					
	Normal[c]		*Premalignant*[d]		*Malignant*[e]	
	Ki-ras	*Ha*-ras	*Ki*-ras	*Ha*-ras	*Ki*-ras	*Ha*-ras
1	1.0	0.3	19	31	6.3	4.5
2	2.0	3.5	20	8.0	3.5	14
3	2.0	2.3	9.5	9.1	19	9.0
4	1.7	1.0			7.0	7.6
5	2.3	1.0			3.5	1.5
6	1.0	1.0			6.5	1.5
7	1.0	1.3			7.0	1.5
8	4.1	1.5	22	8.0		
9					5.0	1.5
10					4.4	1.5
11					6.5	11
12					1.9	14
13					3.0	6.3
CHB					6.5	2.0
HL60					13	1.5
K562					8.0	1.5

[a]The autoradiographs (**Figure 1**) were scanned and the concentrations of HiHi3 (v-Ki-*ras*) or (v-Ha-*ras*) specific RNAs are given at arbitrary units for each probe.

[b]Histological examination was carried out in part of the specimen and the remaining tissue was stored in liquid nitrogen until RNA and DNA were isolated. No. 1–12, colorectal carcinoma; No. 13, a breast carcinoma; CHB, an established cell line from an adenocarcinoma of the colon; HL60, a promyelocytic and K562 an erythroleukaemic cell line.

[c]Colorectal mucosa.

[d]1–2 Colorectal polyps (predominantly tubular), 3, Colorectal polyp (metaplastic) and 8, colorectal polyp (tubulovillous).

[e]Adenocarcinoma of the colorectum.

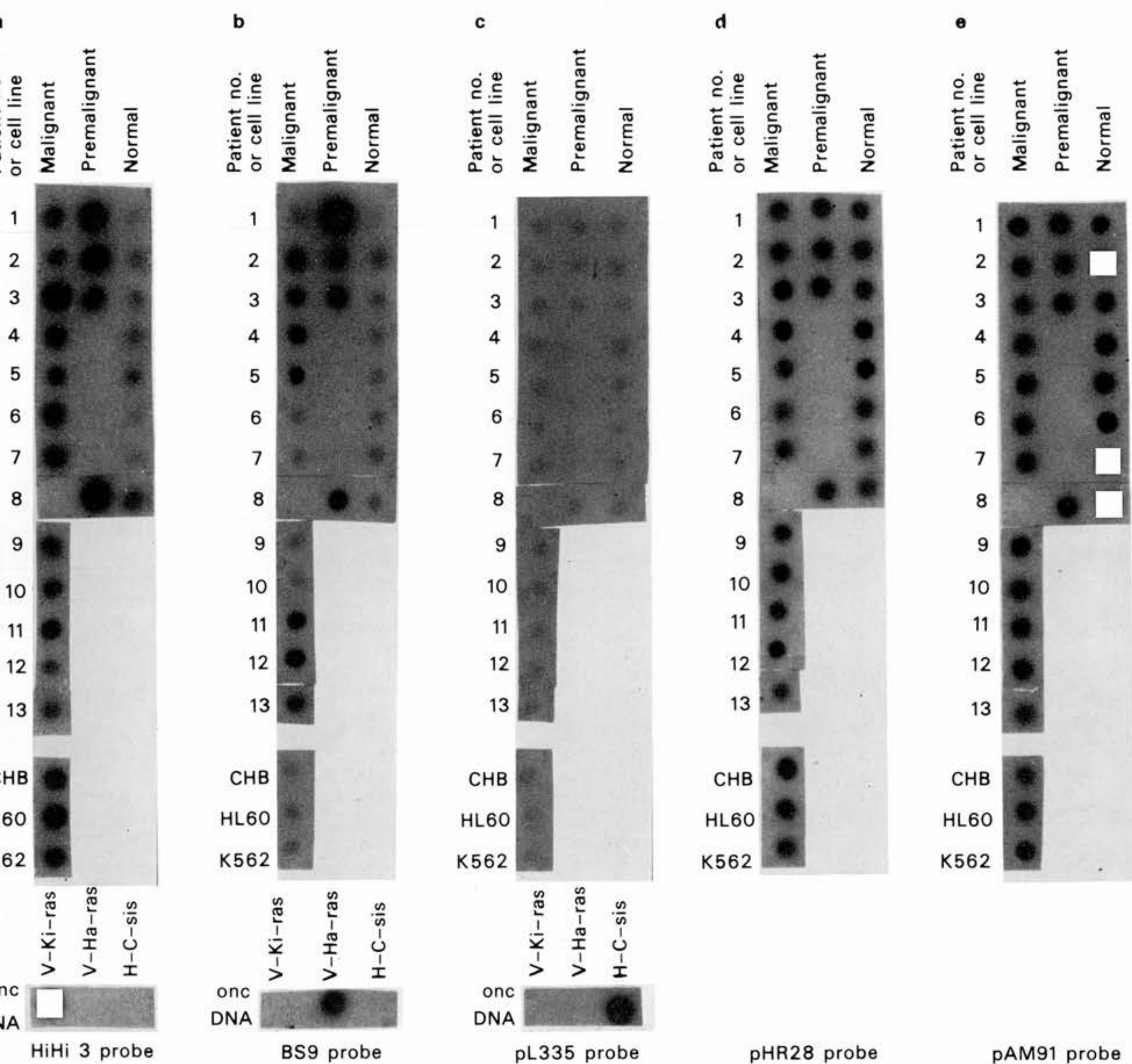


Figure 1 RNA spot hybridization analysis of (a) Ki-*ras*; (b) Ha-*ras*; (c) *sis*; (d) rRNA and (e) actin gene expression in human cells. Extraction of RNA from cells and spotting on to nitrocellulose is described in **Materials and methods**. Malignant: 1–12 = colorectal adenocarcinomata; 13 = breast adenocarcinoma; CHB = an established cell line from an adenocarcinoma of the colon; HL60, a promyelocytic and K562 an erythrolaeukemic cell line. Premalignant: 1–2 = predominantly tubular adenomatous polyps; 3 = metaplastic polyp, and 8 = tubulo villous polyp. Normal: histologically normal colorectal mucosa removed from colectomy specimens several centimetres distant from tumour site.

was examined. The relative levels of Ha-*ras* transcripts varied between 8.0–31 and 4.5–14 × higher in premalignant and malignant tissues respectively as compared to normal colorectal mucosa for the same first three patients as described above (Figure 1 and Table I). In patient No. 1 RNA from two separate polyps (P1 and P2) was examined gave similar results. In other malignant tumours there was some variation in the degree of elevation of Ki and Ha-*ras* expression (Table I). Oncogene expression in some but not all of the premalignant lesions was in fact significantly higher in comparison to the corresponding malignant tumours. Among the latter a more marked variation in expression was observed. Finally in the four other samples, a breast carcinoma RNA (No. 13), an established cell line from adenocarcinoma of the colon (CHB), a promyelocytic (HL60) and an erythroleukaemic (K562) cell line, expression of Ki-*ras* was elevated, but Ha-*ras* RNA levels were increased only in patient No. 13. As an additional control to check the relative amount of RNA from each sample spotted on to nitrocellulose, the filter was hybridized with pHR28, (a human ribosomal), or pAM91 (a mouse actin) DNA probe. As shown in the autoradiographs (Figure 1d and 1e) and confirmed by scanning the dots, there is no substantial difference in the amount of ribosomal or actin RNA present in these samples.

Northern blot hybridization analysis

Northern blot hybridization analysis was carried out to measure the sizes of c-*onc* related transcripts. A v-Ki-*ras* probe, HiHi3 recombinant (Ellis *et al.* 1981), revealed the presence of one main band of ~5.8 kb in total cellular RNA (Figure 2a and c). In several cases a much less intense band of ~2.2 kb was seen as well as some other nondiscrete hybridization probably due to degradation or possibly to cross hybridization with other *ras* gene family transcripts. As shown in Figure 2b, d after scanning across this 5.8 kb band, the ki-*ras* related transcript was found in higher amounts in premalignant and malignant tissues of the colorectum as compared to normal mucosa. A similar sized major transcript was also found in RNA from HL60 and K562 cells. As shown in Figure 2e when a v-Ha-*ras* probe, BS9 recombinant (Ellis *et al.*, 1980) was used, the same size band of 5.8 kb was also present, again more intensely in some premalignant and malignant tissues as compared to normal colorectal mucosa (Figure 2f). These results confirm and extend the spot hybridization analyses. The nature of these transcripts was further investigated by isolating polyA$^+$ RNA and Northern blot hybridization analysis. As shown in Figure 2g, i, Ki-*ras* and Ha-*ras* related transcripts of 5.8 kb in size were again found to be elevated in premalignant and malignant tissues as compared to normal colorectal mucosa. We also detected the 5.8 kb sized transcripts using pT24-C3 (Santos *et al.*, 1982), a recombinant carrying a 6.6 kb Bam HI human DNA fragment containing the whole bladder carcinoma oncogene c-Ha-*ras* 1 (data not shown). The exact nature of these transcripts is still incompletely understood. However mechanisms accounting for varied Ki-*ras* transcripts in terms of alternative splicing patterns have been recently described which include the generation of such a 5.8 kb species (Shimizu *et al.*, 1983; McGrath *et al.* 1983). It seems most likely that the same sized transcript seen with the Ha-*ras* probe represents in fact hybridisation with products of another member of the *ras* gene family. Northern blot hybridization analysis of total of polyA$^+$ RNA using a *sis* probe failed, however, to show any discrete RNA transcripts.

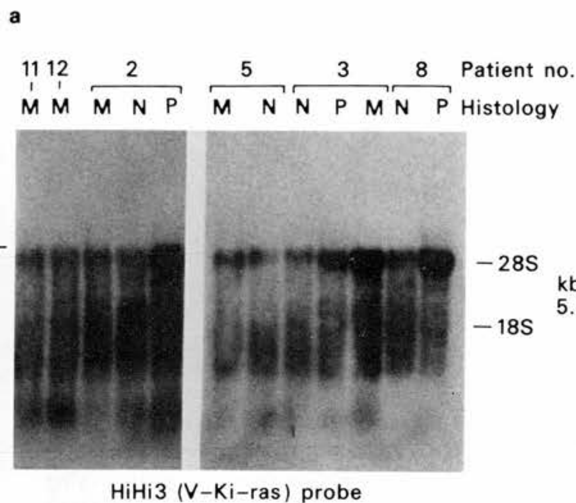
Discussion

Other studies with RNA from human cells have demonstrated the presence of 1.2 kb Ha-*ras* related transcripts in the T24 human bladder carcinoma cell line (Goldfarb *et al.*, 1982) and two ~6.0 kb Ha-*ras* related transcripts in human haematopoietic cell lines (Westin *et al.*, 1982b). More recently, using an N-*ras* probe 3 different sized transcripts of 5.8, 2.2 and 1.5 kb have been found in normal human fibroblasts and established human cell lines and it has been claimed that the 2.2 kb transcript is related to the N-*ras* oncogene (Hall *et al.*, 1983).

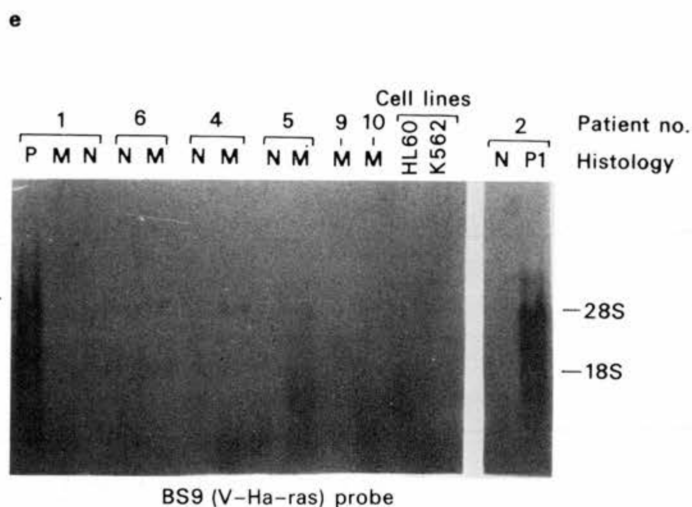
The human genome contains at least four genes homologous to the transforming genes of Kirsten

Figure 2 Northern blot hybridization analysis of transcripts related to human Ki-*ras* and Ha-*ras* oncogenes in RNAs from samples of normal premalignant and malignant tissues of the colorectum. Total RNAs were isolated as described in **Materials and methods.** Poly A$^+$ RNA was isolated using an oligo(dT)-cellulose Type 3 from Collaborative Research Inc. (Spandidos & Paul, 1982). In (a), (c) and (e), 20 μg of total cell RNA and in (g) and (i) poly A$^+$ RNA isolate from 100 μg total cell RNA were analyzed in 1% agarose-formaldehyde-containing gels, blotted on to nitrocellulose and hybridized with *onc* probes. The HiHi3 (Ellis *et al.*, 1981) recombinant containing the v-Ki-*ras* sequences was used as a probe in panels (a), (c) and (g). The BS9 (Ellis *et al.*, 1980) recombinant containing the v-Ha-*ras* sequences was used in (e) and (i). N = normal colorectal mucosa, P = premalignant polyps, M = malignant adenocarcinomata. The autoradiographs are shown in (a), (c), (e), (g) and (i) and the scans across the 5.8 kb bands in (b), (d), (f), (h) and (j).

a



e



b

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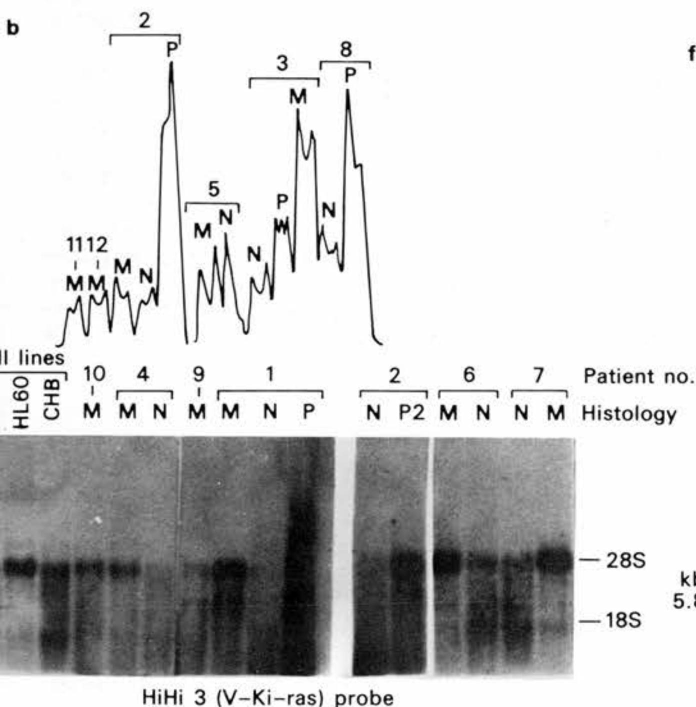
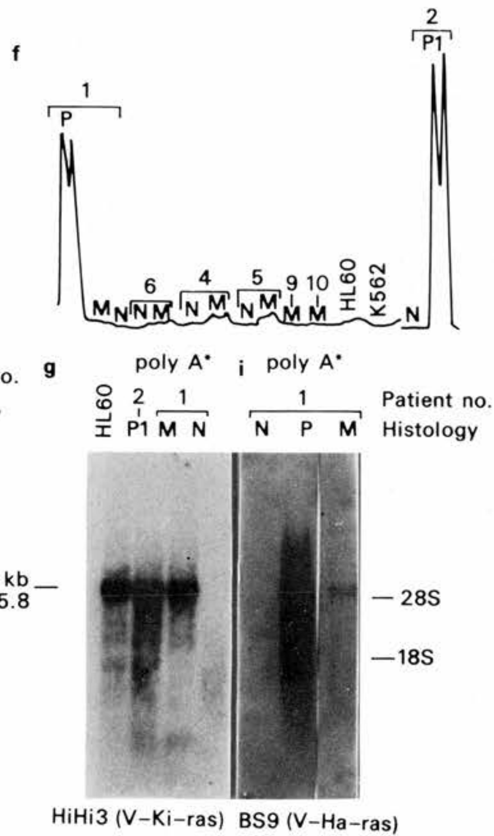
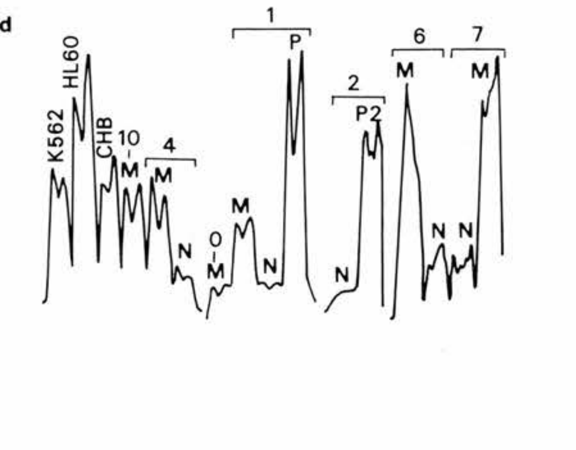


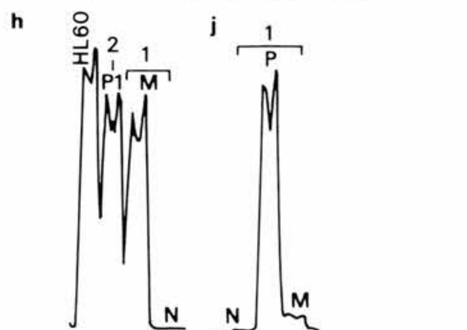


d

h

j





and Harvey murine sarcoma viruses (Chang *et al.*, 1982) which are dispersed in different chromosomes (O'Brien *et al.*, 1983). Moreover, recent transfection studies have revealed the presence of a distantly related N-*ras* oncogene (Hall *et al.*, 1983). Our results demonstrate that cellular sequences related to the transforming genes of Kirsten and Harvey murine retroviruses are actively transcribed in human tissues. The demonstration that Ki-*ras* and Ha-*ras* related transcripts are elevated in premalignant and malignant tissues as compared to normal colorectal mucosa shows that the expression of these *onc* genes is associated with the transformed state of the cells and suggests that elevated expression of these genes in premalignant state(s) may be critical in the process of carcinogenesis. The fact, however, that only a relatively small proportion of these premalignant polyps progress to frank malignancy although elevated oncogene expression was observed in all, suggests that, consistent with the concept of carcinogenesis being a multi-step process (Spandidos, 1983) the elevation observed here is not in itself sufficient to produce malignant change. Parallel studies in this laboratory, of carcinogen induced mouse skin papillomata, which have a similar potential for malignancy, have also demonstrated elevated Ha-*ras* oncogene expression and in addition, the DNA from these tumours has acquired transforming activity in transfection assays (Balmain *et al.*, 1984).

The cellular homologues of several retroviral oncogenes have been shown to exhibit tissue-specific patterns of transcriptional activity (Westin *et al.*, 1982*a*, *b*, Gonda *et al.*, 1982). Expression of c-*onc* genes during mouse development (Muller *et al.*, 1982; 1983) and liver regeneration (Goyette *et al.*, 1983) has lent further support to the hypothesis that cellular oncogenes play a role in normal developmental processes. Abnormal expression of these genes could be directly involved in the development of the transformed phenotype of tumour cells. Although meaningful *in vivo* studies of proliferation rates in these tumours involving repeated sampling and labelling would present obvious ethical problems, the most recent studies on human material *in vitro* comparing malignant tumours to normal mucosa using stathmokinetic (Pritchett *et al.*, 1982) and ^{3}H-Thymidine labelling (Bleiburg *et al.*, 1976) techniques suggest that cell birth rate and turnover time respectively, were only very slightly increased in tumours or not significantly different. Similar findings have been reported for premalignant polyps in human (Weisburger *et al.*, 1975), and in experimentally induced rodent tumours, where similar proliferation rates for benign and malignant tumours were reported (Sunter *et al.*, 1980). In the tumours, furthermore, the growth fraction is probably actually lower than in the normal mucosa. Thus the marked elevation of *ras* related transcripts we observe would not appear to be comparable to the two–three fold increase seen in regenerating liver (Goyette *et al.*, 1983).

Gene amplification seems not to be involved in the generation of elevated *onc* transcript levels found in our present study since Ha-*ras* and Ki-*ras* related DNA sequences in the various tissues were at approximately the same level when examined by DNA spot hybridization analysis (data not shown). However, oncogene amplification remains a possibility particularly since we have observed such a phenomenon in DNA from a different adenocarcinoma of the colon (our unpublished results). Such a phenomenon has recently been described for c-*myc* (Collins & Groudine, 1982; Dala-Favera *et al.*, 1982) and Ki-*ras* (Schwab *et al.*, 1983).

Since our results demonstrate that both the premalignant and malignant tumours examined here are characterized by elevated levels of *ras* family transcripts and if the gene product is unaltered, the question obviously arises as to the nature of the further event(s) involved in the acquisition of the malignant phenotype and their relationship with the changes in gene expression observed here. To address this question may require the use of further assay systems, although as an initial step it will clearly be of interest to clone the *ras* genes involved directly from the tumours since they may not readily be detected in transfection experiments, and such studies, as well as transfection studies using DNA from these tumours, are currently in progress in our laboratory.

We thank members of the Department of Pathology, Glasgow Royal Infirmary, for their co-operation and in particular Professor R.B. Goudie and Dr F.D. Lee for helpful advice, and Messrs. C.S. McArdle, I.G. Finlay and C.S. Morran of the Department of Surgery, Glasgow Royal Infirmary for facilitating the supply of specimens. The pHR28 ribosomal probe was prepared by A. Sproul. We are also indebted to Drs J. Paul, J. Neil, A. Balmain, G.D. Birnie and N.M. Wilkie of the Beatson Institute for helpful discussions. I.B.K. is currently a CRC Research Fellow in the Department of Surgery, Glasgow Royal Infirmary and during the initial stages of this work was McGhie Cancer Research Fellow, Department of Pathology, Glasgow Royal Infirmary. The Beatson Institute is supported by the Cancer Research Campaign of Great Britain.

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Br. J. Cancer (1986), **53**, 231–235

The relation of *ras* family oncogene expression to conventional staging criteria and clinical outcome in colorectal carcinoma

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Summary The elevated levels of *ras*-related cellular RNA in a series of colorectal carcinomata ($n=12$) was correlated with conventional staging criteria (tumour morphology and Dukes' staging) and with clinical outcome with particular reference to the development of metastatic disease. No direct relationship was evident between these parameters suggesting that although abnormal expression of *ras* oncogenes may be critical in the development of malignancy, variations in the level of their expression do not appear to be directly related to clinically evident phenotypic differences.

The characterisation of transforming cellular oncogenes in a variety of human tumours has contributed an initial understanding of cancer and the process of carcinogenesis at the molecular level (for a review see Cooper, 1984), although the extent to which abnormal expression of these genes is directly related to variations in the clinical behaviour of tumours is not yet clear. Elevated levels of transcription of several oncogenes have now been shown in a wide range of tumours (Slamon *et al.*, 1984; Spandidos & Agnantis, 1984; Spandidos *et al.*, 1985) and it has recently been shown that amplification of an N-*myc* oncogene is related to tumour stage in neuroblastoma (Brodeur *et al.*, 1984) whilst a study of c-*myc* expression in a series of human leukaemias showed increased expression to be apparently related to a more immature phenotype (Birnie *et al.*, 1986). Activation of a *ras* oncogene has also been suggested to be related, in a mouse lymphoma, to the generation of a more aggressive tumour variant (Vousden & Marshall, 1984).

Staging of colorectal carcinoma is currently undertaken using Dukes' classification (Dukes & Bussey, 1958) based on the extent of tumour invasion and spread through bowel wall. However, it has long been recognised that this classification constitutes no more than a relatively crude indication of prognostic probability (Woods, 1980), and the clinical outcome for patients within the different stages may vary significantly. Tumour morphology similarly yields only a very broad correlation with prognosis with marked inconsistencies occurring between degree of differentiation and clinical outcome (Finlay & McArdle, 1982). Despite initial enthusiasm regarding the potential of carcinoembryonic antigen as a tumour marker (see NIH Consensus Statement, 1981) it has now been shown that the presence of elevated pre-operative serum levels does not correlate with clinical outcome (Lewi *et al.*, 1984) and that subsequent elevations are not an invariable feature of advanced recurrent or disseminated disease (Finlay & McArdle, 1983). Studies of ploidy in these tumours have shown in general an inverse relation between increasing degrees of aneuploidy and prognosis although this is not absolute (Wolley *et al.*, 1982). We have recently shown, in a study of 'occult' metastatic disease using computerised tomography (Finlay & McArdle, 1982), that the presence or absence of metastatic disease at the time of clinical presentation, long known to be associated with a dire prognosis (Bengtsson *et al.*, 1981), is in fact the most critical prognostic factor, regardless of Dukes' staging and accounts almost entirely for the anticipated pattern of mortality. Studies of the rate of growth of these metastases show that they in fact had been present for a mean period of 3 years prior to surgery. This raises the possibility that these tumours may behave consistently from an early stage as metastasising or non-metastasising variants (Finlay *et al.*, 1982). However the phenotypic determinants or, possibly, features of a host response which might be responsible for such variation remain poorly understood and there is in particular no means of predicting the metastatic potential of a tumour, clearly of importance in determining further therapeutic strategies.

Since we have previously reported a variable elevation of expression of *ras* family oncogenes in a

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Received 8 June 1985; and in revised form, 7 October 1985.

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series of premalignant and malignant tumours of the colorectum as compared to normal colonic mucosa (Spandidos & Kerr, 1984), it was obviously of interest to determine whether the variation in expression we observed was related in any way to conventional staging criteria and whether, in particular, this could be used to predict the clinical behaviour of these tumours.

Materials and methods

Random blocks from a series of tumours and corresponding colonic mucosa were dissected out from operative specimens and snap frozen in liquid nitrogen. Our procedure for extraction of RNA for spot hybridisation assays has already been described in detail. (Spandidos & Kerr, 1984). Briefly 10 μg aliquots of total cellular RNA were hybridised sequentially on nitrocellulose paper with ^{32}P-labelled nick-translated HiHi3 (Ellis *et al.*, 1980) and BS9 (Ellis *et al.*, 1981) recombinant probes containing the viral Kirsten *ras* and viral Harvey *ras* DNA sequences and, subsequently, as controls to check the quantity of RNA present, with the pL335 (Dalla Favera *et al.*, 1982), pAM91 (Minty *et al.*, 1982) and pHR28 probes containing the human cellular *sis*, mouse actin and human 28S ribosomal DNA sequences respectively. Similarly, the quality of our RNA preparations were checked by formaldehyde-agarose gel electrophoresis, followed by ethidium bromide staining, blotting onto nitrocellulose and hybridisation to DNA probes.

Quantitation of the spot hybridisation reactions following autoradiographic exposure was performed by densitometric scanning as previously described (Spandidos *et al.*, 1981) and our figures were based on an arbitrary value obtained when the *ras* probes were hybridised with normal mucosa.

Histopathological reports were based on standard 6 μm paraffin embedded sections stained with haematoxylin and eosin.

Patients in the study were followed up as outpatients at 3 monthly intervals with biannual ultrasonography and further radiological investigations as indicated.

Results

It is evident from Figures 1 and 2 that the degree of elevated expression of Kirsten or Harvey *ras* related sequences in our series does not appear to correlate with either tumour morphology or Dukes' staging since the range of values obtained, ranging from ~2 to 20 fold for the Kirsten to 1.5 to 14 fold for the Harvey-related sequences, clearly overlaps all histological grades and Dukes' stages.

Table I Relation of Ki and Ha-*ras* related oncogene expression to age, sex and clinical outcome.

Patient	*Age*	*Sex*	*Follow-up in months*	*Status*	*Ki*-ras	*Ha*-ras
1	52	M	27	ND	6.3	4.5
2	83	F	(9)	ND[a]	3.5	14
3	62	F	(19)	DD[a]	19.0	9.0
4	61	F	33	LD	7.0	7.6
5	60	M	(5)	DD[a]	3.5	1.5
6	54	F	(6)	DD[a]	6.5	1.5
7	81	F	(4)	DD[a]	7.0	1.5
8	72	M	24	ND	9.0	1.5
9	75	M	36	ND	5.0	1.5
10	78	F	(—)	ND[a, c]	4.4	1.5
11	79	M	(—)	(post op.) —[a, b]	6.5	11
12	51	F	(8)	(post op.) DD[a]	1.9	14

Status of patients at follow-up or death was assessed and abbreviated as follows: ND—no evidence of disease; DD—disseminated disease; LD—local disease; [a]dead; [b]not known; [c]post-mortem assessment.

Although follow-up is in some cases relatively short it is, however, known that of those who will ultimately develop disseminated disease the majority would be expected to do so within a period of two years (Finlay *et al.*, 1982*c*). The information in Table I thus shows that the level of expression of these sequences does not appear to be related to clinical outcome in general nor to the development in particular of metastatic disease.

Discussion

Our results therefore indicate that, although some elevation of Kirsten and Harvey-related *ras* oncogene expression is seen in all these tumours, variation in the amounts of RNA homologous to the *ras* probes is not related to clinically apparent phenotypic variation nor clinical outcome. If the hypothesis relating to a heterogeneity of cells with metastatic potential in a primary tumour (Hart & Fidler, 1981), currently held by several authors to be extremely contentious (Weiss *et al.*, 1983; Alexander, 1983), were indeed true in this tumour type, then random sampling could obviously not be expected to identify such variation. We have, however, already presented evidence showing that these tumours appear to behave consistently as metastasising or non-metastasising variants (Finlay *et al.*, 1982). In addition, although a heterogeneous

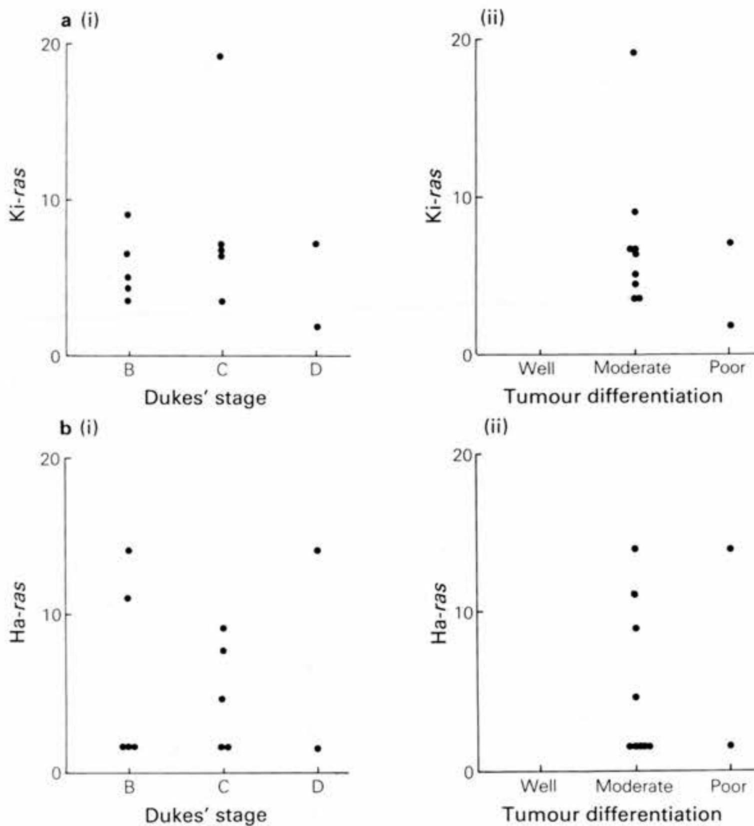

Figure 1 Relation of (a) Ki-*ras* and (b) Ha-*ras* related oncogene expression to Dukes' stage (i) and degree of tumour differentiation (ii). In one case histology was not available.

pattern of p21 expression has been reported in formalin-fixed paraffin-embedded sections of these tumours by immunocytochemical techniques (Thor *et al.,* 1984), we (Kerr *et al.,* 1985) and others (Williams *et al.,* 1985) have demonstrated, using the monoclonal antibody Y13-259 (Furth *et al.,* 1982) on frozen sections of these tumours, widespread positive staining of tumour cells with little reactivity of underlying stroma. It is unfortunately not possible to quantitate expression using such techniques. Thus, although some variation within individual cells cannot be excluded, it is clear from the evidence based on presently available methods of study that neither the presence of *ras* oncogene expression nor observable differences in its level can be used to predict variations in clinical behaviour in these tumours.

These findings are consistent with a recently published study which showed similar elevations of p21 protein levels in these tumours but which was also unable to correlate these absolutely with conventional staging criteria, although, interestingly, it was shown that metastatic tumour contained relatively low levels of p21 (Gallick *et al.,* 1985).

Although activity of a number of cellular proto-oncogenes has been demonstrated in a variety of tissue types and at various developmental stages (Westin *et al.,* 1982*a,b*; Muller *et al.,* 1982, 1983), it is not yet clear in the majority of cases what their exact physiological roles might be. It is widely hypothesised, however, that they may be concerned with cell growth control and regulation (Heldin & Westermark, 1984). Nor is it precisely understood what part they play in the process of carcinogenesis although they have been identified in several cases as the active transforming genes in 3T3 transfection assays, including an activated Ki-*ras* from a colonic carcinoma (Pulciani *et al.,* 1982). We have previously shown elevated expression of *ras* family oncogenes in malignant and premalignant tumours of the colorectum and elevated expression as well as transforming activity of a Ha-*ras* oncogene has been shown in experimentally induced mouse skin carcinomata

and in premalignant papillomata (Balmain *et al.*, 1984). It is also of interest in this context that during experimental *in vitro* transformation of early passage rodent cells, transfection with either an activated, mutated Ha-*ras* oncogene or with a normal Ha-*ras* proto-oncogene linked to an enhancer induces immortalisation alone, and that only when linked to transcriptional enhancers can a mutated form of the gene induce complete malignant transformation (Spandidos & Wilkie, 1984). Such results would be consistent with a role for both quantitative and qualitative changes in *ras* gene expression within an overall multi-step process of carcinogenesis.

If indeed, as we have previously hypothesised, activation of these genes at a premalignant stage may be critical in the process of carcinogenesis but not in itself sufficient, it may be that subsequent event(s) whose nature is not yet clear, perhaps involving activation of a variety of other gene loci and possibly associated with some form of genomic instability, are more directly involved in the expression of a frankly malignant phenotype and its variants. It is, however, possible that variation in the nature of the mutation previously shown to be associated with *ras* activation (Reddy *et al.*, 1982; Shimizu *et al.*, 1983; Santos *et al.*, 1984) may also be significant in this context, although in a series of urothelial tumours recently reported (Fujita *et al.*, 1984) neither the presence of a transforming *ras* oncogene in DNA in a small percentage of cases nor the presence or absence of a documented mutation in these, appeared to be related to tumour stage. Nonetheless, study of such variation and of abnormal expression of cellular oncogenes, already shown in the context of neuroblastoma even in gross tumour specimens to be clinically significant, as well as of other event(s) involved in the generation of malignancy will clearly be important in attempting to define the behaviour of these tumours more fully.

We thank members of the Departments of Pathology and Surgery, Glasgow Royal Infirmary, for their cooperation in obtaining and documenting specimens and Dr G.D. Birnie for critical appraisal of the manuscript. This work was supported by the Cancer Research Campaign of Great Britain.

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