CHROMOSOMAL LOCATION OF RATE-LIMITING ENZYMES IN POLYAMINE BIOSYNTHESIS AND GENE EXPRESSION IN COLORECTAL NEOPLASIA

A Thesis Submitted for
the Degree of M.D. (Doctor of Medicine)
Glasgow University

by

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April 1991

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ACKNOWLEDGEMENTS

The experimental work described in this thesis was carried out in the laboratory of Dr. T. Shows, Department of Human Genetics, Roswell Park Cancer Institute, Buffalo, New York, U.S.A. I gratefully acknowledge the advice and assistance of all those in the laboratory who introduced me to the techniques of molecular genetics, especially Roger Eddy, Linda Haley, W. R. Henry, Mary Byers, Dr. Hiroshi Nakai, Dr. James Tricoli, Dr. Lisa Davies, and Dr. Norma Novak.

I wish to thank Dr. Arnold Mittelman, Dr. Rosemary Elliot, Dr. Lynne Maquat, Dr. Carl Porter, and Dr. Tom Mueller for their helpful advice. I am particularly indebted to Dr. Tom Shows and Professor J. M. Connor for their guidance and criticism during the preparation of this thesis.

Thanks go to Ina Young and Sandy Shows for secretarial help while in Buffalo, and to Kathy Springmeyer, Careen Bresee, Audrey Wurth, and Jean Finn in St. Louis for typing the manuscript.

I wish to thank Dr. Lynne Maquat, Dr. Anthony Pegg, and Dr. Olle Janne for providing probes.

I wish to thank Dr. Carl Porter for permission to use Figures 2 and 3, Dr. Tom Shows for figures 1 and 4, and Cell Press and Dr. Bert Vogelstein for Figure 31.

Special thanks go to Dr. E. Douglas Holyoke for the opportunity to work in the laboratory and to both Dr. and Mrs. Holyoke and Dr. and Mrs. Shows for their hospitality during my two years in Buffalo.

I would also like to acknowledge all the surgeons at Roswell who provided tissue samples.

Surgical Developmental Oncology Service	Dr. N. Petrelli
	Dr. L. Herrera
	Dr. J. Stulc
	Dr. A. Mittelman
Upper GI Service	Dr. H. Douglass
	Dr. H. Nava
Breast Service	Dr. M. Schuh
Soft Tissue/Melanoma Service	Dr. C. Karakousis
	Dr. R. Nambisan

Lastly, thanks to Professor Sir Tom Symington for his invaluable help throughout the years.

DECLARATION

The research described in this thesis was carried out while I was a Fellow in Surgical Oncology at Roswell Park Cancer Institute, Buffalo, New York, U.S.A., from July 1985 to June 1987. The experiments were performed in the Department of Human Genetics under the direction of Dr. Thomas B. Shows.

I personally performed 95% of the Southern analyses described both for the mapping experiments involving the ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC) genes and for the investigation of both genes from various tumour types. Roger Eddy performed the remaining 5%. I carried out the analysis of ODC polymorphisms in human volunteers. All the Northern analyses described were performed by myself.

Regarding the extraction of DNA and RNA from tissue, approximately 80% were carried out by myself; W. R. Henry doing the remaining 20%.

The mouse-human somatic cell hybrids have been established in Dr. Shows' laboratory for a number of years. Linda Haley grew and preserved the cell lines, Mary Byers performed the cytogenetic analysis, and W. R. Henry performed the histochemical staining. The in-situ hybridisation experiments described for chromosome 2 were done by Mary Byers and Dr. Hiroshi Nakai. The ODC activity experiments were carried out in Dr. Carl Porter's laboratory.

Help with statistical analysis was provided by W. Lawrence.

The S-adenosylmethionine data has been published in Cytogenetics Cell Genetics and the data on ornithine decarboxylase mapping and expression has been published in Cancer Research. The mapping data for ODC was initially presented at Human Gene Mapping 9, Paris Conference 1987.

SUMMARY

Colon cancer represents one of the major causes of death in the Western hemisphere, being responsible for over 60,000 deaths per year in the U.S. Over the past 50 years, the hypothesis of the adenoma-carcinoma sequence, which proposes that benign colonic polyps are the precursor lesions of malignancy, has been accepted. Any study of the possible carcinogenic mechanisms in colon cancer therefore should also be directed to the precursor lesions - the benign polyps.

The polyamines putrescine, spermidine and spermine, are low molecular weight aliphatic amines intimately involved in cell proliferation and differentiation. Since the 1970s, it has been noted that polyamine concentration is higher in a number of malignancies when compared to normal tissue. The two main rate-limiting enzymes in the polyamine biosynthetic pathway are ornithine decarboxylase (ODC) which catalyses the conversion of ornithine to putrescine, and S-adenosylmethionine decarboxylase (AdoMetDC) which acts as an aminopropyl donor in the conversion of putrescine to spermidine and spermidine to spermine. ODC and AdoMetDC are highly inducible enzymes, their activity rising early in oncogenesis in certain model systems. Regulation of both enzymes occurs at a number of levels. Since the polyamines are so necessary for normal growth and differentiation it is likely that deregulation of the polyamine biosynthetic pathway is a major factor in carcinogenesis.

Both polyamine content and enzyme activity rise early in rodent colon when exposed to known carcinogens and bile acids. Polyamine concentration is elevated in human colon cancer compared to normal mucosa. Several workers have shown that ODC and AdoMetDC activity is greatly increased in both benign colonic polyps and cancer implying that activation of the polyamine pathway may be involved in the conversion of benign to malignant polyps.

A study of the control of the ODC and AdoMetDC genes starts with a knowledge of the chromosomal loci for these genes.

The purpose of this study was: 1) To map the ODC and AdoMetDC genes; 2) Study expression of the ODC gene in colon neoplasia (carcinoma and polyps) in relation to ODC enzyme activity; 3) Investigate whether any

amplification or rearrangement of the ODC gene was responsible for increased expression; and, 4) Study similar genetic mutations of the AdoMetDC gene in colon neoplasia.

The DNA from 31 mouse/human cell hybrids was digested with *Pst*I, and the DNA from 37 hybrids digested with *Hind*III. Fragments were separated by agarose gel electrophoresis, transferred to nylon filters, probed with a radiolabelled human cDNA ODC clone and autoradiographs obtained. By this method, the ODC gene was mapped to sequences on chromosome 2 and 7q31→qter. In both *Pst*I and *Hind*III digests the sequences on chromosome 7 were less prominent under high stringency conditions. The locus on chromosome 2 was defined by in situ hybridisation: of 1987 metaphase sets examined, 361 silver grains were seen with 16.1% touching chromosome 2. Thirty-one percent of the grains on this chromosome were located at 2p25 with no significant hybridisation to chromosome 7. The locus on 2 was designated ODC1 and that on 7 ODC2.

AdoMetDC was also mapped by somatic cell hybrid techniques: DNA from 38 somatic cell hybrids was digested with *Pst*I and separated in a similar manner to that described for ODC. Two loci for AdoMetDC were found-one on chromosome 6 (referred to as AMD 1), and the other at Xq22 - Xq28 (AMD 2).

The ODC gene locus on chromosome 2 was seen to be polymorphic, 3 consistent banding patterns being seen. Allele frequencies were calculated to conform to the Hardy-Weinberg law with $p = 0.28$ and $q = 0.72$.

ODC gene expression was studied in 18 isolated colorectal tumours and adjacent normal mucosa and 6 polyps. The expression of the housekeeping enzyme triose phosphate isomerase (TPI) was used as an internal control. In all cases a single ODC RNA transcript was seen measuring 2.2 kb. ODC expression was consistently increased in tumours compared to mucosa by a mean of 4.2-fold and in polyps by a mean of 3.2-fold ($p < 0.001$). No correlation was found between ODC expression and site of tumour, stage, or degree of differentiation.

In 15 patients paired samples from the same patient were analysed for both ODC activity and ODC expression. Although all samples showed a significant increase in activity and expression when compared to mucosa, no correlation was seen between the two parameters for the same sample.

ODC expression was also increased in gastric cancer, leiomyosarcoma and liposarcoma, but not to the same degree as in colorectal neoplasia, suggesting tissue specific gene regulation.

ODC DNA analysis was performed on 18 colorectal carcinomas and seven polyps. No amplification of either locus was seen to account for the elevated expression. No consistent rearrangement of the ODC gene was seen in colorectal neoplasia. No amplification of ODC was seen in leiomyosarcoma, liposarcoma, breast, lung, or gastric cancer. Analysis of AdoMetDC DNA was performed on 13 samples of colorectal cancer and five polyps. No amplification or rearrangement was seen.

No correlation was noted between ODC and amplification of NMYC, or between ODC expression and expression of IGF-2.

Elevation of ODC expression and activity seems to be a consistent finding in colorectal carcinoma and polyps in this and other studies. The expression of ODC in early polyps suggests a role for ODC in the early events of oncogenesis. The ODC gene is influenced by hypomethylation and mutated ras genes, both of which are known to occur early in the cascade of colon cancer formation proposed by Vogelstein. The stereospecific interaction of the polyamines with DNA may evoke further genetic deregulation along this cascade.

The precise role of ODC regulation in colorectal neoplasia is still unclear. It is apparent that increased expression is not due to amplification of the gene as in other systems, but may be due to altered transcription rate or mRNA stability. Similarly, it is unclear how elevated activity is produced at the tissue level. As no correlation was found between expression and activity, post transcriptional mechanisms must also be in operation, such as altered forms of ODC protein. An example would be the GTP-activatable form of ODC which has recently been isolated from colon tumours.

1. INTRODUCTION

1.1 Epidemiology of Colon Cancer

Colorectal cancer is a major cause of disease in the Western world. There are estimated to be 157,000 new cases in the United States in 1991 and 60,000 deaths due to the disease. Although the disease is common in the U.S., it is even more prevalent in Scotland.[1-3]

Environmental factors are thought to play an important part in the incidence of colorectal cancer. Immigrants from Japan and black Africa, where the disease is relatively infrequent, show an increased risk when living in the U.S.[2] The low incidence in some underdeveloped countries[3] has prompted interest in possible aetiological factors. In the early 1970s, D.P. Burkitt proposed that the higher intake of fibre in the diet in several African countries, with consequent decreased transit time through the gut, was responsible for altered colorectal cancer rates. This was attributed to a possible dilutional effect on carcinogens in the stool.[4,5]

Faecal bile acids are thought to be carcinogenic to the colon mucosa. Bile acids can act as tumour promoters in animal models.[6] Hill, et al.,[7] found a higher faecal bile acid concentration in patients with large bowel cancer when compared to patients with other diseases, although this result could not be repeated by Murray, et al.[8]

The bile acids produced by the liver are converted by intestinal flora to secondary bile acids which are carcinogenic.[9] A higher ratio of anaerobic to aerobic bacteria in the colon has been demonstrated in countries with a higher incidence of colon cancer.[7,10]

Dietary intake of fat and red meat is also positively correlated with colon cancer incidence.[11,12] It is possible that high fat and low fibre intake act together in increasing the risk of colon cancer. Diets high in fat also cause a higher excretion of bile acids.[9]

Alcohol intake has also been associated with high rectal cancer rates.[2,6] On the other hand, increasing selenium[2,6] and calcium[13] in the diet may have a protective role against colon cancer. Lipkin and Newmark[14] demonstrated lower proliferative activity in colonic crypts

when oral calcium supplements were administered.

It appears then that multiple environmental factors may influence the incidence of colon cancer in a particular population. Strong[15] has proposed that the response to environmental agents may differ because of genetic variation, cancer being a genetic disease at the cellular level. Hill, et al.,[16] in particular reference to colorectal cancer, put forward a hypothesis that the environmental carcinogen can only cause progression from normal mucosa to neoplastic polyp to carcinoma (the adenoma-carcinoma sequence) when the person is genetically predisposed to the disease.

1.2 The Adenoma-Carcinoma Sequence

The importance of the study of colonic polyps in relation to colonic cancer rests on the increasing evidence that these are the precursor lesions which progress to carcinoma.

The word polyp is derived from the Greek polypous meaning morbid excrescence. The classification of neoplastic polyps currently used divides adenomas into tubular, villous and tubulo-villous types.[17-19] The incidence of malignancy is increased in villous compared to tubular adenomas, the percentage found containing malignancy being 41 and 5, respectively.[19] Cuthbert Dukes recognised that there was a strong correlation between "simple tumours" and subsequent development of colon cancer.[20] The chance of malignant transformation also increases with the size of the polyp-polyps 1-2 cm in diameter have a 10% incidence of carcinoma, whereas 46% of polyps over 2 cm will contain malignancy.[19,21] Malignant potential also increases as the degree of dysplasia progresses from mild to severe.[19] Most of the work detailing the adenoma-carcinoma sequence was performed by Basil Morson at St. Mark's Hospital in London. This data is supported by Shinya and Wolff[22] and Love.[23] Cole and McKalen[24] injected tritiated thymidine into a patient with familial polyposis prior to colectomy and found that the normal zone of proliferating cells lies at the bases of the crypts of Lieberkuhn. Development of the adenoma is preceded by a hyperproliferative period when the zone shifts to the upper part of the crypt. Autoradiographs of adenomas reveal the labelled cells to be situated at the periphery of the polyp. Thus,

in adenomas, there is a loss of normal growth control mechanisms.[25] This finding was also observed by Deschner and Lipkin.[26]

Alterations of cell kinetics in neoplasia has been divided into stages by Deschner[27,28] and Lipkin.[29] In Stage I, there is an extension of the proliferative component to the luminal surface of the crypt. A Stage II abnormality comprises a shift of DNA synthesis from the base to the upper and middle thirds of the glands. Bleiberg, et al.,[30,31] also using tritiated thymidine, found a larger S phase duration in colonic cancer and polyps compared to normal tissue.

It is now generally accepted that an adenoma-carcinoma sequence exists, that polyps should be regarded as premalignant lesions, and that polyp removal therefore is a method of cancer prevention. The continuum of epithelial neoplasia begins with an adenoma of varying degrees of dysplasia, this is followed by the appearance of cytologically malignant cells, but still confined to the crypt. This can be classified as an intraepithelial carcinoma.[32] The malignant cells then invade the lamina propria which separates the crypts of Lieberkuhn, termed intramucosal carcinoma. It is only when the mucosa is breached that the tumour becomes invasive and potentially metastatic.

1.3 The Staging of Colorectal Cancer

In 1932, Sir Cuthbert Dukes at St. Mark's Hospital developed a staging system for carcinoma of the rectum.[33] Stage A represented those cases where the carcinoma was limited to the wall of the rectum; in Stage B, the carcinoma had spread through to the extra-rectal tissues; and in Stage C, regional lymph nodes were involved. Survival decreased as the stage of disease became more advanced.

This staging system has also been applied to colon cancer and several modifications of Dukes' original schema have been used. The staging system used in this thesis is that proposed by the Gastrointestinal Tumour Study Group (GITSG).

Colon[34]

A - Invasion of the mucosa.

B_1 - Invasion of the muscularis mucosa (sub-mucosa).

B_2 - Penetration to or through the serosa without nodal involvement.

C_1 - One to four nodes involved.

C_2 - Five or more nodes involved.

Rectum[35]

A and B_1 - As for colon cancer.

B_2 - Extension of the tumour through the rectal wall without nodal involvement.

C_1 and C_2 - As for colon cancer.

In this system, the depth of penetration of the bowel wall did not affect the classification of stage C tumours.

1.4 The Genetics of Colorectal Neoplasia

Although environmental factors are important in the oncogenesis of colon cancer, they are believed to operate on the underlying genetic predisposition for the disease.[15,16] The contribution of inherited factors to the origin of colorectal cancer is poorly understood, but begins with the well-defined autosomal dominant conditions. Familial polyposis, now called familial adenomatous polyposis, is a condition in which the colon and rectum are carpeted with hundreds of polyps.[36] Carcinoma will develop in 100% of affected persons. In 1882, Harrison Cripps described polyposis in a brother and sister.[37]

Following Cripps' report, Smith,[38] Bickersteth,[39] and Handford[40] described cases of multiple polyposis of the lower bowel. Both Smith's and Bickersteth's cases appeared to be familial in origin. The case Handford described was notable in that carcinoma occurred simultaneously with multiple polyps.

Cockayne, in 1927,[61] was the first to suggest a Mendelian dominant mode of inheritance. In 1930, Dukes[42] put forward the idea that in polypsis coli, colon cancer itself is not inherited, rather it is a tendency to epithelial proliferation leading to polyps and later to cancer which is inherited. Lockhart-Mummary later also

embraced this idea.[43]

In 1939,[44] Lockhart-Mummary and Dukes made the insightful hypothesis that polyposis arose as a result of gene mutation. Part of our understanding of the adenoma-carcinoma sequence begins with an observation of the natural history of familial polyposis.

In 1950, E. J. Gardner produced the first of several papers concerning the syndrome which bears his name and the genetics of colon cancer became more complex.[45] In this syndrome, polyposis is associated with extra-colonic manifestations such as epidermoid cysts, desmoid tumours, and osteomas.

Blair and Trempe[46] and later Lewis, et al.,[47] described congenital hypertrophy of the retinal pigment epithelium in Gardner's syndrome.

Various other inherited syndromes associated with multiple adenomatous polyps have been described, for example, Turcot's syndrome, associated with neurogenic tumours[48]; Oldfield's syndrome, associated with multiple sebaceous cysts[49]; and Muir-Torre syndrome, associated with multiple skin tumours and other malignancies[50-52]. Cowden's[53] syndrome and Peutz-Jeghers[54,55] syndrome also result in multiple polyposis, but of the hamartomatous, not adenomatous type. Cronkite-Canada syndrome[56] results in inflammatory polyps and is occasionally associated with colorectal cancer.[54]

Gardner has raised the issue that familial adenomatous polyposis and Gardner's syndrome may be variations of the same disease, transmitted by the same genetic defect.[57]

Alfred Warthin, in 1913, was the first to recognise the phenomenon of the cancer family.[58] Since then, Lynch has studied numerous kindreds extensively with the recognition of two types of hereditary non-polyposis colorectal cancer: cancer family syndrome or Lynch syndrome II comprises early onset proximal colonic cancer and extra-colonic adenocarcinomas and Lynch syndrome I (hereditary site specific colon cancer) which has all of the above characteristics but is not associated with extra-colonic cancer.[59-67] Aside from the autosomal dominant conditions, colon cancer exhibits familial aggregations, in that family members of the index case appear to have a higher incidence of the disease.

Duncan and Kyle[68] investigating familial colon cancer in

northeast Scotland, found 16% of patients with colon cancer had a first degree relative with the disease, but did not find them to be younger than patients without a family history. Lovett,[69,70] in London, found a three-fold increase in death rate from intestinal cancer among relatives over that which would be expected in the general population, confirming what Macklin had found in an Ohio population.[71]

Not only does the tendency to form multiple polyps appear to be inherited: Richards and Woolf[72,73] have noted that solitary polyps of the colon and rectum also appear to be inherited in a mendelian dominant pattern in some cases.

Although it has been said that approximately 5% of colon cancer is inherited,[74] Burt, et al.,[75] presented evidence that sporadic adenomas and colon cancers are inherited much more commonly. Their analysis concerned a large Utah kindred with no recognisable inheritance pattern. One or more adenomatous polyps was found in 21% of family members but in only 9% of controls. This excess of discrete polyps and cancers suggested to them the presence of an autosomal dominant gene for susceptibility. They conclude that a major step would be the genetic mapping of the locus for susceptibility to colon cancer.

Cytogenetic abnormalities observed in colorectal cancer specimens may lead investigators to the locus for this gene.

Numerous chromosomal abnormalities have been seen in colon cancer. These include 12q-, +7, +8, +12, 17(q11)[76], partial trisomy of 1 q[77] monosomy 17p, monosomy 18, monosomy 20q, trisomy 13, monosomy 1p, and trisomies X and 8q.[78] Levin and Reichmann[79] also report trisomy 13, 19, 20, 21, loss of 17 and structural alterations of 1 and 5. Abnormalities of chromosomes were also seen by Shabtai, et al.[80] In polyps, the most frequent karyotypic change is +8 and -20.[81] Attempts have been made to characterise chromosomal changes in familial polyposis and Gardner's syndromes.[82] Increased tetraploidy was seen in both conditions and has also been seen in non-hereditary adenomata.[83]

1.5 Gene Mutation and Activation in Colon Cancer

Altered expression of several genes occurs in colon neoplasia. Increased Ki-ras and Ha-ras oncogene expression occurs in both colorectal polyps[84] and carcinomas.[84,85] C-fos and c-myc expression is also increased in colon carcinomas.[85] Tricoli, et al.,[86] found significantly increased insulin-like growth factor 2 (IGF-2) mRNA levels in 50% of rectal and rectosigmoid cancers but not in cancers from other sites in the colon. The activation of Ki-ras in colon carcinoma occurs by two different point mutations of the same codon.[87]

Altered DNA methylation results in altered expression of mRNA.[88] Increased ras oncogene expression in colon cancers may also be due to hypomethylation. Feinberg and Vogelstein[89] demonstrated hypomethylation of the Ha-ras oncogene in five of seven colon cancers. Ki-ras hypomethylation occurred less frequently.

1.6 Gene Mapping and Its Importance in Cancer Research

Knowledge of the chromosomal locus of a gene is important in studying aspects of genetic regulation. Certain specific chromosomal aberrations have been associated with various cancers and are believed to be essential for oncogenesis to occur.[90] An example would be Burkitt's lymphoma in which a translocation of a region of chromosome 8 occurs. The breakpoint on chromosome 8 appears to be crucial for the disease.[91-93] This area involves the locus for c-myc, and its rearrangement to the vicinity of one of three immunoglobulin loci on chromosome 14, 2 or 22 results in deregulation of the myc gene. A similar activation of the oncogene c-abl, located on chromosome 9 occurs in chronic myeloid leukemia, in which a 9; 22 translocation occurs. Such a translocation occurs in over 90% of cases.[90-93]

The trigger for these chromosomal translocations may be their proximity to known fragile sites. Expression of fragile sites occurs by altering the concentration of folic acid and thymidine in culture media.[92] Certain fragile sites coincide with breakpoints involved in the rearrangements of several neoplasias. C-mos has been mapped to

8q22, the breakpoint in acute myeloblastic leukemia; the proto-oncogene c-ets-1 has been mapped to 11q23-q25, a region involved in a breakpoint in acute monoplastic leukemia. Both 8q22 and 11q23 are the sites of heritable fragile sites.[94]

Gene mapping also allows study of diseases in which oncogenesis may occur by the deletion of chromosomal material. In 1971, Knudson developed the two hit theory of neoplasia in which he stated that mutations of both the maternal and paternal chromosomes are necessary for neoplasia to occur.[95] In the familial case, a germ-line or constitutional mutation is followed by a somatic mutation of the normal chromosome, which unmasks the disease locus. In sporadic cases, two somatic mutations at the disease locus would account for the disorder. His hypothesis was subsequently shown to be true for both retinoblastoma[96,97] and Wilms tumour.[98] This loss of genetic material has been termed, "loss of heterozygosity". The deletion in retinoblastoma occurs at 13q14, to which has also been mapped the gene for esterase-D, an enzyme deficient in retinoblastoma patients. The Wilms tumour locus has been mapped to 11p13 where the gene for catalase also resides. Again, deficiencies of this enzyme occur in some cases of Wilms tumour.[90]

Thus, the knowledge of the gene map is vital to understanding deregulation of certain genes and is one of the first steps to understanding the role of a gene in disease.

Gene amplification is another genetic alteration which is involved in neoplasia and tumour progression. Multiple gene copies of the oncogene N-myc which is located at 2p23-24 occur in neuroblastoma. Amplification of N-myc is related to advanced stage and poorer prognosis in this disease.[99] Mouse adrenocortical tumours contain amplified c-Ki-ras oncogene sequences. Amplified c-myc (normally present on chromosome 8) has been found on chromosome X in the colon cancer cell line COLO-320.[100] Gene amplification may also predict response to therapy, for example, resistance to methotrexate is determined by amplification of the dihydrofolate reductase gene.[101]

1.7 DNA Polymorphisms

Based on studies of the β-globin cluster in humans, it has been

estimated that the human genome contains 3 x 10^7 sequence variants or one every 100 base pairs.[102] This would result in approximately 10^6 sequence polymorphisms per chromosome. These variations between individuals have been termed restriction-fragment-length-polymorphisms (RFLPs)[103] and can be identified on Southern analysis.[104] Polymorphic loci can result from additions or deletions of DNA, genetic rearrangements, or base-pair substitutions at the restriction endonuclease recognition site. The identification of RFLPs for a given gene may be useful not only as a marker but in understanding the phenotypic expression of that gene. Polymorphic markers can be transmitted in a Mendelian fashion.

Figure 1 illustrates how RFLPs are detected. DNA is isolated from several individuals and digested with a restriction endonuclease; separation of the fragments in an agarose gel and subsequent Southern blotting[104] and hybridisation with a radiolabelled probe results in a different blotting pattern for each individual. Because of altered restriction sites in these individuals, different areas of DNA are recognised by the probe. It can be seen that person A and person C are both homozygotes, whereas B is the heterozygote.

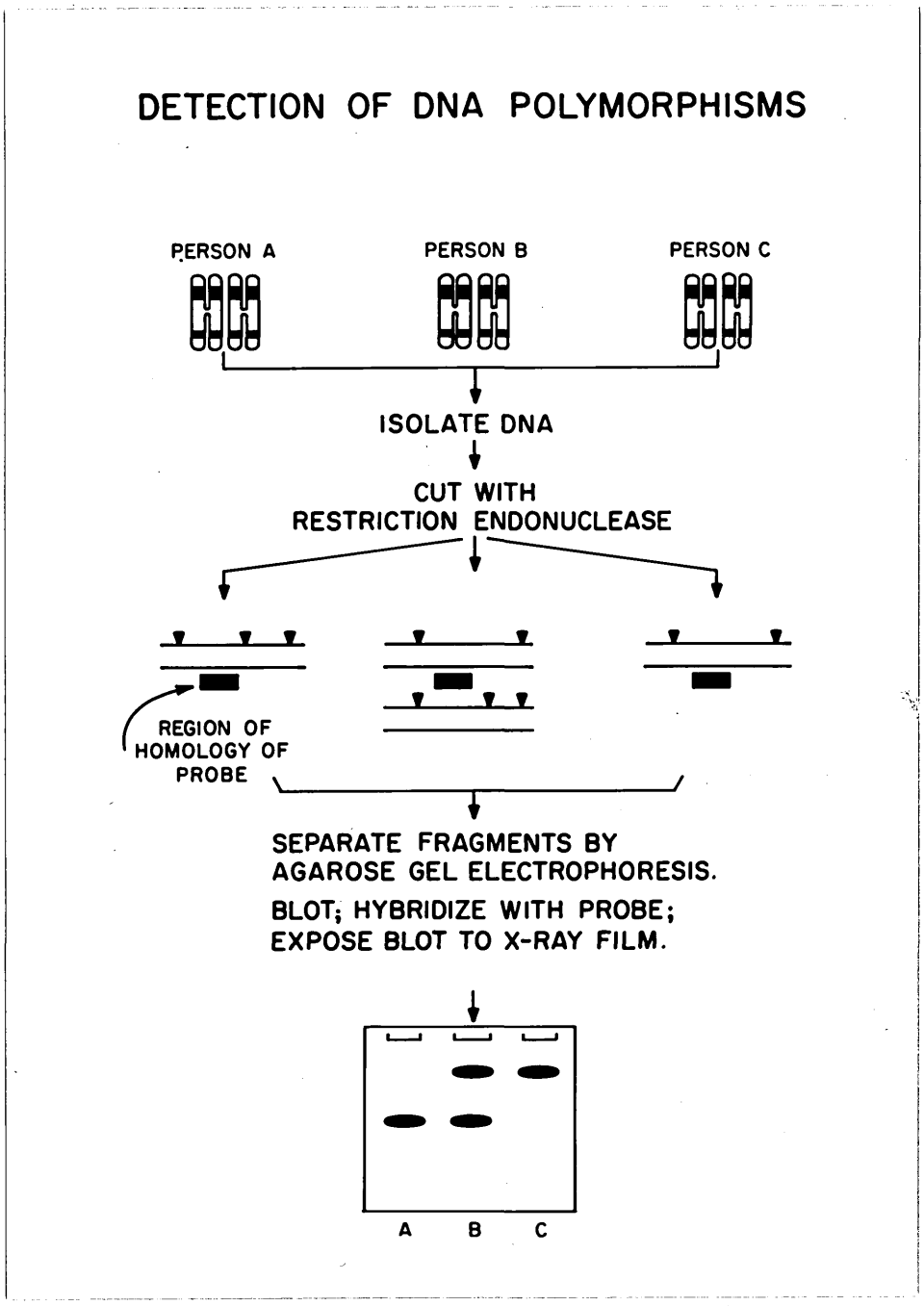

Figure 1

Detection of DNA Polymorphisms in Humans

DNA is isolated from individuals, cleaved with restriction endonuclease, and fragments blotted using the Southern technique. The blot is hybridised to a radiolabelled probe which recognizes a specific site of DNA (indicated by black bar). The restriction sites are indicated by arrows. The normal pattern of restriction sites is seen in person A. In person C, a restriction site has been lost due to mutation (e.g., single base pair substitution DNA or rearrangement). Thus, the probe recognizes a fast migrating band in person A, a slowly migrating band in person C, and in B, the heterozygote, both slow and fast bands.

1.8 The Polyamines in Cancer

Cancer is characterised by uncontrolled growth and spread of abnormal cells. Normal cellular mechanisms of growth become disordered and deregulated. A group of low molecular weight aliphatic amines - the polyamines - have been shown to be essential for normal cell growth and proliferation.[105-111] The polyamines comprise putrescine, spermidine, and spermine.

Putrescine

$H_2N\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}NH_2$

Spermidine

$H_2N\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}NH\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}NH_2$

Spermine

$H_2N\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}NH\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}NH\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}NH_2$

Anthony von Leeuwenhoek, in 1678,[112] was the first to recognise a crystalline substance in semen which precipitated: he termed this 'semenstuf'. Vauquelin was studying the clotting properties of semen and noted crystallisation of a substance.[113] It was not until 1924 that spermine and spermidine were synthesised by Rosenheim.[114] The precursor of spermidine, putrescine, was isolated from vibrio cholera in 1887.[115]

The enzyme ornithine decarboxylase (ODC, EC 4.1.1.17) catalyses the first and rate limiting step of polyamine biosynthesis, namely the conversion of ornithine to putrescine.

The polyamines appear to be essential for periods of proliferation in a tissue. An enhanced accumulation of putrescine in regenerating liver in response to tissue loss was found to be due to a 100- to 500-fold increase in ornithine decarboxylase activity.[110] Increased ornithine decarboxylase activity is also seen in kidneys following contralateral nephrectomy.[110] Further support for the importance of the polyamines in cell division was provided by the observation that polyamine synthesis is enhanced during the late G, and early S phases of the cell cycle.[111]

A major breakthrough in polyamine research was the discovery by Metcalf, et al.,[116] of an irreversible inhibitor of ornithine decarboxylase, alpha difluoromethylornithine (DFMO). Since then, the biosynthetic pathway of the polyamines has been elucidated (Figures

2 and 3).[109,117]

S-adenosylmethionine decarboxylase (AdoMetDC, EC 4.1:1.50) introduces decarboxylated S-adenosylmethionine into the pathway, where it acts as a donor of aminopropyl groups for the conversion of putrescine to spermidine and spermidine to spermine. S-adenosylmethionine is essential to polyamine biosynthesis in its decarboxylated form. The aminopropyl transferases spermidine synthase (EC 2.5.1.16) and spermine synthase (EC 2.5.1.22) (3 and 4 in the diagrams) are regulated by the availability of decarboxylated S-adenosylmethionine and thus AdoMetDC regulates a key step in the pathway.

Both ODC and AdoMetDC are under strict negative control by the polyamines.[117,118] Polyamine metabolism appears to be cyclic in nature (Figure 3). Through consecutive N'-acetylation and oxidation reactions, spermine can be converted to spermidine and spermidine to putrescine. In the diagram, (5) represents spermidine/spermine N'-acetyltransferase and (6) polyamine oxidase.

S-methylthioadenosine (MTA), a by-product of the polyamine pathway is also a feedback inhibitor of spermidine and spermine synthases. MTA is cleaved by MTA phosphorylase restoring the adenine and methionine pools.

Both ornithine decarboxylase and S-adenosylmethionine decarboxylase are highly inducible, for example by trophic agents such as androgens, growth hormone and oestrogens. The stimulation of ODC usually peaks four hours after the administration of hormone,[110] or after hepatectomy.[118] ODC has one of the shortest half-lives of any enzyme (10-30 minutes),[118] AdoMetDC having a half-life of 20 minutes to two hours.[119]

The functions of the polyamines which have been described are manifold,[110,120] and include membrane stabilisation, stabilisation of DNA, association of tRNA, stimulation of both DNA and RNA synthesis, binding of tRNA to chromosomes, and initiation of translation. The possible effects on DNA and protein synthesis are of great interest, and would explain the pivotal nature of the polyamines in cell growth. Many of the effects are attributable to the physiochemical attributes of the polyamines - the amino groups are largely protonated and thus impart a flexibility of structure which allows

interaction with the DNA molecule.[106] In one model of the stereospecific interactions, polyamines bridge the minor groove of DNA with the positively charged amino groups of polyamines interacting with the negatively charged phosphate groups of the DNA backbone.[121] Research on the role of polyamines in cancer began with Hamalainen in 1947 when he made the pioneering observations of increased spermine content of postmortem organs from patients who had died of different types of neoplasm.[122,123] In 1971, Russell found increased urinary excretion of polyamines in patients with metastatic cancer.[124]

Further work by Russell and colleagues revealed urinary polyamines to be an indicator of response to therapy as levels fall following extirpation of the tumour.[124,125] CSF polyamines have been used to measure response to treatment of medulloblastoma.[126]

High urinary polyamine content has been described for a variety of human malignancies including tumours of the gastrointestinal tract, respiratory system, urinary system, female and male reproductive system, breast, central nervous system and bone.[123] Elevated serum polyamines are also seen in the presence of several different cancers.[123]

The mouse skin tumour model of initiation and promotion has provided evidence of the essential role of ODC in carcinogenesis. In 1976, O'Brien[127] found a 250-fold increase in ODC activity five hours after application of 12-O tetradecanoylphorbol-13-acetate (TPA) accompanied by a smaller, slower rise in AdoMetDC activity. His observations led him to believe that induction of ODC was an obligatory event in mouse skin carcinogenesis.

The addition of 1% DFMO to the drinking water of mice subjected to multiple applications of TPA reduced the incidence of papilloma formation by 90%.[128]

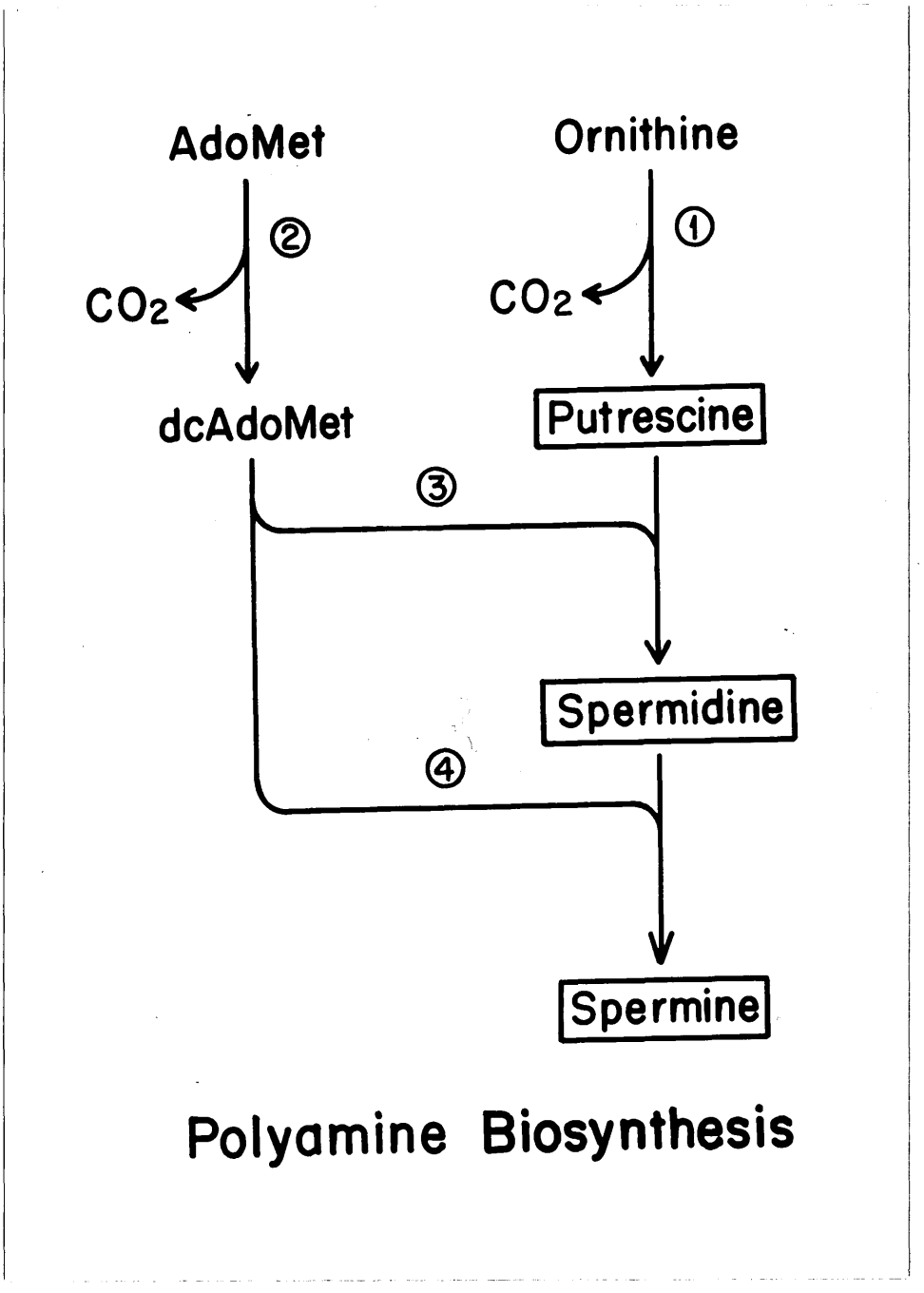

Figure 2

The Polyamine Biosynthetic Pathway

Ornithine decarboxylase (ODC) (1) catalyses the formation of putrescine from ornithine. S-adenosylmethionine decarboxylase (AdoMetDC) (2) catalyses the reaction yielding decarboxylated S-adenosylmethionine (dcAdoMet) from S-adenosylmethionine (AdoMet) which then acts as an aminopropyl donor to form spermine and spermidine: (3) spermidine synthase (4) spermine synthase.

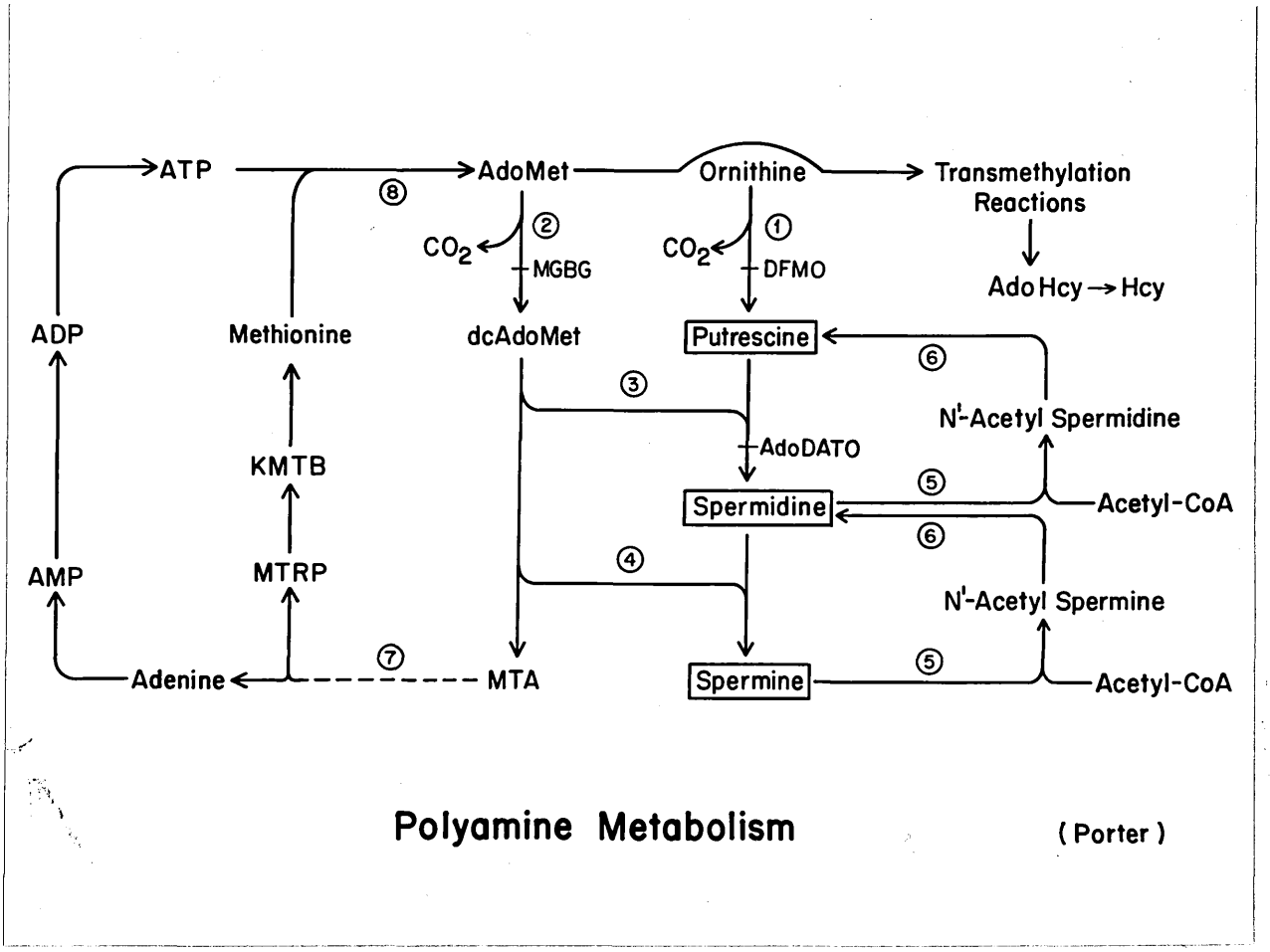

Figure 3

The Cyclic Nature of Polyamine Metabolism

1) Ornithine decarboxylase (ODC).
2) S-adenosylmethionine decarboxylase (AdoMetDC).
3) Spermidine synthase.
4) Spermine synthase.
5) Spermidine/Spermine N'-acetyltransferase.
6) Polyamine oxidase.
7) MTA phosphorylase.
8) AdoMet synthetase.

AdoHcy - adenosylmonocysteine.

HCY - homocysteine.

KMTB - 2-keto-4-methylthiobutyrate.

MTRP - 5-methylthioribose-1-phosphate.

Inhibitors

DFMO - α-difluoromethylornithine - irreversible inhibitor of ODC.

MGBG - methylglyoxal-bis-(guanyl-hydrazone) - non specific inhibitor of AdoMetDC.

AdoDATO (S-adenosyl-1,8,-diamino-3-thioctane) - transition state analogic inhibitor of spermidine synthase.

1.9 Regulation of ODC and AdoMetDC

Because of their importance in cell growth and neoplasia the regulation of ODC and AdoMetDC have been the subject of intense research in the past 15-20 years, although AdoMetDC regulation has lagged behind. Such study was greatly facilitated by the cloning and later sequencing of the mouse gene for ODC,[129-134] and the cloning of the bovine[135] and rat[136] AdoMetDC gene.

It is known that amplification of the ODC gene occurs in certain cell lines resistant to DFMO[129,137] and arginine.[138] Alhonen-Hongisto, et al., have shown that alterations in ODC gene copy number and gene rearrangements are related to altered tumourigenicity in Ehrlich ascites-carcinoma cells.[139] Overproduction of ODC is not always associated with gene amplification however; in an L1210 leukaemia cell line, a 60-fold excess of ODC was accompanied by only a two-fold gene amplification.[140] McConlogue and colleagues have shown in several variants of cell lines which overproduce ODC that altered translatability of the ODC gene also occurs.[141]

Multiple levels of regulation of ODC occur. The down regulation of ODC by the end product of the reaction - the polyamines-occurs both at the level of translation[142-144] and by post-transcriptional[145,146] and post-translational[147] modifications. One of the post-translational modifications known to regulate ODC is the binding of ODC to an inhibitory protein which has been termed antizyme.[148-154] Down regulation also occurs by changes in the rate of enzyme turnover,[155] modification of the protein by phosphorylation,[156] conversion from an active to inactive form,[157,158] antienzyme binding causing enhanced enzyme breakdown,[159,160] and induction of specific enzyme binding leading to enhanced breakdown.[161]

In the androgen-stimulated kidney[162] and in transformed mouse fibroblasts,[163] increased ODC activity was found to be due to an increase in the amount of ODC protein. The administration of the carcinogen 12-O-tetradecanoylphorbol-13-acetate (TPA) to hamster fibroblasts and mouse skin led to increased amounts of both mRNA and protein.[164,165] In the mouse, two RNA transcripts exist measuring 2.2 kb and 2.7 kb in size. They differ because of heterogeneity at the

3′ end.[166]

Regulation of AdoMetDC activity also occurs at multiple levels. AdoMetDC activity decreases in response to the administration of exogenous spermidine.[119] This effect varies depending on the tissue studied,[167] and it has been suggested that AdoMetDC exists in different forms in different tissues.[168]

Treatment with DFMO will result in an increase in both AdoMetDC protein[169] and mRNA.[170] Part of the increase in protein is accounted for by a slowing of degradation of the protein,[169] but increased synthesis of mRNA also occurs.[117] Increases in AdoMetDC activity due to trophic agents occurs by increased protein content due to stabilisation of the protein.[171]

The regulatory mechanisms of ODC and AdoMetDC in solid tumours have not been studied to date.

1.10 Polyamines and the Gastrointestinal Tract

Increased urinary polyamine levels have been reported for patients with colorectal cancer both disseminated[172-174] and localised.[175] Elevated serum polyamines have also been noted.[176-177]

The importance of polyamine biosynthesis in normal gastrointestinal tract physiology has been elucidated by Luk and others. For example, ornithine decarboxylase is increased in the intestine recovering from a chemotherapeutic insult,[178] following jejunectomy,[179] and during the intestinal adaption response to lactation.[180] The normal maturation and recovery of the intestinal mucosa is abolished by DFMO leading to villous atrophy.[178,181,182]

Measurements of the polyamine content of colorectal cancers and polyps compared to normal mucosa revealed statistically significant increases in all types of colorectal neoplasia. Polyamine content in adenomas was as high as in carcinomas and tended to be greater for lesions having severe rather than moderate dysplasia.[183,184] Kingsnorth and colleagues[185] found mean spermine and spermidine content of colorectal cancer specimens to be more than three times that of control mucosa and independent of site of tumour, stage of disease, and histological grade.

LaMuraglia, et al., have found a corresponding increase in ODC

activity in both cancers and polyps of up to 320% of control. Similar elevations were noted for both benign and malignant neoplasms.[186,187] A salient paper by Luk and Baylin[188] noted that ornithine decarboxylase activity was also higher in dysplastic compared to non-dysplastic polyps from familial polyposis patients. Porter, et al.,[189] found a gradient of increased enzyme activity, the activity in polyps being between that of mucosa and carcinoma.

An association between ODC induction and tumour promotion also appears to operate in the colon. Ball has found a significant rise in ODC activity in rodent colon after dimethylhydrazine administration.[190] Takano[191] and Rozhin[192] have both reported early and significant increases in ODC activity following intrarectal instillation of N-methyl-N-nitro-N-nitrosoguanidine (MMNG) or tumour-promoting bile salts.

Bile salts also result in stimulation of ODC activity in gastric mucosa.[193] The intrarectal administration of unsaturated fatty acids (also believed to be tumour promoters in the colon) resulted in a 23-fold to 49-fold increase in ODC activity.[194]

Luk, et al.,[195] observed that the azoxymethane-treated rat model of colonic carcinogenesis appears to be a multi-step process with ODC activity increased four times higher than normal mucosa.

The importance of ODC induction in the causal relationship to the carcinogenic process in the colon has been underlined by observations using the irreversible inhibitor of ODC - DFMO. The concomitant administration of DFMO will markedly reduce the incidence of colon tumours in mice treated with dimethylhydrazine[196] or rats given azoxymethane.[197] DFMO has also been shown to decrease growth of Wilms tumour, renal adenocarcinoma[198] and mammary tumours.[199]

Mouse colon cancer cell line doubling time is increased both in vitro and in vivo by DFMO.[200] The activity of ODC in the colon does not reflect proliferative rate. Ornithine decarboxylase activity is highest in the region of the villous tip and lowest in the crypts, although the crypt is the site of cell division. AdoMetDC activity, however, shows the opposite pattern.[201] Tutton and Barkla[202] found cell proliferation in primary colon tumours to be substantially suppressed by a single dose of DFMO. A much larger dose was required to inhibit normal crypt epithelium in small and large intestines,

however cell proliferation in normal mucosa is known to be faster than in tumours, which characteristically are slow growing.[202,203] They purported that altered regulation of ODC is present in the tumour tissue and that cell proliferation is more ODC-dependent in neoplasia.

In general, the rapid increase of ODC activity is paralleled by an increase in activity of AdoMetDC, although the response is slower and less marked.[119] Porter et al.,[189] found AdoMetDC activity to be high in both colorectal carcinomas and polyps compared to normal mucosa, the mean value for polys being lower than for carcinomas.

1.11 Aims

The aims of this work were several-fold.

1. To map the chromosomal locus of the ODC gene.
2. To map the chromosomal locus of the AdoMetDC gene.
3. To investigate ODC gene polymorphism in tumours.
4. To study ODC mRNA expression in colorectal neoplasia.
5. To correlate mRNA expression with ODC activity in the same sample in order to determine methods of ODC regulation in colorectal neoplasia.
6. To compare ODC mRNA expression in colorectal neoplasia with other solid tumours.
7. To determine if ODC and AdoMetDC gene amplification and/or gene rearrangement occurs in colorectal neoplasia and other solid tumours.
8. To compare ODC mRNA expression with expression of other genes known to be altered in colon neoplasia, e.g., IGF-.2

2. MATERIALS AND METHODS

2.1 Gene Mapping

2.1.1 Somatic Cell Hybridisation

The development of recombinant DNA technology and the use of restriction endonucleases (enzymes derived from bacteria which cut DNA at specific nucleotide recognition sites) has resulted in a logarithmic rise in the number of genes which can be mapped.

The technique of somatic cell hybrid analysis is based on the use of cytogenetics, enzyme marker and recombinant DNA technology.[102,204,205] A summary of mapping by somatic cell hybrid methodology is given in Figure 4. Mouse and human cells are fused using polyethylene glycol. As cells are grown in culture, the entire mouse genome is retained, however, certain human chromosomes are lost. On the same passage, cells are removed and: 1) DNA isolated; 2) homogenised for analysis on starch gel electrophoresis using known enzyme markers - the chromosomal location of which is already known; and, 3) karyotyped to determine which human chromosomes can be recognised. The somatic cell hybrid DNA is digested with restriction endonuclease, separated by electrophoresis in agarose gel and transferred to filters by the Southern technique[104] for hybridisation with the DNA probe to be mapped. The segregation of human-specific bands on the Southern blots is correlated with the results of histochemical staining and karyotyping to assign the gene locus.

A panel of human-mouse hybrids is obtained so that the full human chromosome complement is obtained.

A test blot is usually run where DNA is digested with several different restriction endonuclease, and the enzyme resulting in good separation between human and mouse bands for that gene, is chosen for subsequent mapping experiments.

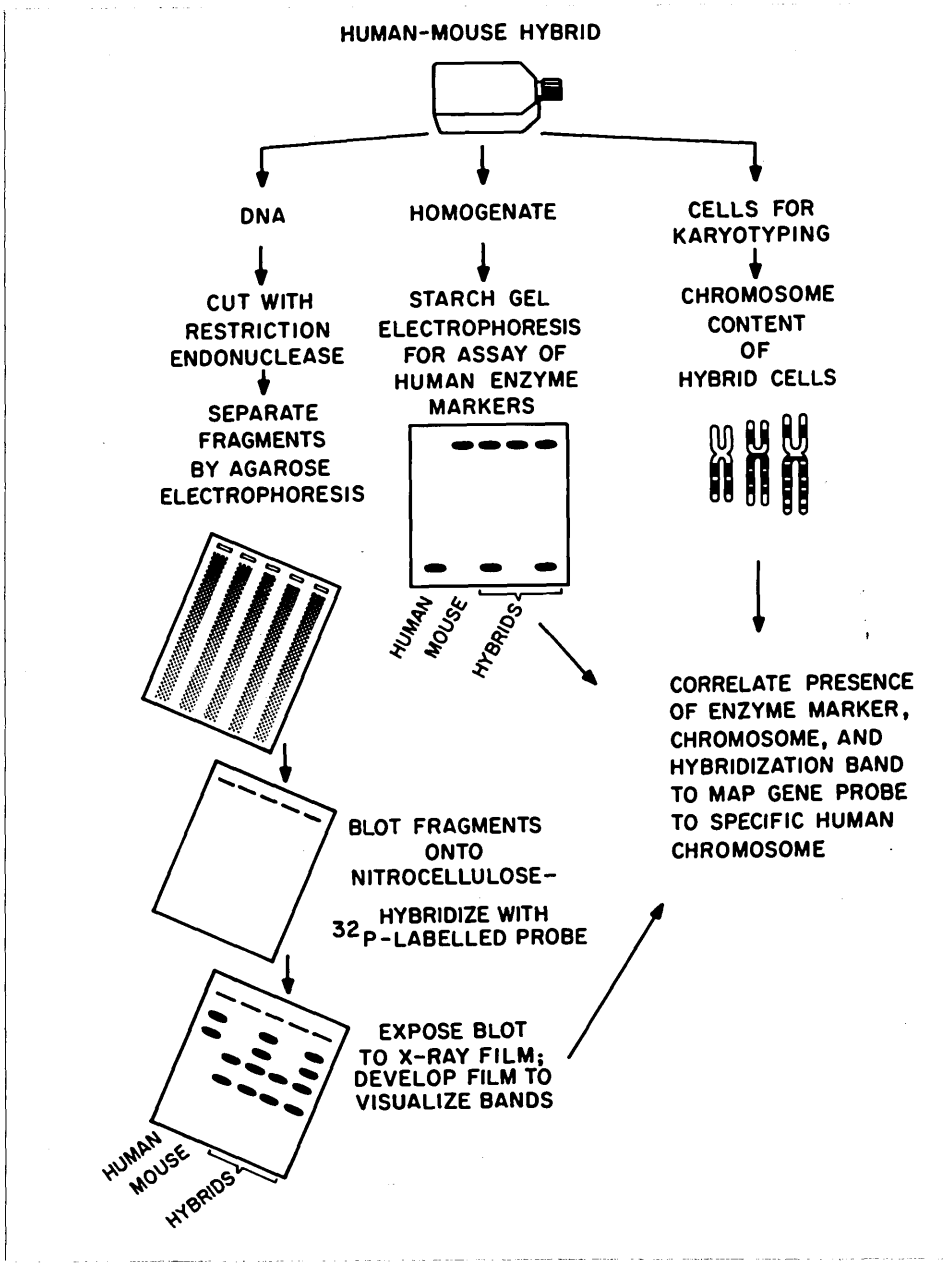

Figure 4

Mapping of Cloned Genes in Human-mouse Somatic Cell Hybrids by Southern Blotting

Clones of cell hybrids containing certain human chromosomes are cultured and harvested. From the same passage cells are used to prepare DNA, cell-free homogenates, and for karyotyping. Human enzyme markers previously assigned to human chromosomes are assayed by histochemical staining. Confirmation of human chromosome content is provided by karyotyping. DNA from each group of cell hybrids is cleaved with restriction endonuclease, giving a unique pattern of human and mouse fragments when hybridised to a radiolabelled gene after Southern blotting. The segregation of human-specific bands on Southern blots is correlated with the results of histochemical staining and karyotyping to assign the gene to a chromosomal locus.

The presence of chromosome translocations within the hybrids, which can be identified karyotypically, allows regional mapping of certain genes.

2.1.2 Mapping the ODC Gene

The human-mouse hybrid cell lines where derived from the following hybrid sets, the human and mouse parental cells given in parenthesis: REW (WI-38 x RAG), WIL (WI-38 x LTP), XER (GM2859 x RAG), JWR (JoVa x RAG), JSR (JoSt x RAG), 1CL (GM1006 x LMTK$^-$, ATR (A1Tr x RAG), TSL (GM2808 x LMTK$^-$), REX (CaVa x RAG), SIR (GM469 x RAG), DUA (DUV x A9),[206] GAR (GM806 x RAG),[207] VTL (VT x LMTK$^-$),[208] DUM (DUV x RAG), NSL (GM2836 x LMTK$^-$), XOL (GM0097 x LMTK$^-$), XTR (GM194 x RAG).

The hybrids had been analysed previously for human chromosome content using enzyme markers[102] and karyotyping techniques.[209,210] Cells were examined on the same passage through cell culture.

Table 1 gives further details of the chromosomes retained in these hybrids.

10 μg DNA from 31 cell hybrids was digested with the restriction endonuclease *Pst*I (Boehringer Mannheim) under the conditions described by the manufacturer. These hybrids were derived from 13 unrelated human and 4 mouse cell lines. The enzyme *Hind*III was used to digest DNA from 37 cell hybrids involving 13 unrelated human and 4 mouse cell lines. 0.1 vol sucrose dye marker was added and digested DNA fragments were separated by electrophoresis in 0.8% agarose in a Tris acetate buffer for 16-24 hours at 33-35v, 40-50 mA. The gels were stained with ethidium bromide (10 mg/ml) and photographed to confirm complete digestion. Gels were denatured for thirty minutes in 0.5 MNaOH and 1.5 M NaCl, then neutralised for thirty minutes in 3MNaCl 0.5 M tris pH 7.0. DNA was then transferred to zetapor (AMF Cuno) in 20 x SSC overnight by the technique described by Southern.[104] Filters were pretreated with 2 x SSC, baked in a vacuum oven at 80° C for 3-4 hours and

washed for 1 hour in 0.1 x SSC, 0.5% SDS (sodium dodecylsulphate) at 65°C.

Prehybridisation took place at 42°C overnight in 50% deionised formamide, 5 x SSC/5 x Denhardt's, 50mM $NaPO_4$ pH 6.5, 500 μg/ml sonicated salmon sperm DNA, and 0.1% SDS.

The ODC probe was labelled with ^{32}P by nick translation and the blots hybridised for 48 hours at 42° C in a solution containing 200 μg/ml sonicated salmon sperm DNA, 50% formamide, 5 x SSC/5 x Derhardt's, 1 x SSC, 20 mM $NaPO_4$ (pH 6.5), 10% dextran sulphate, and 0.1% SDS. Filters were washed under low stringency conditions (1 x SSC; 0.1% SDS) for 1 hour at 60°C. Autoradiographs were obtained after exposure at -70° for 3-5 days. Blots were then washed under conditions of high stringency (0.1 x SSC, 0.1% SDS) at 68°C for 1 hour. After 7-14 days at -70°C, autoradiographs were obtained.

Table 1

Mouse-human Cell Hybrids for Mapping ODC

Hybrid	Human chromosomes retained
ATR-13	1,2,3,4,5,6,7,8,10,12,13,14,15,16,17,18,19
DUA-1CSAZF	7
DUA-3BSAGA	2,7,8,13,14,17
DUA-5BSAGA	3,5,11,14,17,18,21
DUM-13	1,2,3,5,6,7,10,11,12,14,16,17,18,19,20,21,22
GAR 1	3,5,8,10,12,14,15,16,20,X
ICL-15	8,12,17,20,21
JSR-2	3,4,7,13,14,X
JSR-14	2,3,4,5,6,12,13,17,20,21,X
JSR-17S	1,2,3,5,8,9,10,11,12,13,14,15,16,17,18,20,21,22
JWR-22H	4,6,7,10,11,12,13,14,15,17,18,20,21
NSL-5	1,8,10,12,14,16,18,20
NSL-16	3,4,5,7,8,10,12,14,15,16,17,18,20,21
NSL-9	5,8,10,12,13,14,15,16,17,20,21,22
REW-7	1,2,3,4,5,6,7,8,10,11,12,13,14,15,17,18,19,20,21,22,X
REW-10	1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,X
REX-11BSAgB	3,10,14,15,18
REX-11BSHF	3,10,14,18
REX-26	1,2,3,4,7,8,8,10,11,12,14,15,16,17,18,19,21
REX57BSHB	14,17,21
SIR 8	1,2,3,4,5,7,8,9,10,11,12,13,14,15,16,17,18,21,22,X
SIR-11	7,13,21,22,X
TSL-1	3,4,9,10,11,13,14,16,17,18,20,21
TSL-2	2,5,6,10,12,18,20,21,X
VTL-6	2,6,7,8,10,11,15,17,19,20,21,22
VTL-7	11,14,17,19,20,21,22
VTL-17	5,7,10,11,13,14,17,20,21
VTL-8	13,15,17,20,21,22
WIL-1	8,12,14,17,18,21,X
WIL-2	8,12,15,17,21,X
WIL-5	4,8,10,17,18,21,X
WIL-6	2,4,5,6,7,,8,10,11,14,17,18,20,21,X
WIL-7	2,3,5,6,8,10,11,13,14,17,18,21,X

Table 1 (continued)

Mouse-human Cell Hybrids for Mapping ODC

WIL-8X	3,4,5,7,8,10,11,12,14,17,18,19,20,21,X
WIL-8Y	3,6,7,8,10,11,14,15,16,17,18,19,20,21,22,X
WIL-14	2,7,8,10,14,15,17,X
WIL-13	3,5,17,18,21,22
WIL-2CSAZ	8,10,12,17,21
XER-11	1,3,4,5,6,7,8,9,10,12,15,16,17,18,19,20,21,22
XOL-6	5,6,7,10,11,12,14,17,19,20,22
XOL-9	2,3,4,6,12,15,17,18,19,21,22,X
XTR-22	2,4,5,6,8,10,11,15,18,19,20,21,22,X
XTR-3BSAgB	9,12,20,21

2.1.3 Mapping the AdoMetDC Gene by Somatic Cell Hybrid Techniques

DNA was obtained from somatic cell hybrids. DNA from 38 somatic cell hybrids involving 14 unrelated human cell lines and four mouse lines was digested with the restriction endonuclease *Pst*I (see Table 2). 10 μg of each sample was digested at 37°C for three hours according to the manufacturer's instructions (Boehringer-Mannheim). The reaction was stopped by heating to 65°C for 10 minutes. 0.1 vol sucrose dye marker was added and the samples loaded on a gel. Separation, transfer, baking, pretreatment and hybridisation was as described for ODC mapping. The filters were rinsed briefly in 2 x SSC, 0.1% SDS at room temperature, then washed individually three times for 20 minutes in 0.1 x SSC, 0.1% SDS at 50°C. Dried filters were exposed to Kodak XAR 5 X-ray film with an intensifying screen for 3-10 days.

The AdoMetDC probe used in these experiments was randomly primed.

Table 2

Mouse-human Cell Hybrids for Mapping AdoMetDC

Hybrid	Human Chromosomes Retained
ATR-13	1,2,3,4,5,6,7,8,10,12,13,14,15,16,17,18,19
DUA-3BSAGA	2,7,8,13,14,17
DUA-5BSAGA	3,5,11,14,17,18,21
DUM13	1,2,3,5,6,7,10,11,12,14,16,17,18,19,20,21,22
GAR 1	3,5,8,10,12,14,15,16,20,X
ICL 15	8,12,17,20,21
JSR 14	2,3,4,5,12,14,17,18,20,21
JSR17-S	1,2,3,5,8,9,10,11,12,13,14,15,16,17,18,20,21,22
JWR-22H	4,6,7,10,11,12,13,14,15,17,18,20,21
JWR 26C	2,3,4,5,6,7,9,10,11,12,14,15,16,17,18,20,21,X
NSL-5	1,8,10,12,14,16,18,20
NSL-9	5,8,10,12,13,14,15,16,17,20,21,22
NSL-16	3,4,5,7,8,10,12,15,16,17,18,20,21
REW-5	1,2,3,4,5,6,7,8,10,11,12,13,14,15,17,18,19,21,22,X
REW 11	4,7,11,12,13,16,20,21,22,X
REX-11BSAgB	3,10,14,15,18
REX-11BSHF	3,10,14,18
REX26	1,2,3,4,7,8,9,10,11,12,14,15,16,17,18,19,21
SIR-8	1,2,3,4,5,7,8,9,10,11,12,13,14,15,16,17,18,21,22,X
SIR 11	2,9,13,21,X
TSL-1	3,4,9,10,11,13,14,16,17,18,20,21
TSL-2	2,5,6,10,12,18,20,21,X
VTL-6	2,6,7,8,10,11,15,17,19,20,21,22
VTL-7	11,14,17,19,20,21,22
VTL-8	13,15,17,20,21,22
VTL-17	5,7,10,11,13,14,17,20,21
WIL 1	8,12,14,17,18,21,X
WIL-2CSAZ	8,10,12,17,21
WIL 5	4,8,10,17,18,21,X
WIL 6	2,4,5,6,7,8,10,11,14,17,19,20,21,X
WIL 7	2,3,5,6,8,10,11,13,14,17,18,21,X
WIL 8X	3,4,5,7,8,10,11,12,14,17,18,19,20,21,X

Table 2 (continued)

Mouse-human Cell Hybrids for Mapping AdoMetDC

WIL 8Y	6,7,8,10,11,14,17,18,20,21,X
WIL 14	1,3,5,7,8,10,12,14,15,17,X
XER-7	1,2,3,4,5,6,7,8,9,10,12,13,14,15,18,19,X
XER 11	1,3,4,5,6,7,8,9,10,12,15,16,17,18,19,20,21,22
XTR-3BSAgH	1,3,4,5,9,10,12,20,21
XTR 22	2,4,5,6,8,10,11,15,18,19,20,21,22,X

2.1.4 Mapping the ODC Gene by In Situ Hybridisation

The method of in situ hybridisation combines molecular techniques with high resolution chromosome banding. The method used in Dr. Shows' laboratory combines two modifications of the technique by Harper and Saunders.[211] Longer prometaphase chromosomes are obtained using bromodeoxyuridine as a cell-synchronizing agent, and a Hoechst 33258/Giemsa chromosome staining method results in high-resolution chromosome banding.[212]

2.2 DNA Extraction (Cells)

Approximately 10^8 cells were removed from plastic flasks by trypsinisation and washed several times with serum-free medium. DNA was extracted as described in Naylor, et al.[213]

2.3 Experiments on ODC DNA Polymorphism in Human Blood Samples

Samples of whole blood were obtained from Red Cross volunteers. White blood cells were isolated from heparinised blood using the dextran sedimentation method described by Skoog and Beck.[214] 3% dextran, MW 228,000, pH 7.3, was added 2:1 to normal blood for a total volume of 5 ml. Red cell sedimentation occurred in 18-20 min. The overlying supernatant plasma-dextran suspension of leukocytes and platelets was removed without disturbing the red cells. Leukocytes were selectively sedimented by centrifuging the supernatant at 800-1200 RPM for 7-10 min. The resulting leukocyte pellet was frozen in liquid nitrogen for later DNA extraction, or used immediately.

2.4 Study of ODC Expression in Tumours

2.4.1 Tissue Procurement

Surgical samples were obtained with the help of the Department of Surgical Oncology Service of Roswell Park Cancer Institute. Patients gave signed consent prior to surgery that

their operative specimens may be used for research within the Institute. Shortly after resection, the samples were taken to the Pathology Department where a staff pathologist dissected samples of tumour and adjacent normal mucosa. In the case of colon, rectum, and stomach, mucosa was dissected from submucosa, the sample being taken from grossly normal tissue approximately 5 cm distant from the neoplasm. Tissue was frozen in liquid nitrogen and stored at -70°C for later extraction of DNA and RNA.

2.4.2 RNA Extraction

Tissue samples were removed from -70°C and placed in liquid nitrogen. A portion equivalent to 0.5-1 cm^3 was removed, wrapped in clear kitchen wrap and pulverised into small fragments. Fragments were added to 15 ml 4M guanidine isothiocyanate and homogenised using a Polytron. After filtering with sterile gauze, the volume was made up to 15 ml and 3 g ultrapure caesium chloride added and dissolved. The mixture was layered onto a 3.5 ml 5.7 M cushion of calcium chloride in a SW 41 polyallomer tube.[215] Specimens were centrifuged in a Beckman ultracentrifuge at 30,000 rpm at 20°C for 18 hours. The supernatent was removed with a pipette and the RNA pellet dissolved in 1 ml 10 mM Tris pH 7.4, 5 mM EDTA, 1% SDS. The RNA solution was decanted into a falcon tube, the SW 41 tube rinsed and the rinse solution pooled with the RNA solution. Extraction was performed with an equal volume of 4:1 chloroform:butanol. Phases were separated by spinning for 10 minutes at 2000 rpm. The top phase was removed and the organic phase reextracted with an equal volume of the Tris/EDTA/SDS buffer. To the RNA solution was added 0.1 vol Na acetate pH 5.2 and 2.2 vol 100% ethanol. Afer 2-12 hours, at -20°C the solution was centrifuged at 9500 RPM for 15 minutes at 4°C. The pellet was dissolved in filtered water and reprecipitated in sodium acetate and ethanol as above. The final RNA pellet was dissolved in filtered water and quantified by absorbance.

2.4.3 Dot Blots

Dot blot Northern analysis was performed according to the procedure of Kafatos, et al.[216] Briefly, this involved incubating the RNA samples with SSC and formaldehyde, then spotting 10 μg of denatured total RNA onto zetabind (AMF Cuno) using a Schleicher and Schuell apparatus.

2.4.4 Northern Analysis

10 μg RNA was ethanol precipitated using 0.1 vol 3.0 M sodium acetate pH 5.2 and 2.2 vol cold 100% ethanol. The pellet was resuspended in 3.7 μl 0.2 micron filtered water to which was added 2.7 μl deionized glyoxal (40%),[217] 8 μl spectrograde DMSO, 1.6 μl 0.1 M NA phosphate pH 6.8. Samples were incubated at 50°C for 60 minutes. After cooling to room temperature, 4 μl of RNA loading buffer was added to each sample (50% glycerol, 0.01 M Na phosphate, pH 7, and 0.4% bromophenol blue). Samples were loaded onto a 1.2% agarose gel in 0.01 M Na phosphate, pH 6.8, and run at 95 volts for four hours with the buffer recirculated from cathode to anode to maintain equal pH. RNA was transferred overnight onto zetabind and dried at room temperature. Shortwave ultraviolet light for three minutes exposure crosslinked the RNA to zetabind and allowed localisation of the 18S and 28S bands.

Northern blots were prehybridised for five minutes at 65°C in 1%.BSA, Na_2 HPO_4/NaH_2 PO_4 0.5 mM, Na_2 EDTA 1 mM, 7% SDS, pH 7.0. Radiolabelled probe was added, total counts being 10 x 10^6 cpm/ml and blots were hybridised overnight at 65°C in the same solution used for prehybridisation.

The blots were removed and washed twice in buffer A (see Appendix) at 65°C for 10 minutes each. Blots were washed three times in buffer B at 65°C, 10 minutes each. After a very brief wash in ddH_2O, blots were dried and exposed to film.

To quantify the amount of ODC expression, Northern blots were also hybridised with the cDNA probe for the enzyme triose phosphate isomerase (TPI). TPI is a housekeeping enzyme

involved in glycolysis, gluconeogenesis and the pentose phosphate shunt. Swartout, et al.,[218] have also used TPI as an internal control for experiments on haematologic malignancies.

2.5 Measurement of ODC Activity

ODC activity was measured using the technique of Seely and Pegg.[219]

2.6 Experiments on ODC and AdoMetDC DNA Gene Amplification and Deletion in Colorectal Neoplasia and Other Tumours

2.6.1 DNA Extraction (Tissue)

0.5 g of frozen tissue was thawed and minced into small pieces with a scalpel. The sample was homogenised using a Dounce apparatus on ice in 10 mM Tris-HCl, pH 8.0 10 mM EDTA, 150 mM NaCl. The homogenate was decanted into a Falcon tube and the homogeniser rinsed with fresh buffer which was pooled with the homogenate. The volume was made up to 10 ml and Proteinase K added to a concentration of 1 mg/ml. SDS was added to a final concentration of 1% and the tubes incubated overnight at 37°C. Extraction was performed once with an equal volume of phenol (10,000 RPM 10 min.) and once with chloroform:iso amyl alcohol (24:1). The aqueous phase was decanted with a large bore pipette and to this added 1/10 vol 3M sodium acetate and 1 vol isopropanol. After inverting the tube several times DNA floated to the surface where it could be collected with a hooked pipette. DNA was rinsed in 70% ethanol and dissolved in 1-2 ml of 10 M Tris-HCl, pH 8.0, 10 mM EDTA. Quantitation was achieved by reading absorbance at A-260.

2.6.2 Southern Analysis

10 μg DNA was digested with restriction endonucleases run on 0.8% agarose in tris acetate buffer and transferred to zetapor by the Southern technique. Prehybridisation and

hybridisation were as described for somatic cell mapping. Filters were rinsed briefly at room temperature in 2 x SSC 0.1% SDS then washed at 50° in 0.1 x SSC 0.1 % SDS three times. Autoradiographs were obtained after two to seven days at -70°.

2.7 Probes and Labelling Methods Used

2.7.1 ODC

The human ODC cDNA clone (pODC 10/2H) was a kind gift from O. Janne. The clone is 1,825 nucleotides long, with an open reading frame of 1,383 nucleotides and is inserted into the *Eco*RI site of pBR 322.

2.7.2 AdoMetDC

The rat cDNA cloned by Pegg, et al.,[136] was used in the experiments. The 172 bp *Eco*RI fragment is located approximately 370 bp from the 5′ end. Significant homology is present to the bovine AdoMetDC sequence reported by Mach, et al.[135]

2.7.3 IGF-2

The IGF-2 probe contains a 1046-base pair insert with an open reading frame of 790 nucleotides coding for the 180 amino acid precursor for IGF-2.[86]

2.7.4 TPI

The cDNA clone TPI-5a was a kind gift of L. Maquat.[220,221] The clone comprises the last two nucleotides of the translation initiation coding region, the entire 744 - nucleotide coding region and the 448 nucleotide 3′ untranslated region.

2.7.5 Random Priming

Random priming was used to label linear DNA probes. The following were combined - ^{32}P dATP 200 μCi (after drying in dessicator) DNA probe 200 ng, BSA (10 mg/ml) 1 μl, Hexamer 2.5 μl, buffer 2.5 x (10 μl) Klenow 2 μl, H_2O to 25 μl. The reaction was left overnight and the probe separated on a Sephadex column and frozen.

2.7.6 Nick Translation

DNAse I (5 μl) was activated by mixing with 45 μl activation buffer and set on ice for two hours. The following were combined: 10 x Nick translation buffer 10 μl, BSA (1 mg/ml) 5 μl, 1/10 dATP-dGTP 9 μl, 0.5 μg probe DNA, ^{32}P dCTP 90 pmoles, ^{32}P dTTP 90 pmoles, water to 95 μl. DNASE I was diluted 5 μl in 495 μl and 1 μl of the second dilution added to the mixture. After incubation at 15°C for 10 minutes, 4 μl of E. coli polymerase I was added. Incubation continued at 15° C and time points taken until an adequate number of counts obtained. The reaction was stopped with 0.25 M EDTA buffer and the sample frozen.

The labelled probe was run through a 650 Sephadex column to remove unbound nucleotide and the probe frozen again for later use. 5 x 10^6 counts per blot were used for hybridisation.

2.8 Statistical Analysis

Nonparametric statistics were calculated to avoid any assumption that the data had a Gaussian distribution. Computations were made with SPSS (Statistical Package for the Social Sciences). Levels of RNA and activity were compared among groups based on Dukes stage, tumour primary site, and degree of differentiation using a Mann-Whitney test. Matched pairs of tumour and adjacent normal mucosa from the same patient were assessed for correlation with a Spearman rank-order test and for equality of ODC level with the Wilcoxon Matched Pairs test.

3. RESULTS

3.1 Mapping the ODC Gene

3.1.1 Somatic Cell Hybrids

The chromosomal assignment of ODC was determined by correlating the presence or absence of specific human chromosomes in a panel of human-mouse somatic cell hybrids with ODC sequences in the DNA isolated from these hybrid cell lines. The human ODC DNA probe (pODC 10/2H) hybridised to six *Pst*I fragments of human cell line DNA. These fragments measured 9.9, 8.4, 5.5, 4.6, 2.8, and 2.1 kb in length (See Figure 5).

Under conditions of high stringency, the 9.9 kb band and 2.1 kb band became less prominent (Figure 6). In cell hybrids, these bands in addition to the 8.4 kb and 4.6 kb bands cannot be scored easily due to either overlapping mouse bands or faintness of the signal. Lanes 1, 3, and 5 are positive for the 2.8 kb band; lane 3 positive for the 5.5 kb band; and lanes 1 and 5 contain hybrids positive for the 8.4 kb band. Analysis of 31 mouse-human cell lines and DNA from unrelated individuals demonstrated that the 8.4 and 5.5 kb bands were alleles at the same locus, and these two bands were counted together for scoring purposes.

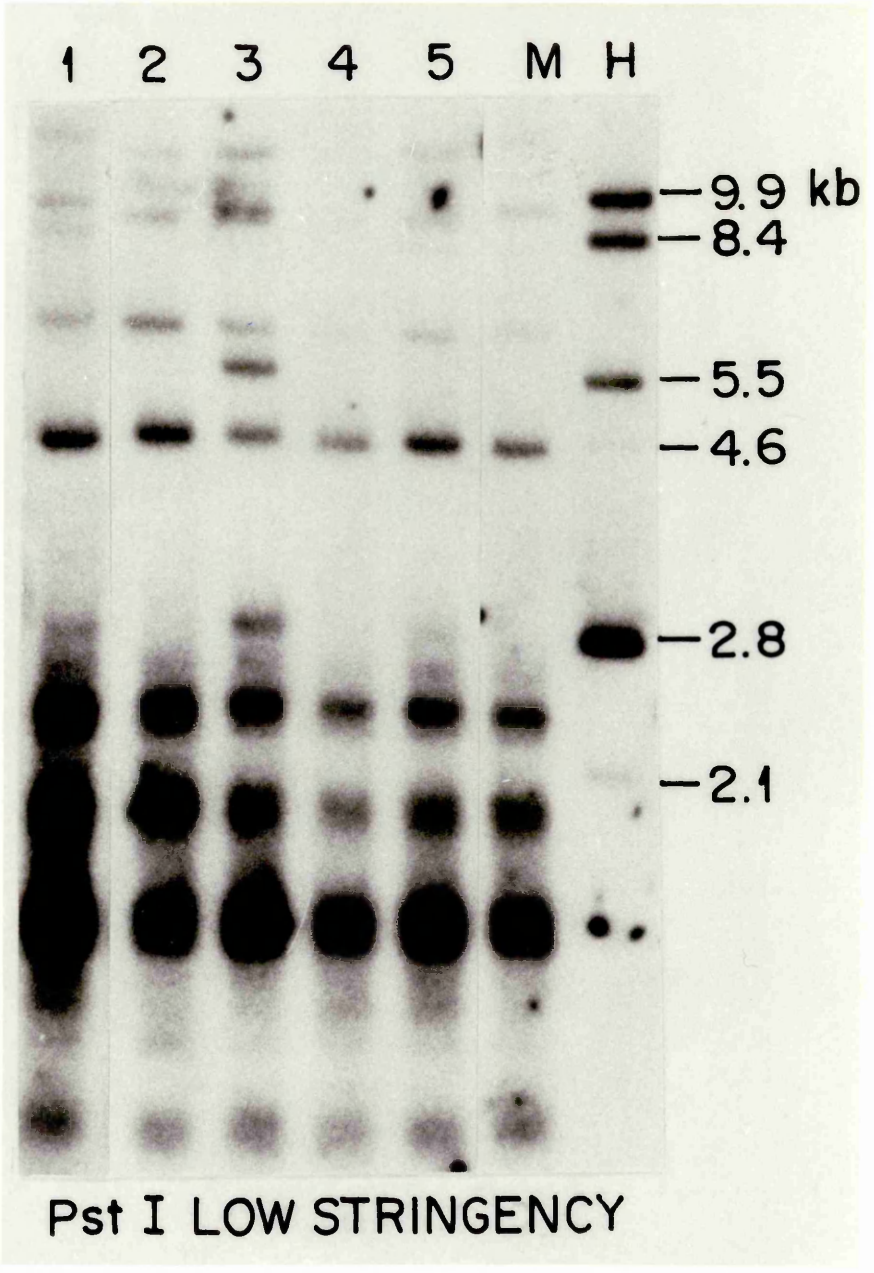

Figure 5

<u>*Pst*I Digest of Human-mouse Hybrid DNA Hybridised With pODC 10/2H - Low Stringency</u>

Southern hybridisation of cDNA probe PODC 10/2H to *Pst*I digests of DNA from human (H), mouse (M), and somatic cell hybrids (lanes 1-5). Low stringency wash conditions; lane 3 is positive for the 5.5 kb band; lanes 1, 3 and 5 are positive for the 2.8 kb band; and lanes 1 and 5 are very weakly positive for the 8.4 kb band and are often difficult to score in cell hybrids. Lengths of DNA bands are indicated in kb on the right of the figure.

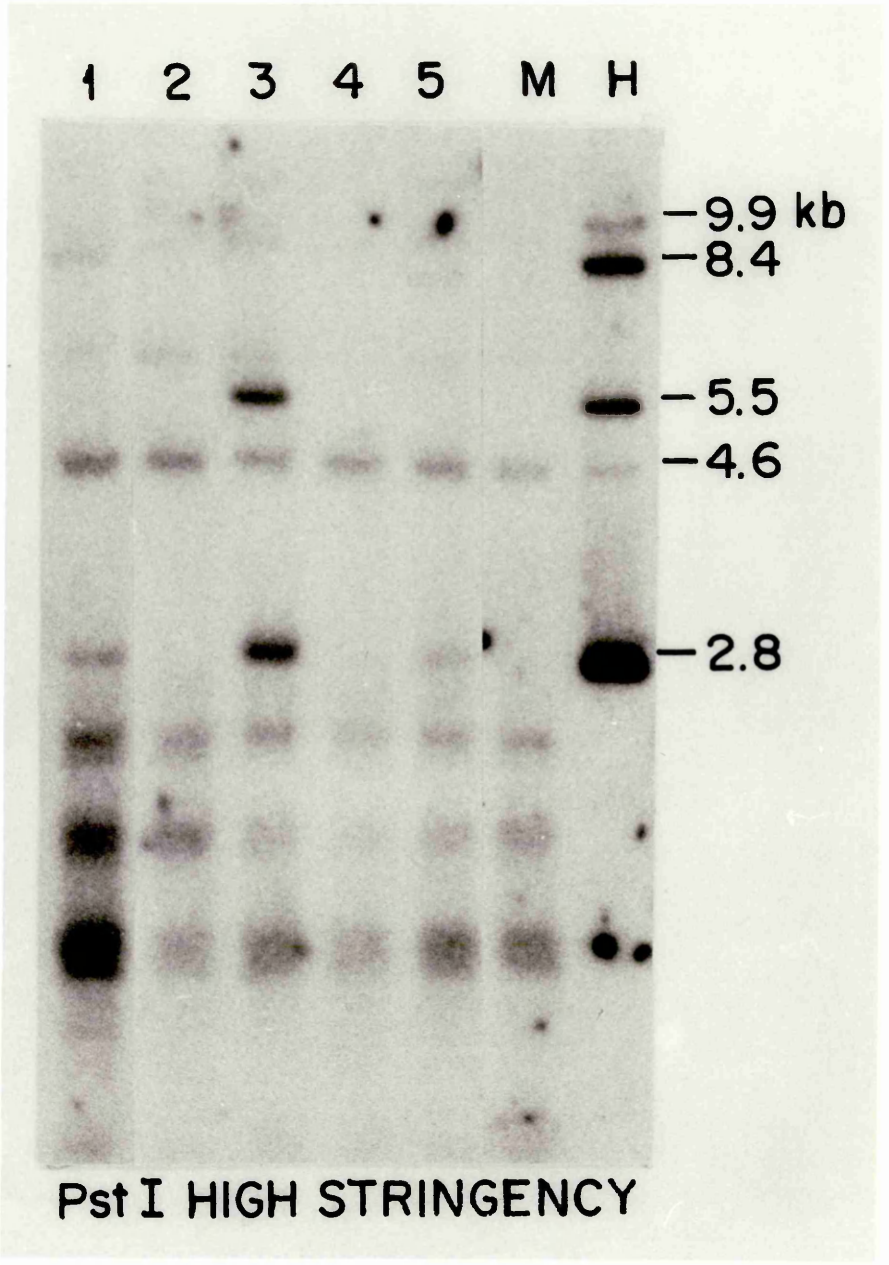

Figure 6

PstI Digest of Human-mouse Hybrid DNA Hybridised With pODC 10/2H - High Stringency

Same blot as Figure 5 but under high stringency conditions: the 9.9 and 2.1 kb bands become less prominent.

As shown in Table 3, the 8.4 and/or 5.5 kb bands segregate together with the 2.8 kb band (only cell hybrids were included that could unequivocally be scored for these 3 bands). These three bands were distributed in cell hybrids that without exception retained human chromosome 2. All other human chromosomes segregated independently of the three bands. Therefore, sequences for these three bands are encoded on human chromosome 2.

Regarding the 9.9 kb band, experiments using the human-mouse hybrid DUA-1CSAZF, which has retained only human chromosome 7, reveal that the 9.9 kb band on *PstI* digests is present in this hybrid (Figure 7) and therefore this band maps to human chromosome 7 (detailed mapping of ODC sequences to chromosome 7 is described below).

3.1.2 In Situ Hybridisation

In situ hybridisation was effective in localising the sequences on to a specific site on chromosome 2 (Figures 8 and 9). Of the 1,987 metaphase chromosome sets examined, 361 attached silver grains were seen with 58 grains (16.1%) touching chromosome 2. Thirty-one percent of the grains on two were located at 2p25. Fourteen grains were distributed on chromosome 7 but never above background levels at any site. No significant distribution of grains was observed on any human chromosome except chromosome 2 at p25. This locus was termed ODC1.

Table 3

(see following two pages)

Legend Table 3

Distribution of the human ODC gene in DNA from cell hybrids digested with *Pst*I. A "t" in the table indicates a chromosome translocation for a particular chromosome, but no intact chromosome is present. Concordant hybrids have either retained or lost the human ODC bands together with a specific human chromosome. Discordant hybrids have either retained the gene, but not a specific chromosome, or the reverse. Percentage of discordancy indicates the degree of discordant segregation for a marker and a chromosome. A 0% discordancy is the basis for chromosome assignment.

Table 3

Segregation of Human Chromosomes and Fragments Hybridising to the ODC Probe in *PstI* Digested DNA From Human-Mouse Cell Hybrids

	Bands kb			HUMAN CHROMOSOMES																							
HYBRID	8.4	5.5	2.8	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Translocations
ATR-13	+	-	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	-	-	-	t	5/X
DUA-1CSAZF	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
DUA-3BSAGA	-	+	+	-	+	-	-	-	-	+	+	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	
DUA-5BSAGA	-	-	-	-	-	+	-	+	-	-	-	-	-	+	-	-	+	-	-	+	+	-	-	+	-	-	
DUM-13	+	-	+	+	+	+	-	+	+	+	-	-	+	+	+	-	+	t	+	+	+	+	+	+	+	t	X/15, 15/X
GAR-1	-	-	-	-	-	+	-	+	-	-	+	-	+	-	+	-	+	+	+	-	-	-	+	-	-	+	
ICL-15	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	+	-	-	+	+	-	-	
JSR-2	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	
JSR-14	+	+	+	-	+	+	+	+	+	-	-	-	-	-	+	+	-	-	-	+	-	-	+	+	-	+	
JSR-17S	+	-	+	+	+	+	-	+	-	t	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	7/9
JWR-22H	+	-	+	t	t	-	+	-	+	+	-	-	+	+	+	+	+	+	-	+	+	-	+	+	-	-	2/1
NSL-5	-	-	-	+	-	-	-	-	-	-	+	t	+	-	+	-	+	-	+	t	+	-	+	-	-	-	17/9,12q+
NSL-16	-	-	-	-	-	+	+	+	-	+	+	t	+	-	+	-	+	+	+	+	+	-	+	+	-	-	17/9
REW-7	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	
REX-11BSAgB	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	+	+	-	-	+	-	-	-	-	-	
REX-11BSHF	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-	t	t	22/X
REX-26	+	-	+	+	+	+	+	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	t	t	22/X
SIR-8	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	
SIR-11	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	+	

Table 3 (continued)

Segregation of Human Chromosomes and Fragments Hybridising to the ODC Probe in *Pst*I Digested DNA From Human-Mouse Cell Hybrids

	Bands kb			HUMAN CHROMOSOMES																							
HYBRID	8.4	5.5	2.8	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Translocations
TSL-2	-	+	+	-	+	t	-	+	+	-	-	-	+	-	+	-	-	-	-	t	+	-	+	+	-	+	17/3 3/17
VTL-6	+	-	+	-	+	-	-	-	+	+	+	-	+	+	-	-	-	+	-	+	-	+	+	+	+	-	
VTL-7	-	-	-	-	-	-	-	-	-	t	-	-	-	+	-	-	+	-	-	+	-	+	+	+	+	-	7q-
VTL-17	-	-	-	-	-	-	-	+	-	+	-	-	+	+	-	+	+	-	-	+	-	-	+	+	-	-	
WIL-2	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-	+	-	-	-	+	-	+	
WIL-5	-	-	-	-	-	-	+	-	-	-	+	-	+	-	-	-	-	-	-	+	+	-	-	+	-	+	
WIL-6	+	-	+	-	+	-	+	+	+	+	+	-	+	+	-	-	+	-	-	+	-	+	+	+	-	+	
WIL-7	+	-	+	-	+	+	-	+	+	-	+	-	+	+	-	+	+	-	-	+	+	-	-	+	-	+	
WIL-8X	-	-	-	-	-	+	+	+	-	+	+	-	+	+	+	-	+	-	-	+	+	+	+	+	-	+	
WIL-8Y	-	-	-	-	-	+	-	-	+	+	+	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	
WIL-14	-	-	-	-	-	+	-	-	-	+	+	-	+	-	-	-	+	+	-	+	-	-	-	-	-	+	
XER-11	-	-	-	+	-	+	+	+	+	+	+	+	+	t	+	-	-	+	+	+	+	+	+	+	+	t	11/X X/11
CHROMOSOME				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	
Concordant no. (+/+)				6	12	8	7	9	9	9	9	3	11	9	9	8	10	7	5	12	9	6	8	11	5	6	
of Hybrids (-/-)				16	18	8	13	12	16	8	8	15	7	12	11	15	6	11	13	6	9	14	9	7	13	8	
Discordant no. (+/-)				6	0	4	6	4	4	3	4	10	2	4	4	5	3	5	8	0	4	7	5	2	7	4	
of Hybrids (-/+)				2	0	10	5	6	2	9	10	1	11	5	7	3	12	7	5	11	9	4	9	11	4	8	
Bands 8.4 and 5.5, 2.8 % DISCORDANCY				27	0	47	35	32	19	41	45	38	42	30	35	26	48	40	42	38	42	35	45	42	38	46	

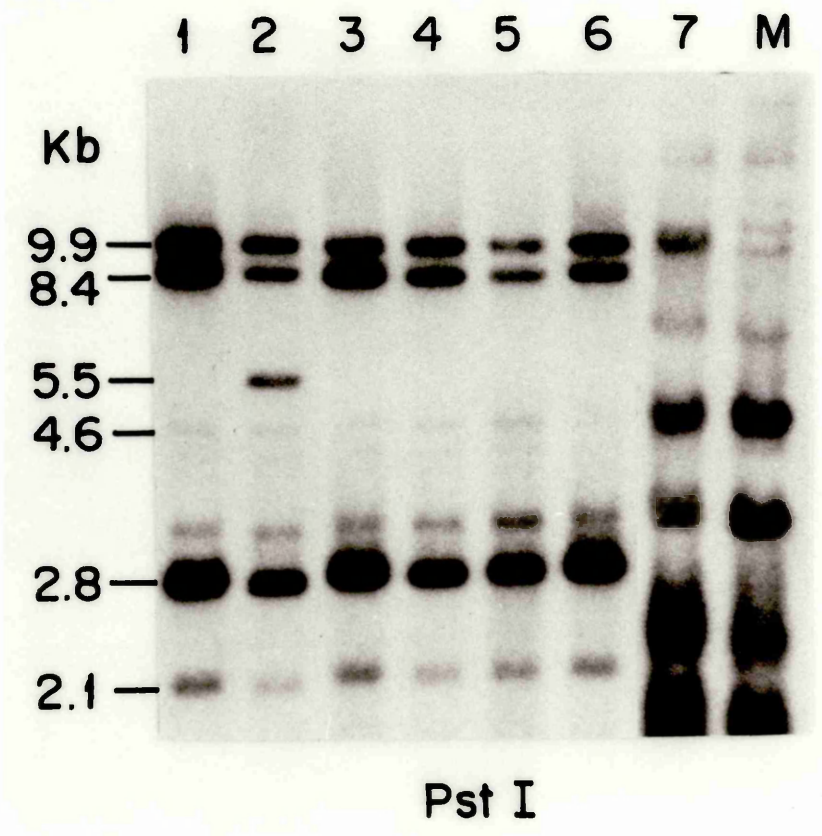

Figure 7

***PstI* Digest Showing Hybrid DUA-ICSAZF Retaining Chromosome 7 Only**

Southern analysis of DNA digested with *Pst*I. Lanes: 1-6 human controls, lane 7: human-mouse hybrid DUA-ICSAZF, which has retained only human chromosome 7 and lane M, mouse control. Lane 7 is positive only for the 9.9 kb band which, therefore, maps to human chromosome 7.

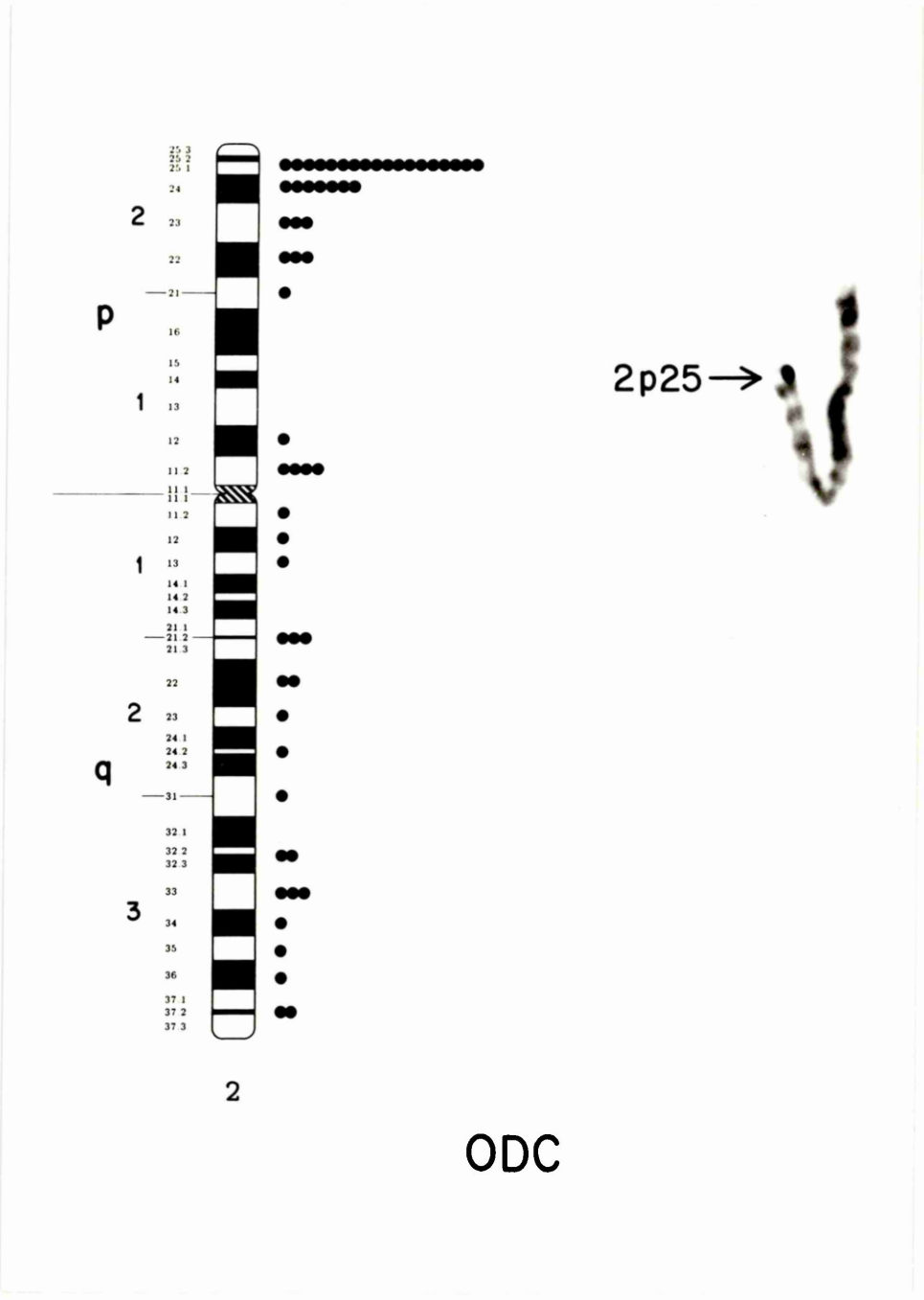

Figure 8

In Situ Hybridisation With pODC 10/2H

Distribution of silver grains localising ODC on chromosome 2 from 198 metaphases. There were 56 metaphase cells with grains on chromosome 2 (29.3%). Thirty-one percent (18/58) of the grains on chromosome 2 are located at 2p25. A representative metaphase chromosome 2 is shown with labelling of 2p25 (arrow). There was no significant accumulation of silver grains above background at any other chromosomal site, including chromosome 7.

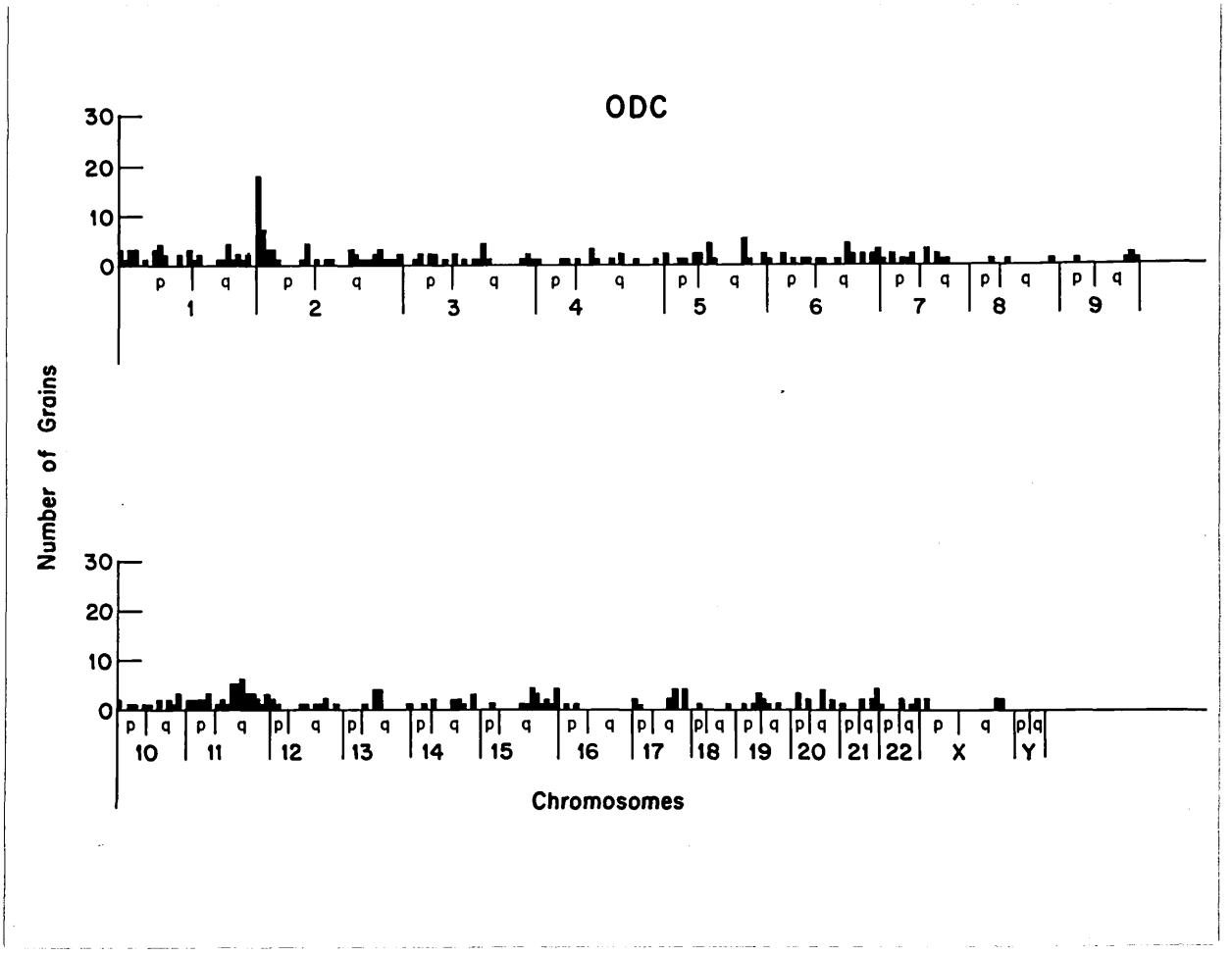

Figure 9

Idiogram Showing the Grain Distribution in 198 Metaphases for the Probe pODC 10/2H

The X axis represents the chromosomes and their elective size proportion, the Y axis, the number of grains. There is a highly significant amount of labelling at 2p25 in comparison to nonspecific background for the other chromosomes.

3.1.3 Chromosome 7 ODC Segment

The DNA of 35 human-mouse hybrids was digested with *Hind*III. Under conditions of low stringency, two fragments of human DNA hybridised to the ODC probe measuring 5.5 and 4.0 kb, respectively (Figure 10). The 4.0 kb band is positive in lane 1 and the 5.5 kb band is positive in lanes 1, 2 and 3. Under more stringent conditions, the 4.0 kb band is less prominent, and the 5.5 kb band is seen more clearly (Figure 11).

Table 4 reveals that the 5.5 kb band is located on chromosome 2, and the 4.0 kb band on chromosome 7. The hybrid JSR-17S with the translocation 7/9: 7pter→7q22:9q23→9pter localised the 4.0 kb band to the q22→qter region of 7. The hybrid VTL-7, which is negative for the 4.0 band, has a deletion on 7 and has retained only the 7pter→7q31 region. These results would indicate a further localisation of the 4.0 kb sequence to the 7q31→7qter region of 7. This locus will be termed ODC2.

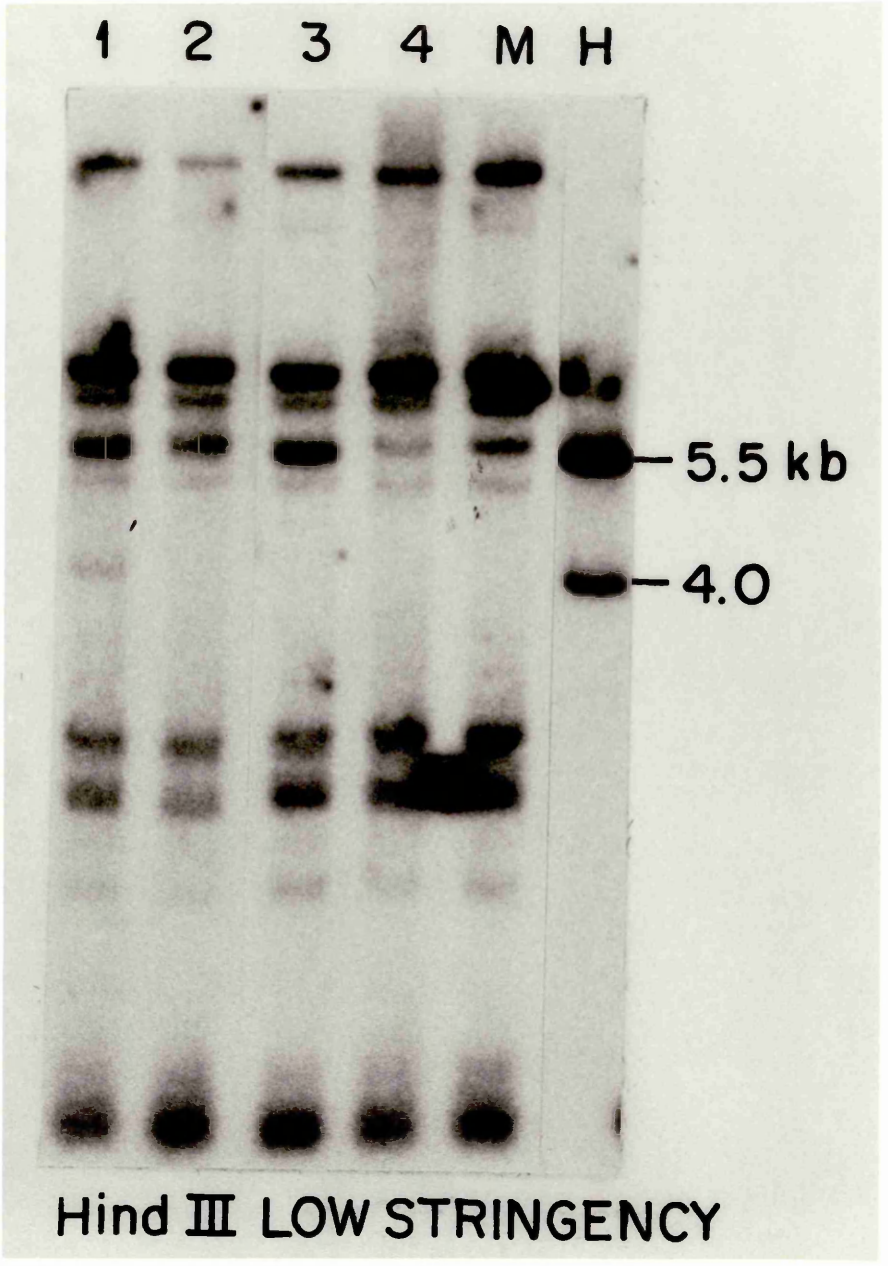

Figure 10

<u>*Hind*III Digest of Human-mouse Hybrid DNA Hybridised With pODC 10/2H - Low Stringency</u>

Southern hybridisation of cDNA probe pODC 10/2H to *Hind*III digests of human (H), mouse (M), and somatic cell hybrid DNA (lanes 1-4) under low stringency wash conditions. Lane 1 is positive for the 4.0 kb band, and lanes 1, 2, and 3 are positive for the 5.5 kb band.

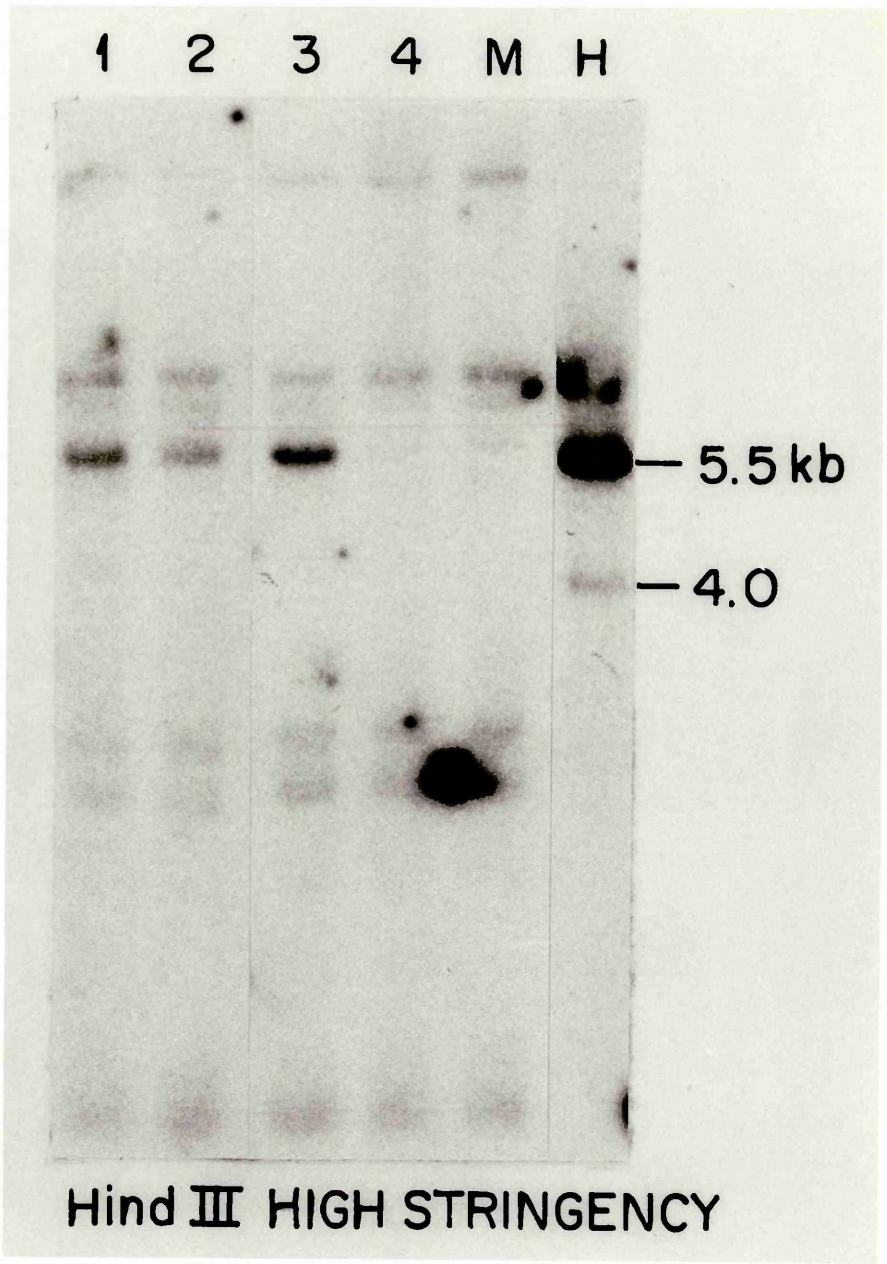

Figure 11

*Hind*III Digest of Human-mouse Hybrid DNA Hybridised With pODC 10/2H - High Stringency

Same blot as Figure 10, under conditions of high stringency. The 5.5 kb band is positive in lanes 1, 2, and 3 and maps to chromosome 2. The 4.0 kb band, positive in lane 1, though seen less clearly under these conditions, maps to chromosome 7.

Table 4

(see following three pages)

Legend Table 4

Distribution of human ODC sequences in cell hybrids digested with *Hind*III. The 5.5 kb band mapped to chromosome 2: the 4.0 kb band mapped to chromosome 7. Cell hybrids JSR-17S and VTL-7, retaining different regions of chromosome 7, localise the 4.0 kb band to the 7q31-qter region of chromosome 7.

Table 4

Distribution of ODC Sequences With Human Chromosomes in *Hind*III Digested Human-Mouse Cell Hybrid DNA

	Bands		HUMAN CHROMOSOMES																							
HYBRID	5.5 kb	4.0 kb	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Translocations
ATR-13	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	-	-	-	t	5/X
DUA-1CSAZF	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
DUA-3BSAGA	+	+	-	+	-	-	-	-	+	+	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	
DUA-5BSAGA	-	-	-	-	+	-	+	-	-	-	-	-	+	-	-	+	-	-	+	+	-	-	+	-	-	
DUM-13	+	+	+	+	+	-	+	+	+	-	-	+	+	+	-	+	t	+	+	+	+	+	+	+	t	X/15 15/X
GAR-1	-	-	-	-	+	-	+	-	-	+	-	+	-	+	-	+	+	+	-	-	-	+	-	-	+	
JSR-14	+	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	-	-	+	-	-	+	+	-	+	
JSR-17S	+	-	+	+	+	-	+	-	t	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	7/9
JSR-14	+	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	-	-	+	-	-	+	+	-	+	
JSR-17S	+	-	+	+	+	-	+	-	t	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	7/9
NSL-16	-	+	-	-	+	+	+	-	+	+	t	+	-	+	-	+	+	+	+	+	-	+	+	-	-	17/9
NSL-9	-	-	-	-	-	-	+	-	-	+	t	+	-	+	+	+	+	+	+	-	-	+	+	+	-	17/9
REW-10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	
REW-7	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	
REX-11BSAgB	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	+	+	-	-	+	-	-	-	-	-	
REX-11BSHF	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-	t	t	22/X
REX-26	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	t	t	22/X
REX-57BSHB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	+	t	t	22/X
SIR-8	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	

Table 4 (continued)

Distribution of ODC Sequences With Human Chromosomes in *Hind*III Digested Human-Mouse Cell Hybrid DNA

	Bands		HUMAN CHROMOSOMES																							
HYBRID	5.5 kb	4.0 kb	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Translocations
TSL-1	-	-	-	-	+	+	-	-	-	-	+	+	+	-	+	+	-	+	+	+	-	+	+	-	-	
TSL-2	+	-	-	+	t	-	+	+	-	-	-	+	-	+	-	-	-	-	t	+	-	+	+	-	+	17/3 3/17
VTL-17	-	+	-	-	-	-	+	-	+	-	-	+	+	-	+	+	-	-	+	-	-	+	+	-	-	
VTL-6	+	+	-	+	-	-	-	+	+	+	-	+	+	-	-	-	+	-	+	-	+	+	+	+	-	
VTL-7	-	-	-	-	-	-	-	-	t	-	-	-	+	-	-	+	-	-	+	-	+	+	+	+	-	7q-
VTL-8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	+	+	+	-	
WIL-1	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	+	-	-	+	+	-	-	+	-	+	
WIL-13	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	+	-	
WIL-2	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-	+	-	-	-	+	-	+	
WIL-2CSAZ	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	+	-	-	-	+	-	-	
WIL-5	-	-	-	-	-	+	-	-	-	+	-	+	-	-	-	-	-	-	+	+	-	-	+	-	+	
WIL-8X	-	+	-	-	+	+	+	-	+	+	-	+	+	+	-	+	-	-	+	+	+	+	+	-	+	
WIL-8Y	-	+	-	-	+	-	-	+	+	+	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	
XER-11	-	+	+	-	+	+	+	+	+	+	+	+	t	+	-	-	+	+	+	+	+	+	+	+	t	11/X X/11
XOL-6	-	+	t	-	-	-	+	+	+	-	-	+	+	+	-	+	-	-	+	-	+	+	-	+	t	1/X
XOL-9	+	-	t	+	+	+	-	+	-	-	-	-	-	+	-	-	+	-	+	+	+	-	+	+	+	X/1
XTR-22	+	-	-	+	t	+	+	+	-	+	-	+	+	-	-	-	+	-	-	+	+	+	+	+	+	X/3
XTR-3BSAgB	-	-	-	-	t	-	-	-	-	-	+	t	-	+	-	-	-	-	-	-	-	+	+	-	t	3/X, 10q-

Table 4 (continued)

Distribution of ODC Sequences With Human Chromosomes in *Hind*III Digested Human-Mouse Hybrid DNA

5.5 kb BAND CHROMOSOME	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
Concordant no. (+/+)	7	13	9	8	9	9	8	9	4	10	8	10	7	8	9	6	11	10	8	8	11	7	7
of Hybrids (-/-)	20	22	11	17	13	19	14	12	17	8	14	12	18	8	14	16	5	11	17	10	5	13	11
Discordant no. (+/-)	5	0	2	5	4	4	4	4	9	3	5	3	6	5	3	7	1	3	5	5	2	5	3
of Hybrids (-/+)	1	0	10	5	9	3	7	10	3	13	7	10	4	14	8	6	17	11	5	12	17	7	6
5.5 kb BAND DISCORDANCY	18	0	37	29	37	20	33	40	36	47	35	37	29	54	32	37	53	40	29	49	54	37	33

4.0 kb BAND CHROMOSOME	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
Concordant no. (+/+)	7	8	10	8	10	8	15	11	4	13	10	10	6	12	9	8	14	10	10	10	11	7	5
of Hybrids (-/-)	18	15	8	15	12	16	18	12	16	9	15	10	15	10	12	16	5	9	17	10	3	11	9
Discordant no. (+/-)	7	7	5	7	5	7	0	4	10	2	4	5	9	3	5	7	1	5	5	5	4	7	5
of Hybrids (-/+)	1	5	9	5	8	4	0	8	3	10	5	10	5	10	8	4	14	11	3	10	17	7	8
4.0 kb BAND DISCORDANCY	24	34	44	34	37	31	0	34	39	35	26	43	40	37	38	31	44	46	23	43	60	44	48

3.2 Mapping the AdoMetDC Gene

The chromosomal assignment of AdoMetDC was determined by correlating the presence or absence of specific human chromosomes in a panel of human-mouse somatic cell hybrids with AdoMetDC sequences in the DNA isolated from these hybrid cell lines.

The rat AdoMetDC probe hybridised to four *Pst*I fragments of human WI-38 fibroblast DNA. The fragments measured 7.2 kb, 5.9 kb, 2.9 kb, and 2.2 kb in length. The probe also hybridised to mouse DNA; a restriction fragment length polymorphism was noted between the RAG and LMTK$^-$ mouse cell lines of BALB/c and C3H origins, respectively (Figure 12, lanes 7 and 8).

Figure 12 demonstrates the banding pattern seen in human WI-38 (lane 9), mouse LTP2a (lane 8), and mouse RAG DNA (lane 7). The 5.9 and 2.9 kb bands were scored. The 7.2 kb band was not scored because of its weak signal and proximity to a faint mouse band. The 2.2 kb fragment overlapped with a mouse band and could not be scored. Lanes 1-6 contain the human-mouse hybrid DNAs. Lanes 1, 2, and 3 are positive for the 5.9 kb band and have retained human chromosome 6; lanes 4, 5 and 6 have lost human chromosome 6 and are negative for the 5.9 kb band. Lanes 1, 3, and 5 are positive for the 2.9 kb band and human chromsome X; lanes 2, 4, and 6 have lost the X chromosome and are negative for the 2.9 kb band.

Table 5 shows that the chromosomal assignment is achieved by correlating the presence of the bands which can be scored (5.9 and 2.9 kb) with the presence of human chromosomes known to be retained in those hybrids. Percent discordancy indicates the degree of discordant segregation for a marker and a chromosome, a 0% discordancy being the basis of chromosome assignment. It can be seen that the 5.9 kb band (designated AMD1) maps to chromosome 6, and the 2.9 kb band (designated AMD2) maps to the X chromosome.

The sequence on X can be further localised on X by the following hybrids: ATR-13 (+) which contains a 5:X translocation retaining Xq22→Xqter. REX-11BSHF (+) and REX-26 (+) also contain the same translocated portion of X. XTR-3BSAgH (-) has a 3:X translocation in which Xq28-Xqter is present. Thus the sequence maps to Xq22-Xq28.

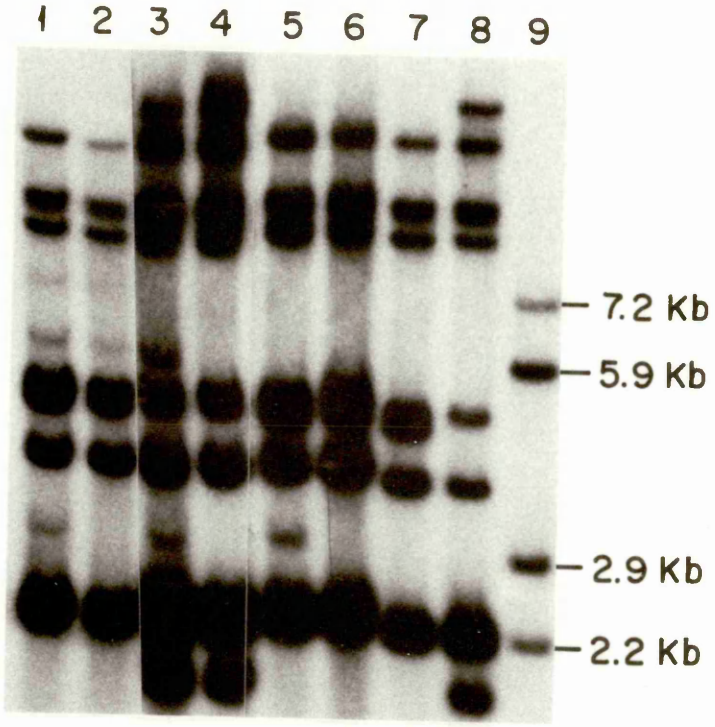

Figure 12

Distribution of AdoMetDC Gene Sequences in Human-mouse Hybrids

Distribution of AdoMetDC (AMD) gene sequences in mouse (lanes 7,8), human (lane 9) and mouse-human cell hybrids (lanes 1-6). DNA was digested with *Pst*I and hybridised with the rat AdoMetDC cDNA probe. Human DNA fragments are seen in lane 9 measuring 7.2, 5.9, 2.9, 2.2 kb. Lanes 1, 2, and 3 contain DNAs from hybrids which have retained human chromosome 6 and are positive for the 5.9 kb band. Lanes 4, 5, and 6 contain hybrids negative for the 5.9 kb band and chromosome 6. Lanes 1, 3, and 5 are positive for the 2.9 kb band and the X chromosome; whereas lanes 2, 4 and 6 have lost the X and are negative for the 2.9 kb band. The 5.9 and 2.9 kb bands segregate independently, as is seen in Table 5.

Table 5

(see following three pages)

Legend Table 5

DNA probe for AdoMetDC (AMD) was hybridised to Southern blots containing *PstI*-digested DNA from the hybrids listed. AMD was determined by scoring presence (+) or absence (-) of human bands in the hybrids on the blots. The table is compiled from 38 cell hybrids containing 14 unrelated human and 4 mouse cell lines. Concordant hybrids have either retained or lost AMD together with a specific human chromosome. Discordant hybrids have either lost the gene but not a specific chromosome, or the reverse. Percent discordancy indicates the degree of discordant segregation for a marker and a chromosome. A 0% discordance is the basis for chromosome assignment. The "t" in the table indicates a chromosome translocation for a particular chromosome, but no intact chromosome is present. The hybrids were characterised by chromosome analysis and by mapped enzyme markers, and partly by mapped DNA probes.

The 5.9 kb band mapped to human chromosome 6. The 2.9 kb band mapped to human chromosome X. The hybrids with X translocations: ATR-13 (+) with the 5pter→5q22→Xqter, REX-11BSHF (+) and REX-26 (+) with the 22pter→22q13::Xq22→Xqter, and XTR-3BSAgH (-) with the 3pter→3q21::Xq28→Xqter, localise the 2.9 kb band to the Xq22→Xq28 region.

Table 5

Segregation of the Human AdoMetDC Gene (AMD) with Human Chromosomes in *Pst*I Digested Human-Mouse Cell Hybrid DNA

	AdoMetDC		HUMAN CHROMOSOMES																							
HYBRID	5.9 kb	2.9 kb	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Translocation
ATR-13	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	-	-	-	t	5/X
DUA-3BSAGA	-	-	-	+	-	-	-	-	+	+	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	
DUA-5BSAGA	-	-	-	-	+	-	+	-	-	-	-	-	+	-	-	+	-	-	+	+	-	-	+	-	-	
DUM-13	+	+	+	+	+	-	+	+	+	-	-	+	+	+	-	+	t	+	+	+	+	+	+	+	t	X/15, 15/X
GAR-1	-	+	-	-	+	-	+	-	-	+	-	+	-	+	-	+	+	+	-	-	-	+	-	-	+	
ICL-15	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	+	-	-	+	+	-	-	
JSR-14	-	-	-	+	+	+	+	-	-	-	-	-	-	+	-	+	-	-	+	+	-	+	+	-	-	
JSR-17S	-	-	+	+	+	-	+	-	t	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	7/9
JWR-22H	+	-	t	t	-	+	-	+	+	-	-	+	+	+	+	+	+	-	+	+	-	+	+	-	-	2/1
JWR-26C	+	+	t	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	-	+	+	-	+	1/2
NSL-5	-	-	+	-	-	-	-	-	-	+	t	+	-	+	-	+	-	+	t	+	-	+	-	-	-	17/9, 12q+
NSL-9	-	-	-	-	-	-	+	-	-	+	t	+	-	+	+	+	+	+	+	-	-	+	+	+	-	17/9
NSL-16	-	-	-	-	+	+	+	-	+	+	t	+	-	+	-	+	+	+	+	+	-	+	+	-	-	17/9
REW-5	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	-	+	+	+	
REW-11	-	+	-	-	-	+	-	-	+	-	-	-	+	+	+	-	-	+	-	-	-	+	+	+	+	
REX-11BSAgB	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	+	+	-	-	+	-	-	-	-	-	
REX-11BSHF	-	+	-	-	+	-	-	-	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-	t	t	22/X
REX-26	-	+	+	+	+	+	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	t	t	22/X
SIR-8	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	

Table 5 (continued)

Segregation of the Human AdoMetDC Gene (AMD) with Human Chromosomes in *PstI* Digested Human-Mouse Cell Hybrid DNA

	AdoMetDC		HUMAN CHROMOSOMES																							
HYBRID	5.9 kb	2.9 kb	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Translocation
SIR-11	-	+	-	+	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	+	-	+	
TSL-1	-	-	-	-	+	+	-	-	-	-	+	+	+	-	+	+	-	+	+	+	-	+	+	-	-	
TSL-2	+	+	-	+	t	-	+	+	-	-	-	+	-	+	-	-	-	-	t	+	-	+	+	-	+	17/3, 3/17
VTL-6	+	-	-	+	-	-	-	+	+	+	-	+	+	-	-	-	+	-	+	-	+	+	+	+	-	
VTL-7	-	-	-	-	-	-	-	-	t	-	-	-	+	-	-	+	-	-	+	-	+	+	+	+	-	7q-
VTL-8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	+	+	+	-	
VTL-17	-	-	-	-	-	-	+	-	+	-	-	+	+	-	+	+	-	-	+	-	-	+	+	-	-	
WIL-1	-	+	-	-	-	-	-	-	-	+	-	-	-	+	-	+	-	-	+	+	-	-	+	-	+	
WIL-2CSAZ	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	+	-	-	-	+	-	-	
WIL-5	-	+	-	-	-	+	-	-	-	+	-	+	-	-	-	-	-	-	+	+	-	-	+	-	+	
WIL-6	+	+	-	+	-	+	+	+	+	+	-	+	+	-	-	+	-	-	+	-	+	+	+	-	+	
WIL-7	+	+	-	+	+	-	+	+	-	+	-	+	+	-	+	+	-	-	+	+	-	-	+	-	+	
WIL-8X	-	+	-	-	+	+	+	-	+	+	-	+	+	+	-	+	-	-	+	+	+	+	+	-	+	
WIL-8Y	+	+	-	-	-	-	-	+	+	+	-	+	+	-	-	+	-	-	+	+	-	+	+	-	+	
WIL-14	-	+	+	-	+	-	+	-	+	+	-	+	-	+	-	+	+	-	+	-	-	-	-	-	+	
XER-7	+	+	+	+	+	+	+	+	+	+	+	+	t	+	+	+	+	-	-	+	+	-	-	-	+	11/X
XER-11	+	+	+	-	+	+	+	+	+	+	+	+	t	+	-	-	+	+	+	+	+	+	+	+	t	11/X, X/11
XTR-3BSAgH	-	-	+	-	+	+	+	-	-	-	+	+	-	+	-	-	-	-	-	-	-	+	+	-	t	3/X, 10q-
XTR-22	+	+	-	+	t	+	+	+	-	+	-	+	+	-	-	-	+	-	-	+	+	+	+	+	+	X/3

Table 5 (continued)

Segregation of the Human AdoMetDC Gene (AMD) with Human Chromosomes in *Pst*I Digested Human-Mouse Cell Hybrid DNA

CHROMOSOME	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
Concordant no. (+/+)	5	10	7	8	10	13	10	9	3	13	9	8	5	9	8	4	10	11	8	9	11	5	8
of Hybrids (-/-)	19	19	12	16	14	25	15	11	16	9	16	10	16	7	16	16	6	12	22	11	6	17	14
Discordant no. (+/-)	6	2	4	5	3	0	3	4	10	0	2	5	8	4	4	9	2	2	5	4	2	8	2
of Hybrids (-/+)	6	6	13	9	11	0	8	14	6	16	9	15	9	18	9	9	18	13	3	14	19	6	8
5.9 kb BAND % DISCORDANCY	33	22	47	37	37	0	31	47	46	42	31	53	45	58	35	47	56	39	21	47	55	39	31

CHROMOSOME	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
Concordant no.(+/+)	8	12	13	12	14	11	13	15	6	18	11	14	7	15	10	8	14	16	9	10	16	6	16
of Hybrids (-/-)	13	12	10	12	10	15	10	9	11	6	10	8	10	5	10	12	2	9	15	4	3	12	16
Discordant no.(+/-)	12	9	6	9	7	10	8	6	15	3	8	7	14	6	10	13	6	5	12	11	5	13	0
of Hybrids (-/+)	3	4	7	5	7	2	5	8	3	11	7	9	7	12	7	5	14	8	2	13	14	5	0
2.9 kb BAND % DISCORDANCY	42	35	36	37	37	32	36	37	51	37	42	42	55	47	46	47	56	34	37	63	50	50	0

3.3 Chromosome 2 Restriction Fragment Length Polymorphism of ODC

*Pst*I digests of leukocyte DNA from 40 unrelated individuals revealed three consistent banding patterns initially suggested by the mapping data (Figure 13). In all individuals, the 9.9 kb band (localising to chromosome 7) is present. The 2.8 kb band on chromosome 2 is also constant, as is the 2.1 kb band. The 4.6 kb band which could not be used for scoring purposes is seen very faintly. The 8.4 kb and 5.5 kb bands mapping to chromosome 2 show a DNA restriction fragment length polymorphism since they segregate between individuals as alleles at the same locus. These phenotypes constitute at least a two-allele locus with the 5.5 kb homozygote in lane 1, the 8.4 kb homozygote in lane 2, and the 8.4/5.5 kb heterozygote in lane 3. Of 40 individuals, 3 corresponded to the 5.5 homozygote, lane 1; 21 corresponded to the 8.4 homozygote, lane 2; and 16 corresponded to the 8.4/5.5 heterozygote, lane 3. The allele frequencies calculated by the Hardy Weinberg Law are 5.5 (p) = 0.28 and 8.4 (q) = 0.72.

3.4 Correlation With N-myc

Since the locus for the oncogene N-myc has been mapped to the tip of the short arm of chromosome 2, 2p24,[99] and these experiements map ODC to 2p25, an experiment was devised to determine if any correlation existed between amplification of N-myc and ODC. Two neuroblastoma cell lines were provided by Dr. P. Rothberg, one exhibiting amplification of N-myc and one without amplification. The DNA was digested with *Pst*I, the fragments separated as described and probed with pODC 10/2H. No amplification of either ODC locus was seen in either neuroblastoma cell line (data not shown).

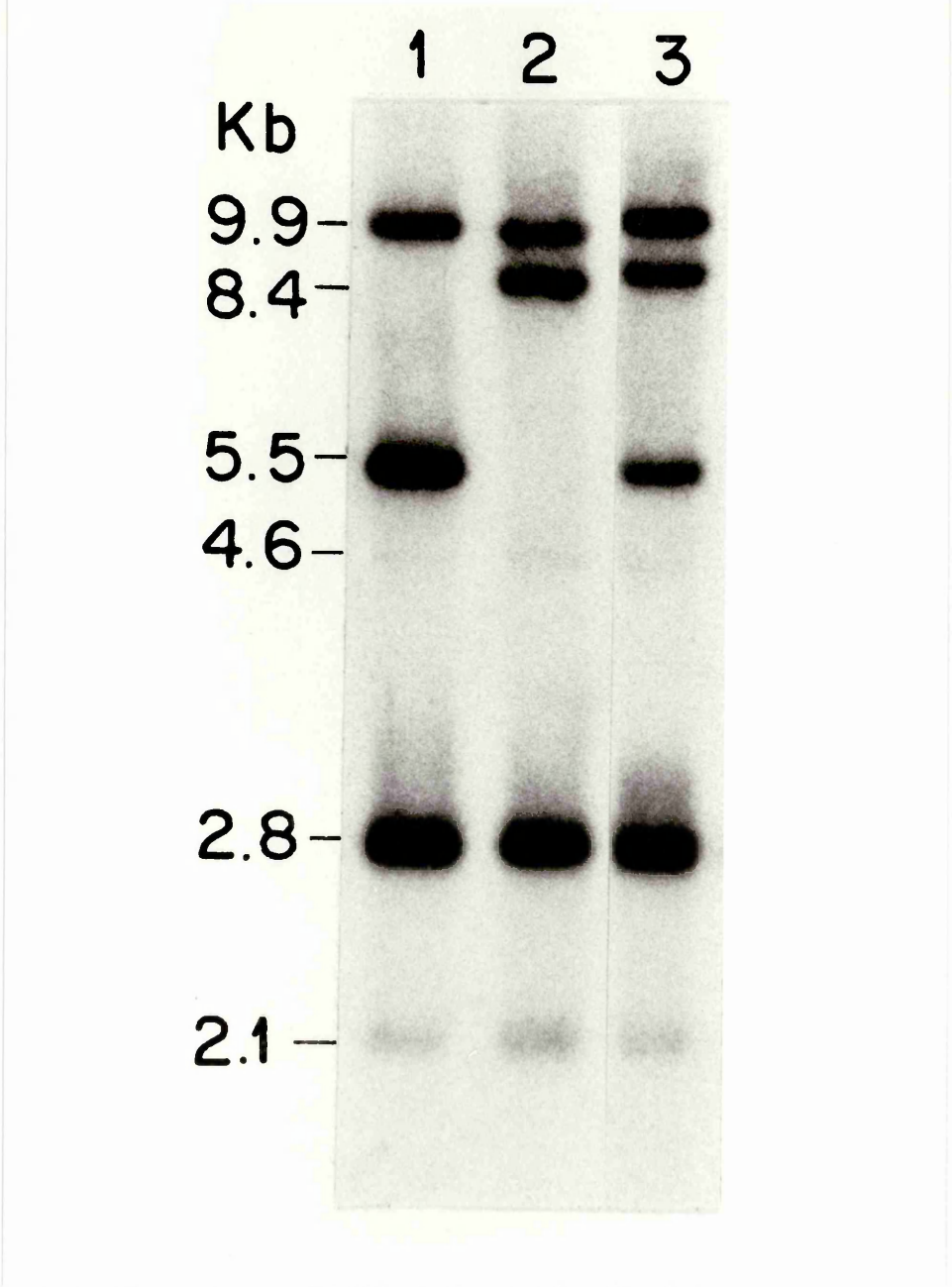

Figure 13

Polymorphism of ODC Among Humans

The banding pattern of DNA hybridisation with pODC 10/2H in human samples after a *Pst*I digest. Only three patterns are seen in 40 samples examined. In all samples, the 9.9 kb band (chromosome 7) and the 4.6, 2.8, and 2.1 bands remain constant. In lane 1, the 8.4 kb band is absent, representing the 5.5 homozygote. Lane 2 depicts the 8.4 homozygote. In lane 3, both bands are present, representing the 5.5/8.4 heterozygote phenotype. This represents a two-allele ODC locus on human chromosome 2.

3.5 The Site of the Functional Gene for ODC

In an attempt to determine which locus, either on chromosome 2 or chromosome 7 harboured the functional gene, an experiment was devised involving mouse-human hybrids in cell culture.

Glyoxal gel analysis was performed comparing mouse and human ODC signals (Figure 14). A slight size difference could be used to distinquish mouse and human mRNA signals in cell culture. The hybrid DUA-1CSAZF, which has retained only human chromosome 7, was grown to 70% confluency as were TSL-2 and DUA-6, which contain human chromosome 2 but not 7. The RNA was run alongside appropriate human and mouse controls in 1.5% agarose. Review of the autoradiographs suggested there to be a human signal from the hybrids containing chromosome 2, and not from the 7-only hybrid, but this result was not conclusive.

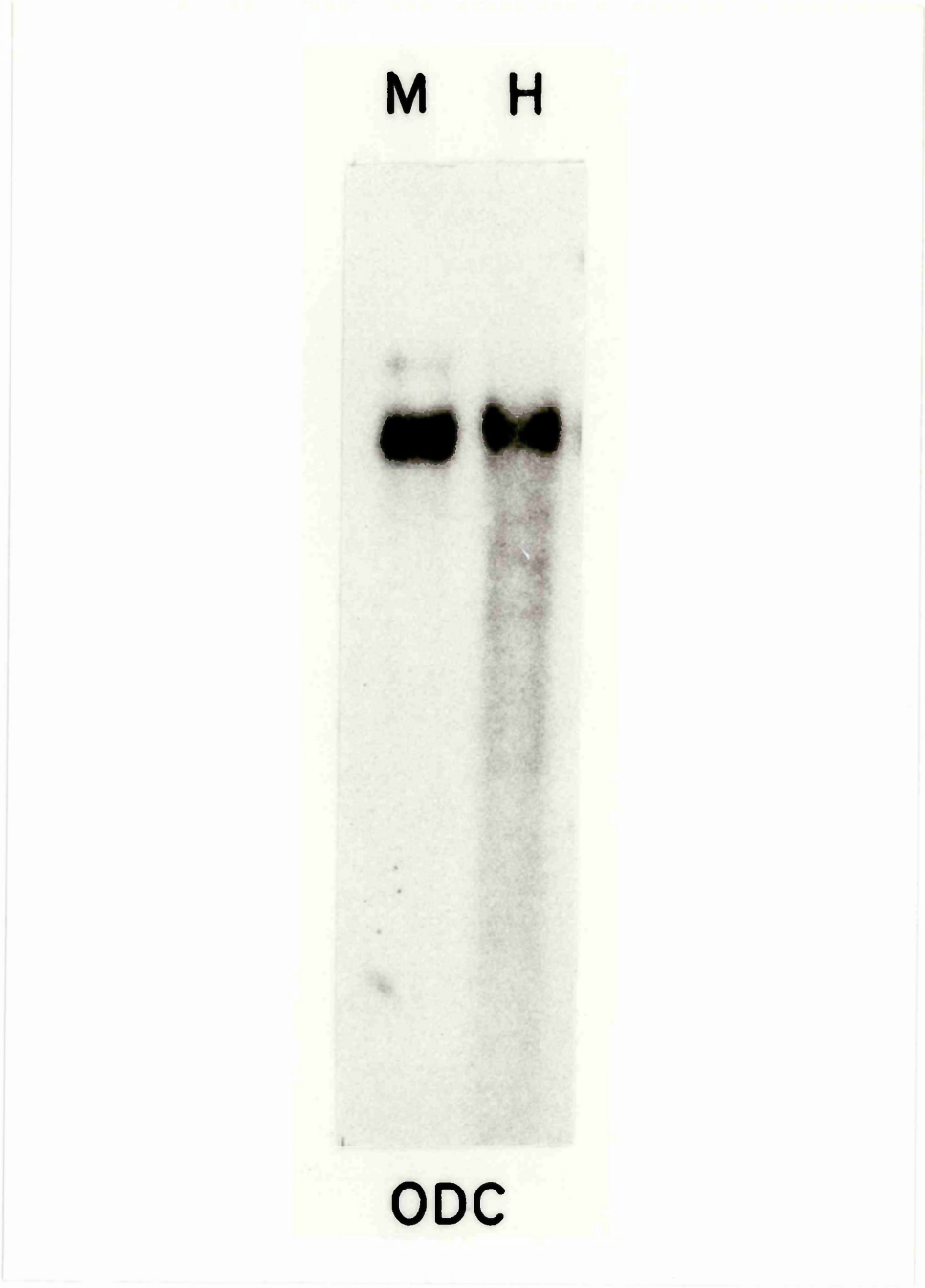

Figure 14

Northern Analysis of ODC Expression in LMTK⁻ Mouse and Human RNA

Northern analysis of ODC expression for LMTK⁻ mouse (M) versus human (H) total RNA. There is a slight size difference between the major mouse band (2.1 kb) and the only human band (2.2). A further mouse signal (2.6 kb) is seen.

3.6 ODC mRNA Expression in Colorectal Neoplasia

An initial dot blot of ODC expression in colonic tumours and paired adjacent mucosa revealed greatly enhanced expression in tumours (Figure 15). Further experiments were therefore performed using glyoxal gel analysis.

ODC mRNA expression was examined in 18 patients with paired samples of sporadic colorectal carcinoma and adjacent, pathologically normal mucosa and in four patients with polyps - two of whom had two polyps. It was found that the mRNA expression of the housekeeping enzyme triosephosphate isomerase (TPI) closely follows the amount of 28S and 18S RNA loaded into each well, and therefore this uniform expression between normal and tumour tissue could be used as an internal control by which expression of ODC could be quantitated. Figure 16 shows mRNA expression of ODC and TPI in four patients. TPI expression remains relatively constant between tumour and normal, whereas ODC shows increased expression in all tumours and polyps examined. In all samples, the ODC message was of constant size: 2.2 kb, and only one transcript was seen by Northern analysis.

Of the 18 colorectal tumours, one of which was a local recurrence, seven were located in the rectum, six in descending colon or sigmoid, two in the transverse, and three in the caecum; 12 were moderately-differentiated, three poorly-, and three well-differentiated. Six were Stage BI, 3 BII, 5 CI, and 4 CII (Table 6). Polyps were tubular or villous with mild-to-moderate atypia (Table 7).

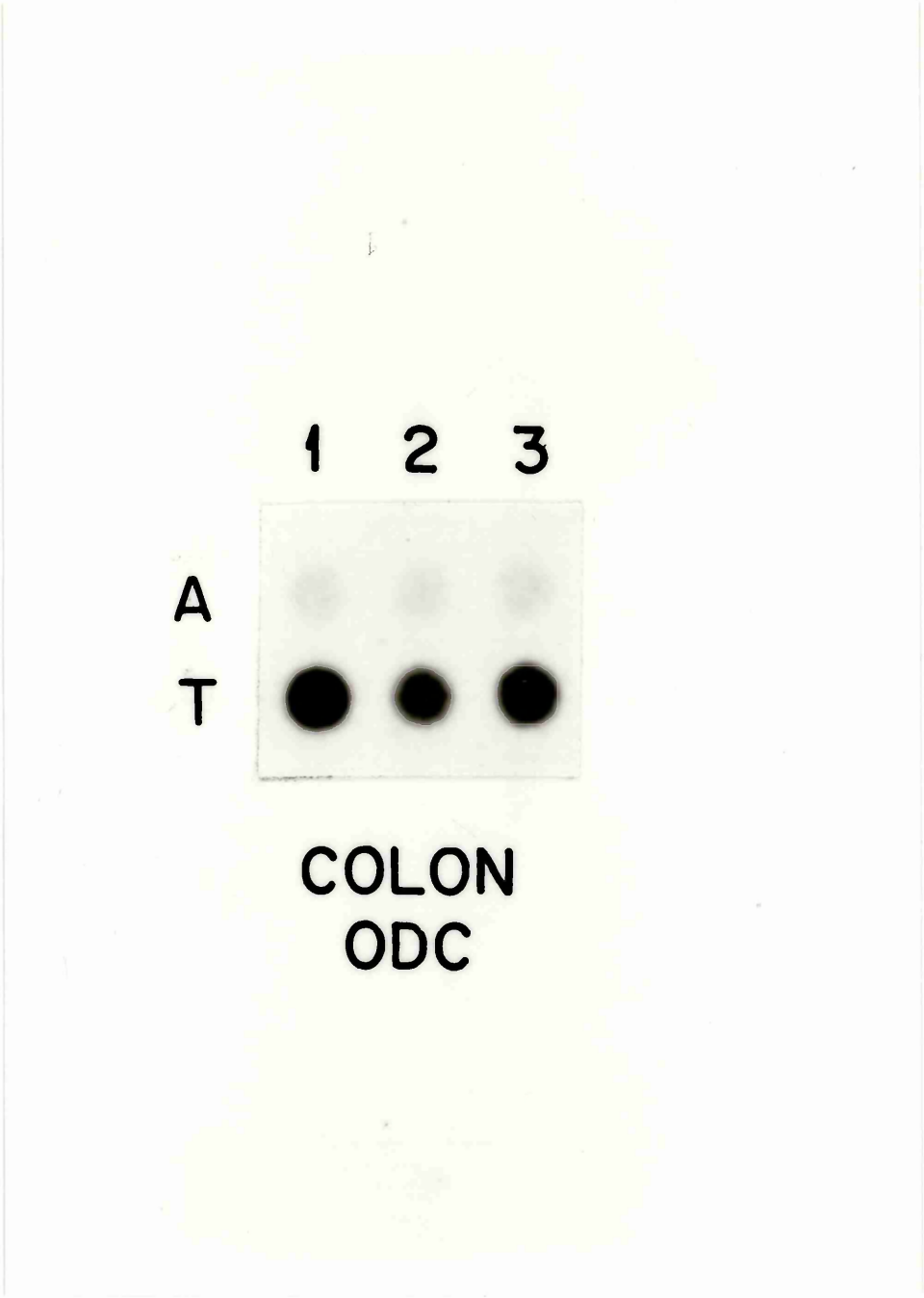

Figure 15

Dot Blot Analysis of Three Colorectal Carcinomas Probed With pODC 10/2H

Dot blot analysis of total RNA isolated from three colon carcinomas (T) and corresponding adjacent normal mucosa (A). Hybridisation of ^{32}P-labeled pODC 10/2H to 10 μg total RNA sample.

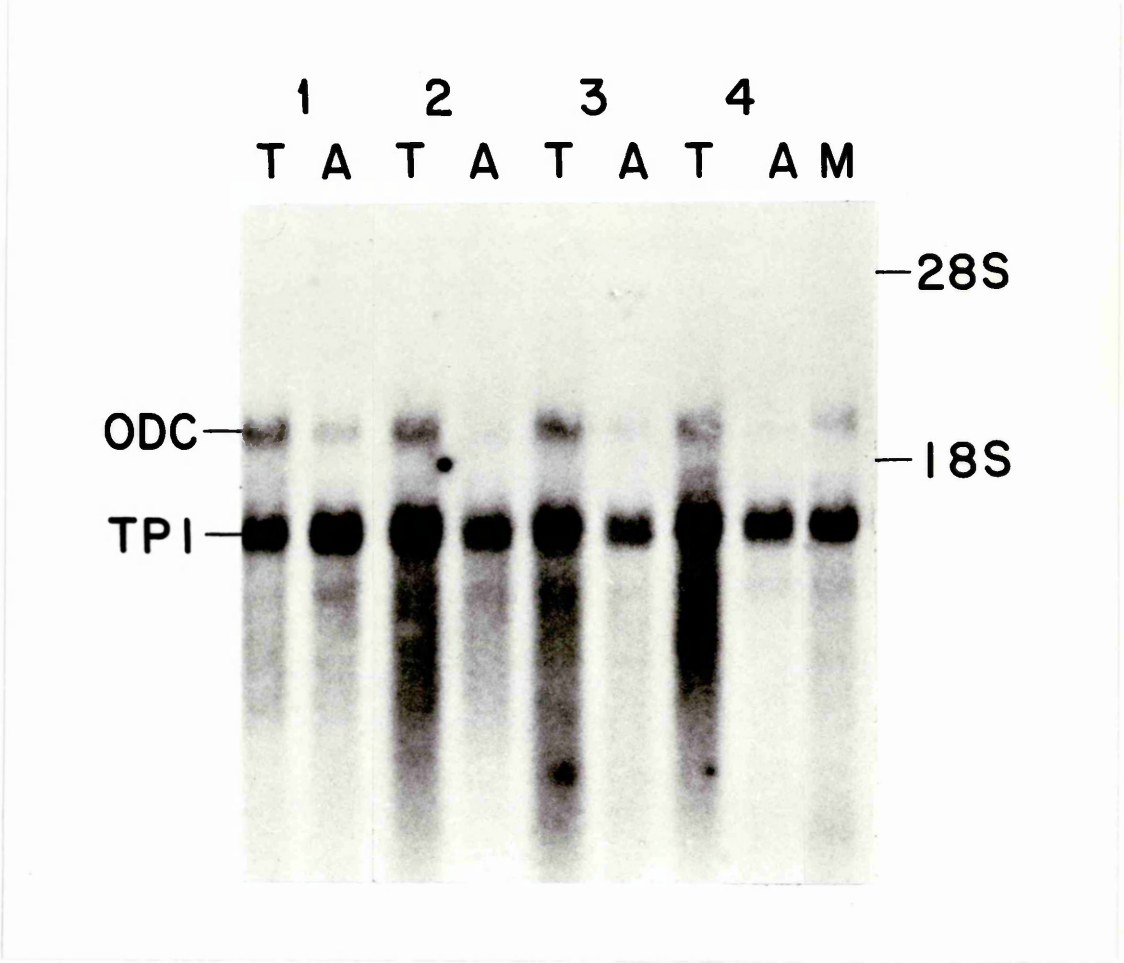

Figure 16

Northern Analysis of ODC mRNA Expression in Four Colorectal Cancers With Adjacent Mucosal Controls

Northern blot of glyoxal gel analysis of tumour (T) and adjacent normal mucosa (A) of four patients with colorectal carcinoma. Lane M represents normal mucosa from a patient who did not have colon cancer. The ODC (2.2 kb) and TPI hybridisation signals are shown. ODC expression is markedly increased in tumours, whereas TPI remains relatively constant between tumour and adjacent mucosa. The position of 28S and 18S ribosomal bands are shown.

Table 6

Colon Carcinoma Specimens From Which ODC RNA Analysed

Patient		Site	Degree of Differentiation	Stage
CC	10	Sigmoid	Moderate	BI
	11	Sigmoid	Well	BI
	12*	Sigmoid	Poor	CII
	14	Sigmoid	Poor	CII
	15	Rectum	Moderate	CI
	17	Caecum	Well	BI
	22	Rectum	Moderate	CII
	27	Caecum	Moderate	BI
	28	Rectum	Moderate	CI
	29	Rectum	Moderate	BI
	30	Caecum	Poor	CII
	31	Transverse	Moderate	CI
	32	Sigmoid	Moderate	CI
	35	Splenic flexure	Moderate	BI
	36	Rectum	Moderate	BII
	37	Descending	Well	BII
	38	Rectum	Moderate	BII
	41	Rectum	Moderate	CI

* = local recurrence

Table 7

Polyp Samples for Which RNA Analysed for ODC Expression

CP2	Ascending colon	Villous with mild atypia
$CP3^{-}$	Caecum	Villous with mild atypia
$CP3^{=}$	Ascending colon	Tubulovillous with mild atypia
FPC5	Rectum	Tubular with moderate atypia
FPC30S	Sigmoid	Tubular with moderate atypia
CC36P	Sigmoid	Villous with mild atypia

RNA expression was expressed as an ODC/TPI ratio for tumour and adjacent normal tissue. The fold increase of tumour compared to adjacent mucosa can therefore be derived. There was a significantly higher ODC mRNA expression in primary tumours than in adjacent mucosa for each paired sample ($p < 0.001$ Wilcoxon matched pairs test). This is graphically demonstrated in Figures 17 and 18. A similar finding was noted for polyps, mRNA expression always being higher than in normal tissue. Of 17 colorectal tumour primaries and one local recurrence, the fold increase of ODC mRNA expression in tumour compared to adjacent mucosa ranged from 1.29 to 12.2 with a median of 3.42 and mean of 4.19 (± 2.756) and a moderate correlation coefficient of .79 ($p < 0.001$, Spearman correlation) (Figure 19). For six polyps, the fold increase ranged from 1.5 to 9.05 with a median of 2.8 and mean of 3.2 (± 1.67).

RNA expression and fold increase was examined with respect to site of tumour (rectum versus elsewhere in the colon), stage of primary tumour (B versus C), and degree of differentation. ODC mRNA expression was independent of these parameters, although there was a suggestion of a difference for adjacent mucosal RNA, the expression being higher in the rectum than the rest of the colon ($p = 0.09$ Mann Whitney) (Figure 20).

3.7 ODC Activity and mRNA Expression

In 15 patients, paired samples from the same patient were analysed for both ODC activity and mRNA expression to investigate whether a direct correlation was present. Although all samples exhibited a significant increase in both ODC mRNA levels and ODC activity when compared to adjacent normal tissue (see Figures 18 and 21), no correlation was found between ODC RNA expression and activity within the same tissue samples (Spearman correlation coefficient 0.068, $p = 0.81$) (Figure 22).

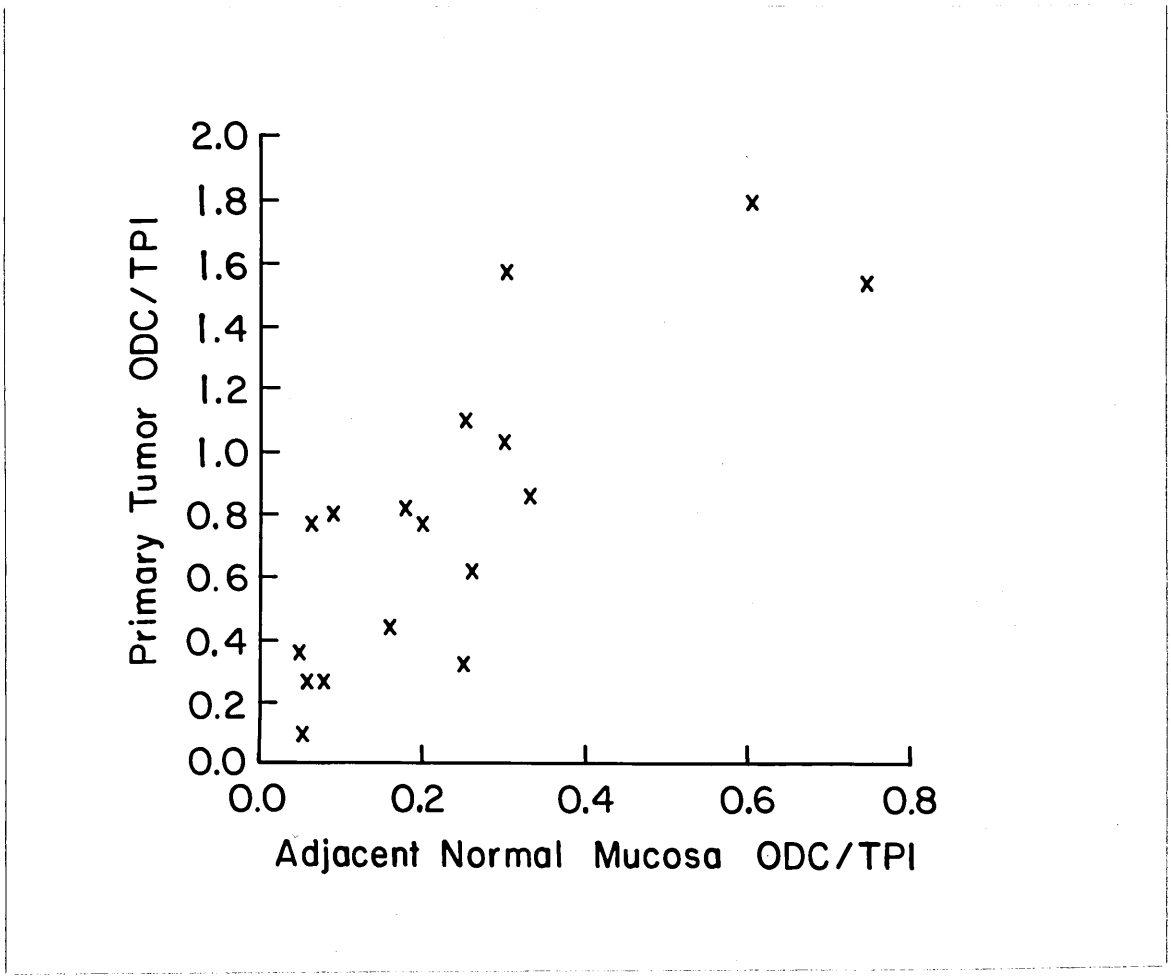

Figure 17

Scattergram Depicting the Relationship of ODC/TPI Ratio in Colorectal Primary Tumours Versus Adjacent Normal Tissue

Scattergram depicting the relationship of ODC/TPI ratio for colorectal primary tumours versus adjacent normal mucosa. ODC RNA expression is consistently higher in tumour than mucosa ($p < 0.001$, Wilcoxon matched pairs test).

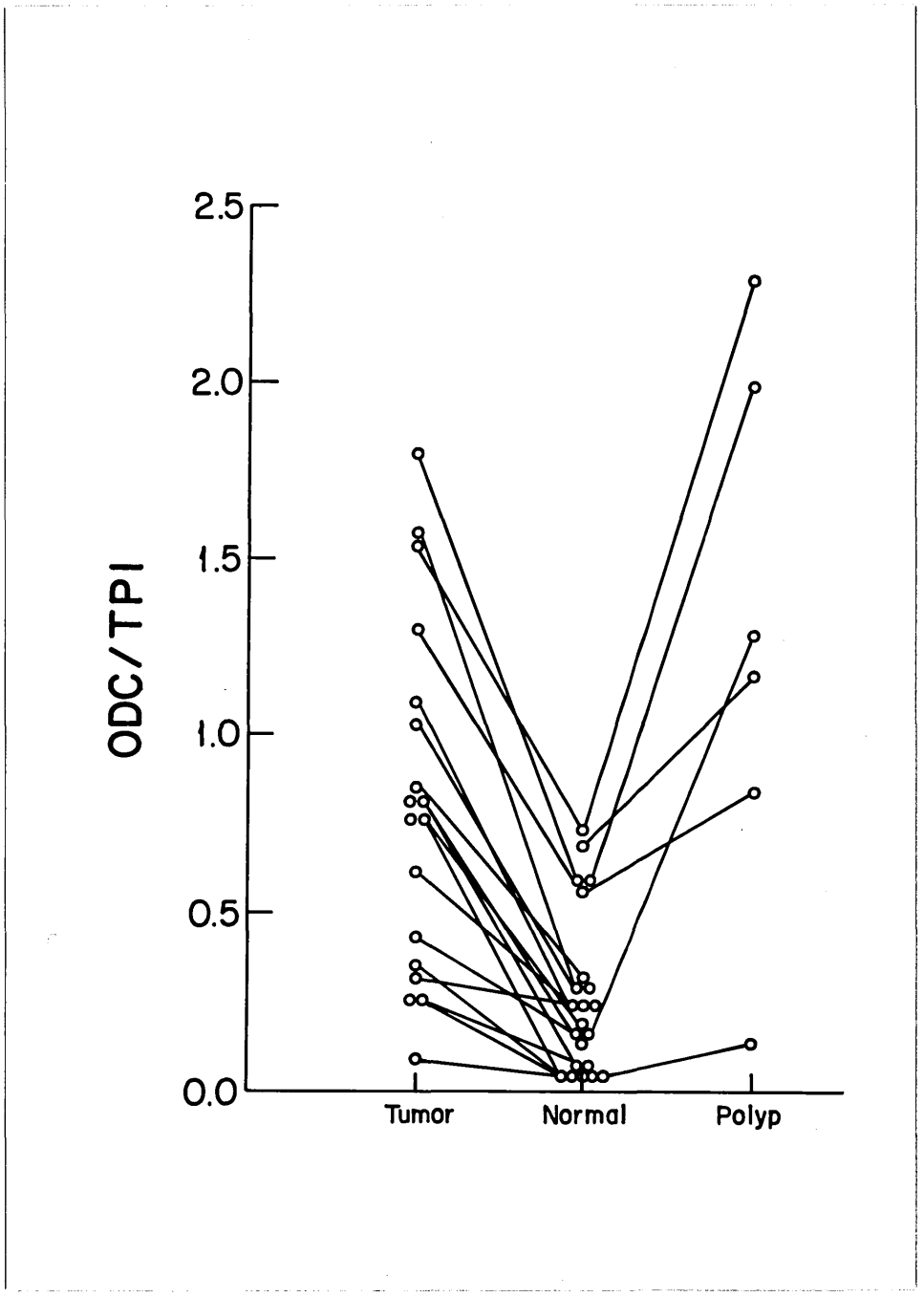

Figure 18

Comparison of ODC Expression for Cancers, Polyps, and Adjacent Mucosa

Graph depicting ODC/TPI ratio for colorectal tumours and polyps with corresponding normal adjacent mucosa as control.

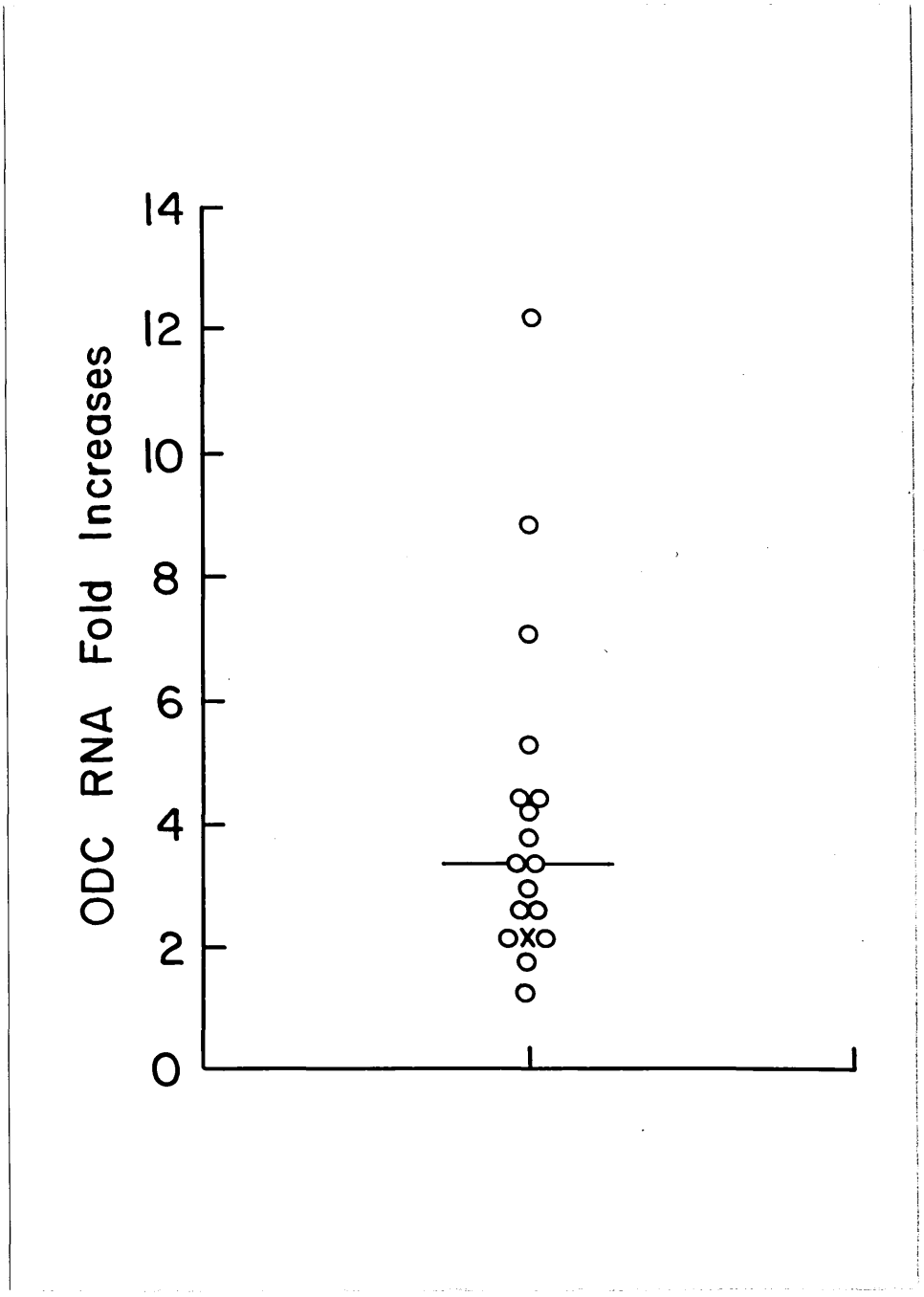

Figure 19

ODC Fold Increase in RNA Expression for Colorectal Tumours

Graph showing fold increases of ODC RNA expression of colorectal primary tumours (O) and one local recurrence (X). Median 3.42, mean 4.19.

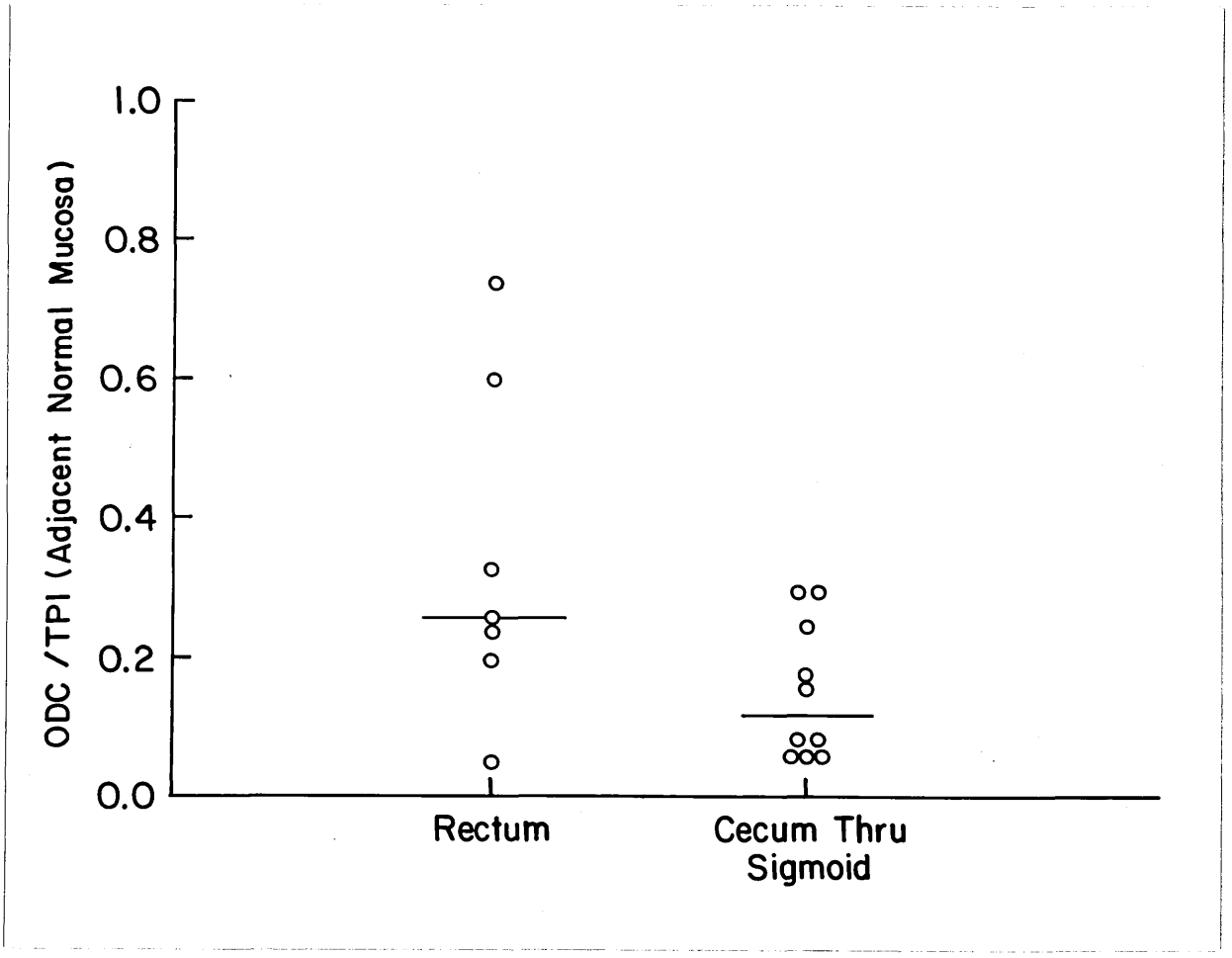

Figure 20

ODC/TPI Ratio for Adjacent Normal Mucosa of Rectum Versus Rest of Colon

ODC/TPI ratio for adjacent normal mucosa from rectum versus rest of colon. There is a suggestion of a higher value for rectal mucosa.

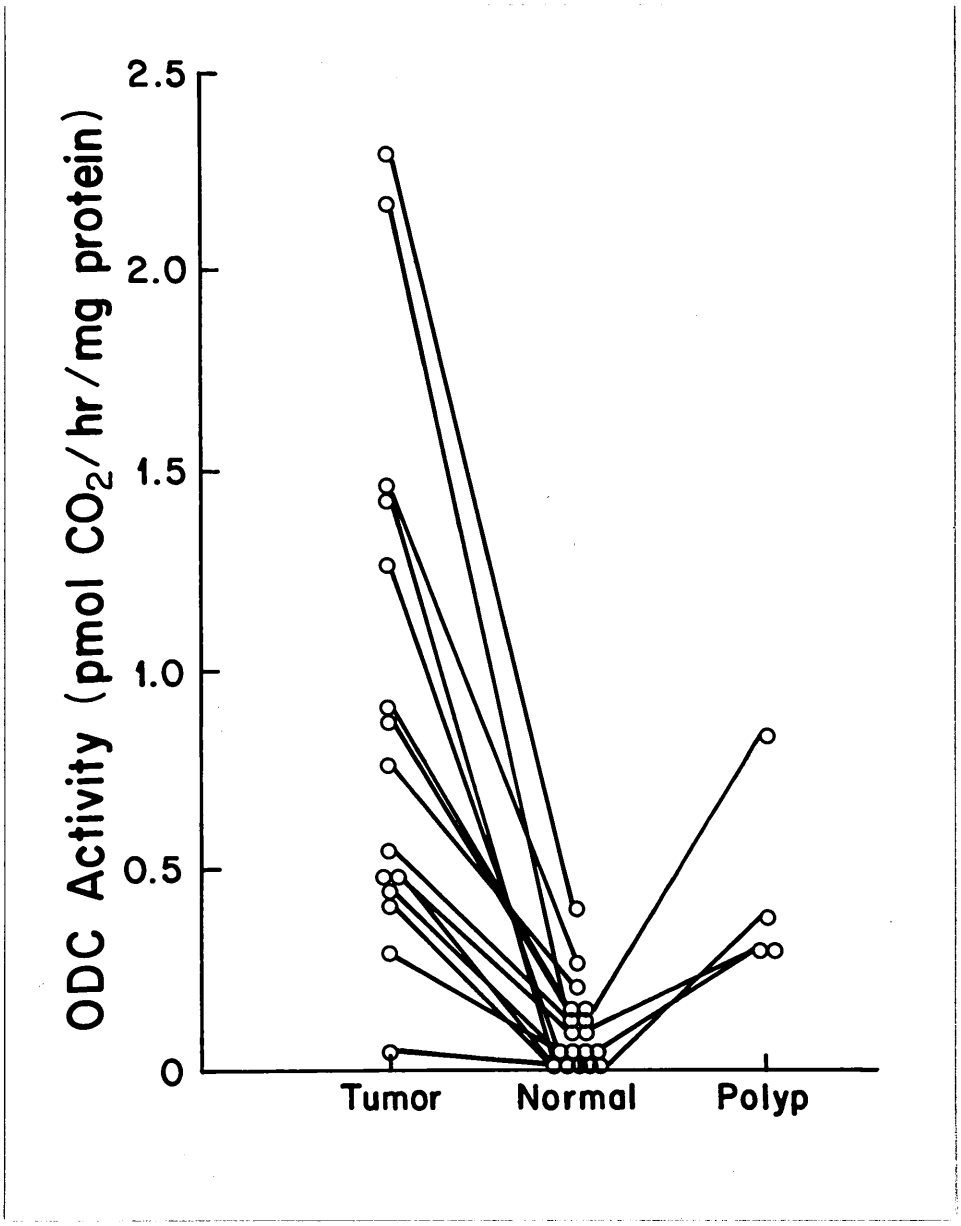

Figure 21

ODC Activity in Colorectal Tumours, Polyps, and Adjacent Mucosa

Comparison of ODC activity (pmol CO_2/hr/mg protein) in colorectal tumours and adjacent normal mucosa and benign polyps and adjacent normal mucosa. Activity data are available for 15 out of 18 tumour and mucosa samples depicted in Figure 18 and four out of six polyps and mucosa samples.

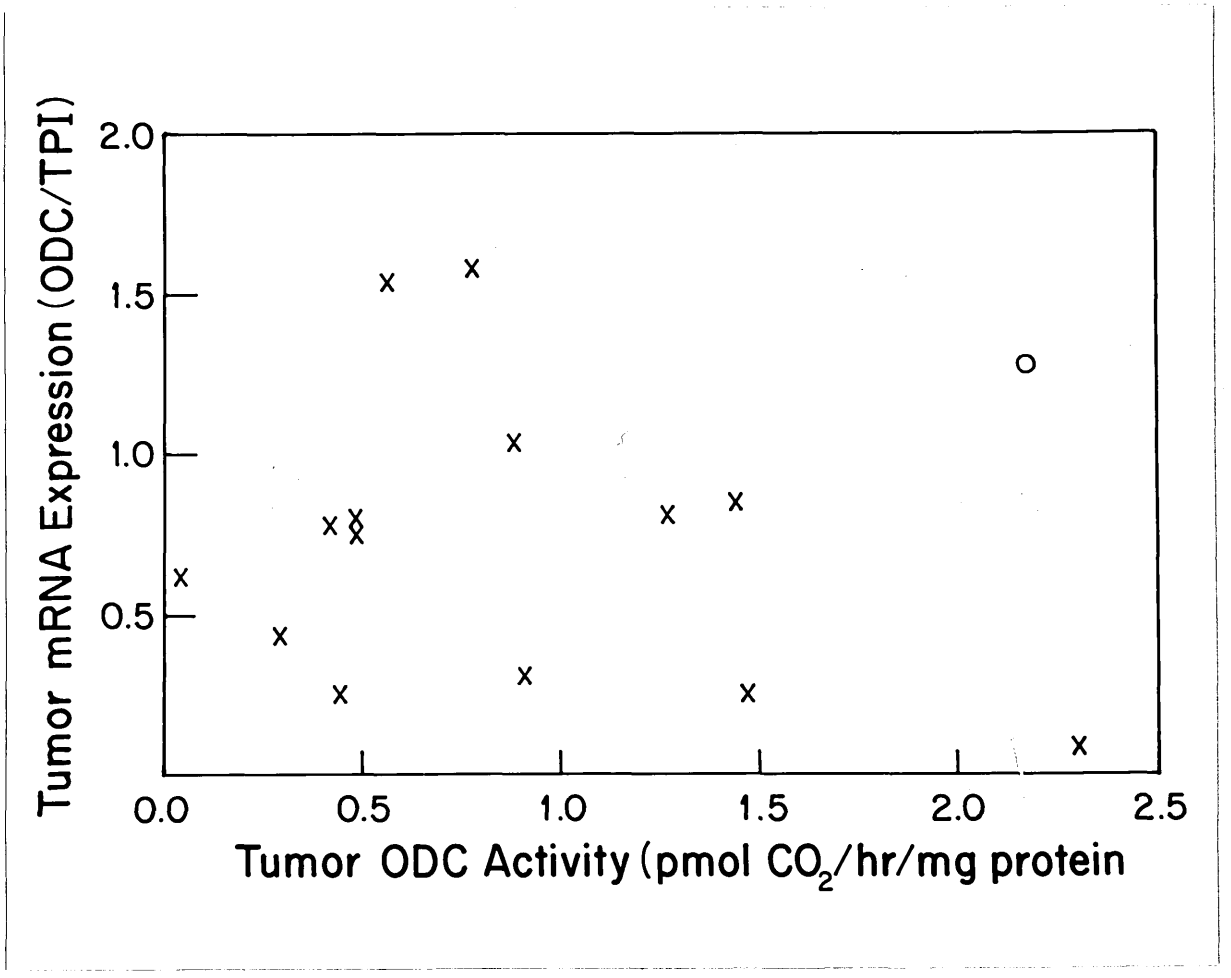

Figure 22

Scattergram Depicting Tumour ODC Activity Versus mRNA Expression

Scattergram showing tumour ODC activity versus mRNA expression in the same sample. 0 = local recurrence, x = primary tumour. No statistical correlation is found.

3.8 ODC Expression in Other Tumours Examined by Northern Analysis

RNA was also extracted from three gastric tumours, two leiomyosarcomas and two liposarcomas. In all tumour types, a single transcript of 2.2 kb was seen (Figure 23), and, again, there was elevation of ODC RNA expression in tumour compared to normal tissue (normal controls were not available for sarcomas). However, it was noted that the ODC/TPI ratio for gastric tumours and adjacent controls and for sarcomas was generally lower than for colorectal samples (Figure 24), although the number of samples examined was small. This is also reflected in the fold increase of tumour RNA expression over adjacent tissue, the mean being 1.87 for gastric tumours and 4.2 for colorectal tumours (Figure 25).

3.9 ODC DNA Analysis in Colorectal Neoplasia

In all, 18 colorectal carcinomas and seven colorectal polyps were examined. The tumour was well-differentiated in one patient, moderately-differentiated in 12, and poorly-differentiated in five. Site was distributed as follows: rectum seven, sigmoid/descending colon four; transverse three, and caecum four. Stage of the primary tumour (GITSG) was BI in four patients, BII in five, CI in six, and CII in three (Table 8). Of the seven benign polyps, six were villous adenomas with mild-to-moderate atypia and one was recorded as a tubular adenoma with atypia (Table 9). Tumour or polyp DNA was run alongside matched pathologically normal adjacent mucosa DNA from the same patient and from normal controls. No amplification of either ODC locus was seen in either carcinoma or polyp with either *Pst*I or *Hind*III digests after Southern blot analysis (Figure 26).

DNA was also analysed from two samples of leiomyosarcoma, five liposarcomas, three infiltrating ductal breast carcinomas, one lung carcinoma, and six gastric carcinomas, along with corresponding controls - no amplification of the ODC gene was seen.

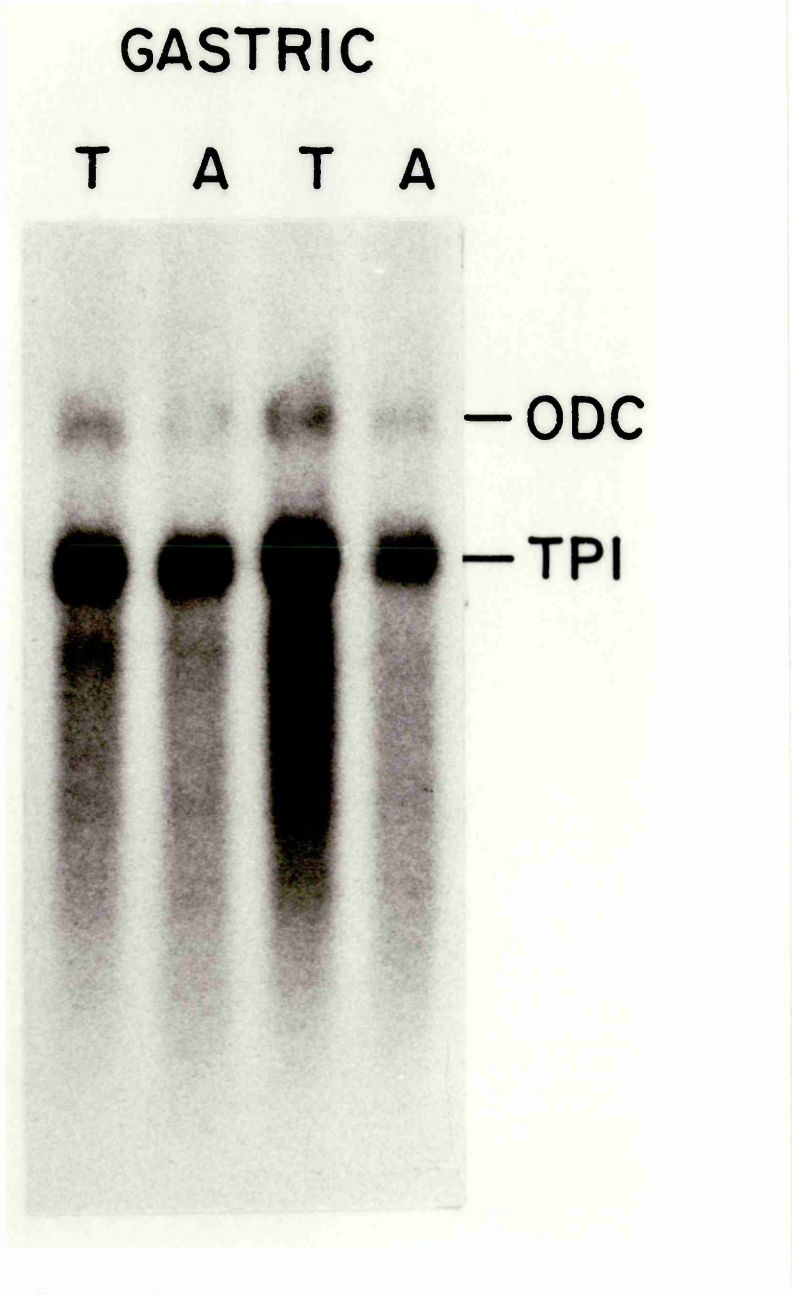

Figure 23

Northern Analysis of ODC Expression in Two Gastric Cancers

Glyoxal gel analysis of ODC RNA expression in gastric tumours from two patients (T) and corresponding adjacent mucosa (A). ODC and TPI signals are seen. A single ODC transcript, 2.2 kb size, is seen.

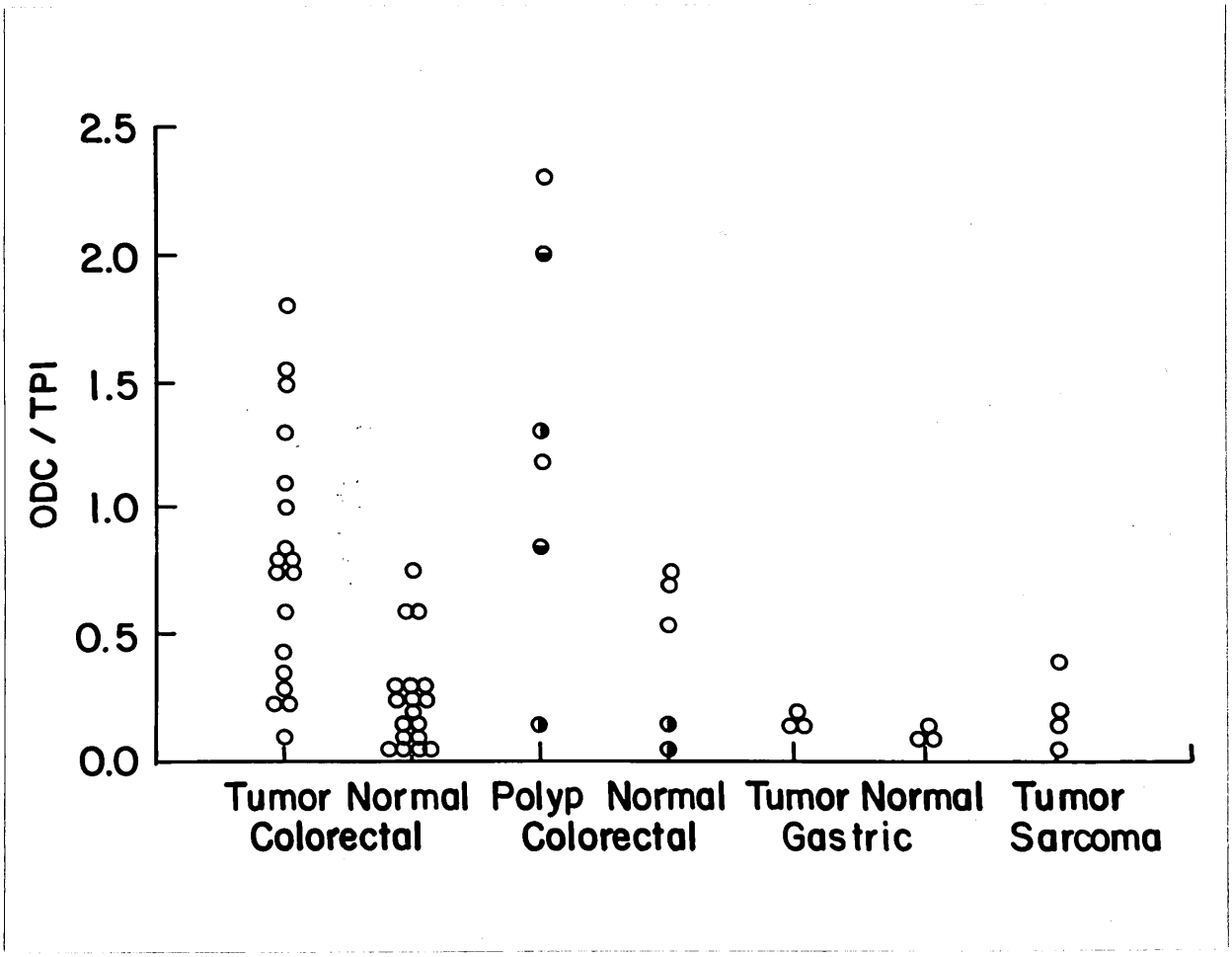

Figure 24

Graph Depicting ODC/TPI Ratio for Colorectal Neoplasia, Gastric Tumours, and Soft Tissue Sarcomas

Graph depicting ODC/TPI ratio for colorectal neoplasia and controls, gastric tumours and adjacent mucosa and for soft tissue sarcomas. ODC expression is generally higher in colon neoplasia compared to gastric cancer and sarcomas.

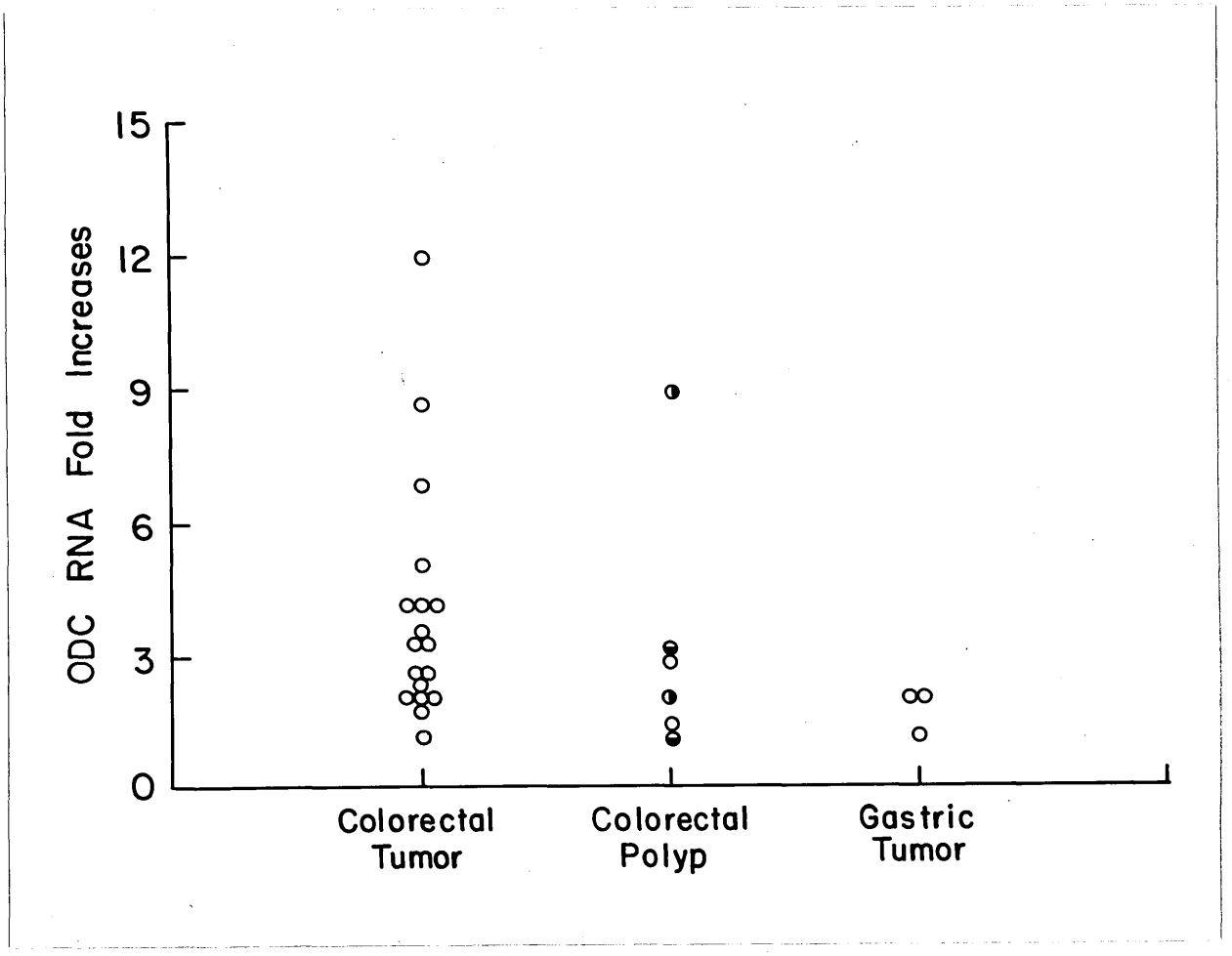

Figure 25

Fold Increase of Neoplasia Over Mucosa for Colorectal Tumours, Polyps, and Gastric Cancers

Fold increases of neoplasia over mucosa for colorectal tumours, polyps, and gastric carcinomas. Mean fold increase for gastric cancer was 1.87 compared to 4.2 for colon tumours.

Table 8

Data on Colorectal Cancer Specimens From Which ODC DNA Analysed

Patient	Site	Degree of Differentiation	Stage
CC 14	Sigmoid	Poor	CII
15	Rectum	Moderate	CI
17	Caecum	Well	BI
22	Rectum	Moderate	CII
26	Sigmoid	Poor	BI
27	Caecum	Moderate	BI
28	Rectum	Moderate	CI
30	Caecum	Poor	CII
31	Transverse	Moderate	CI
32	Sigmoid	Moderate	CI
36	Rectum	Moderate	BII
38	Rectum	Moderate	BII
39	Splenic flexure	Poor	BII
41	Rectum	Moderate	CI
48	Rectum	Moderate	BII
49	Transverse	Moderate	CI
50	Sigmoid	Moderate	BI
51	Caecum	Poor	BII

Table 9

Data on Polyp Specimens from Which ODC DNA Analysed

Patient	Site	Type
CP 1	Rectum	Villous with mild atypia
CP 2	Ascending colon	Villous with mild atypia
CP 3	Caecum	Villous with mild atypia
CP 4	Caecum	Villous with mild atypia
FPC 5	Rectum	Tubular with moderate atypia
CC36P	Sigmoid	Villous with mild atypia
CC52P	Sigmoid	Tubular with atypia

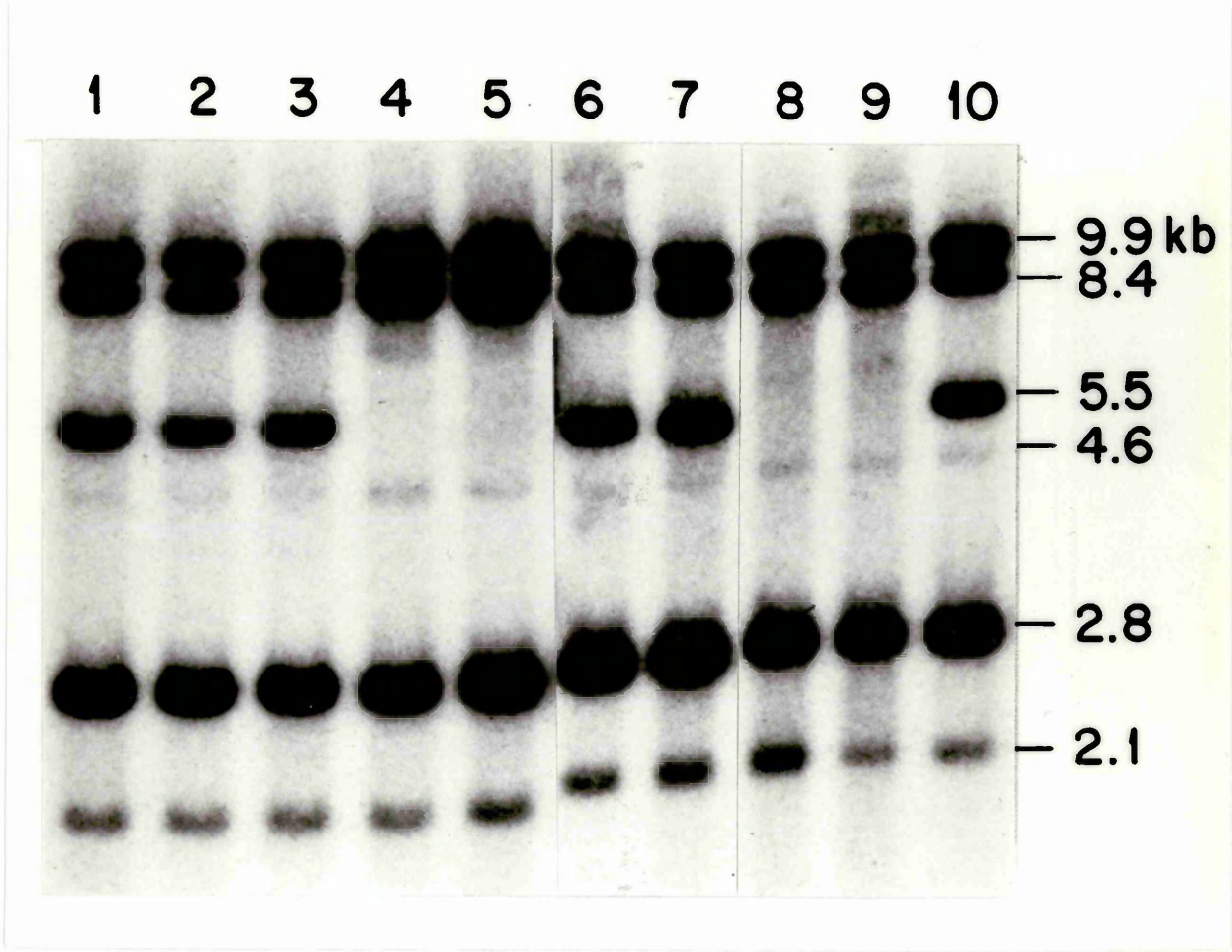

Figure 26

Southern Analysis of Tumour, Polyp, and Adjacent Mucosa Probed With pODC 10/2H, 10 μg of DNA in Each Lane

Lane 1	CC 36	Rectal carcinoma
Lane 2	CC 36P	Sigmoid adenoma from same patient as lane 1
Lane 3	CC 36′	Adjacent rectal mucosa from same patient as lane 1
Lane 4	CP 3	Caecal polyp
Lane 5	CP 3′	Adjacent mucosa from ascending colon
Lane 6	CP 1	Rectal polyp
Lane 7	CP 1′	Adjacent rectal mucosa
Lane 8	CC 39	Carcinoma splenic flexure
Lane 9	CC 39′	Adjacent mucosa
Lane 10		MLD - a human control

No amplification of either ODC locus is seen.

In one patient, CC15, there was a difference between tumour and mucosal DNA in the chromosome 2 DNA polymorphic band region (Figure 27). The pathologically normal adjacent mucosa of this patient revealed a new rare allele of the polymorphism described above. The band for this new allele co-migrates with the original faint 4.6 kb band (Figure 27, lane A). This patient is, therefore, designated an 8.4/4.6 heterozygote at this locus as compared to the 8.4/5.5 heterozygote in the adjacent control lane and the 8.4 homozygote in the far right control lane. The patient's tumour retains the 8.4 kb allele in the polymorphic region and a new 6.6 kb band appears, suggesting that the chromosomal region encoding the 4.6 kb band found in the normal tissue may be altered in the tumour yielding the 6.6 kb band. DNA from this tumour and adjacent mucosa samples were also digested with *Hind*III, *Xba*I, *Eco*RI, *Bam*HI, and *Hin*fI, but the restriction fragment length polymorphisms and the rearrangement in the tumour was not revealed using these restriction enzymes.

Two of the six gastric carcinomas examined also revealed polymorphism at the chromosome 2 locus (Figure 28). In lane 3, a faint 8.4 kb band is seen in the tumour tissue which is absent in the corresponding adjacent mucosa from that patient (lane 4), and in lanes 5 and 6, the tumour tissue exhibits the homozygote picture whereas the normal mucosa (lane 6) reveals a faint 8.4 kb band suggesting the heterozygote. These samples were also digested with the enzymes mentioned above but no RFLPs were seen. CC15 and the two gastric carcinomas mentioned all showed one transcript measuring 2.2 kb when examined by RNA analysis.

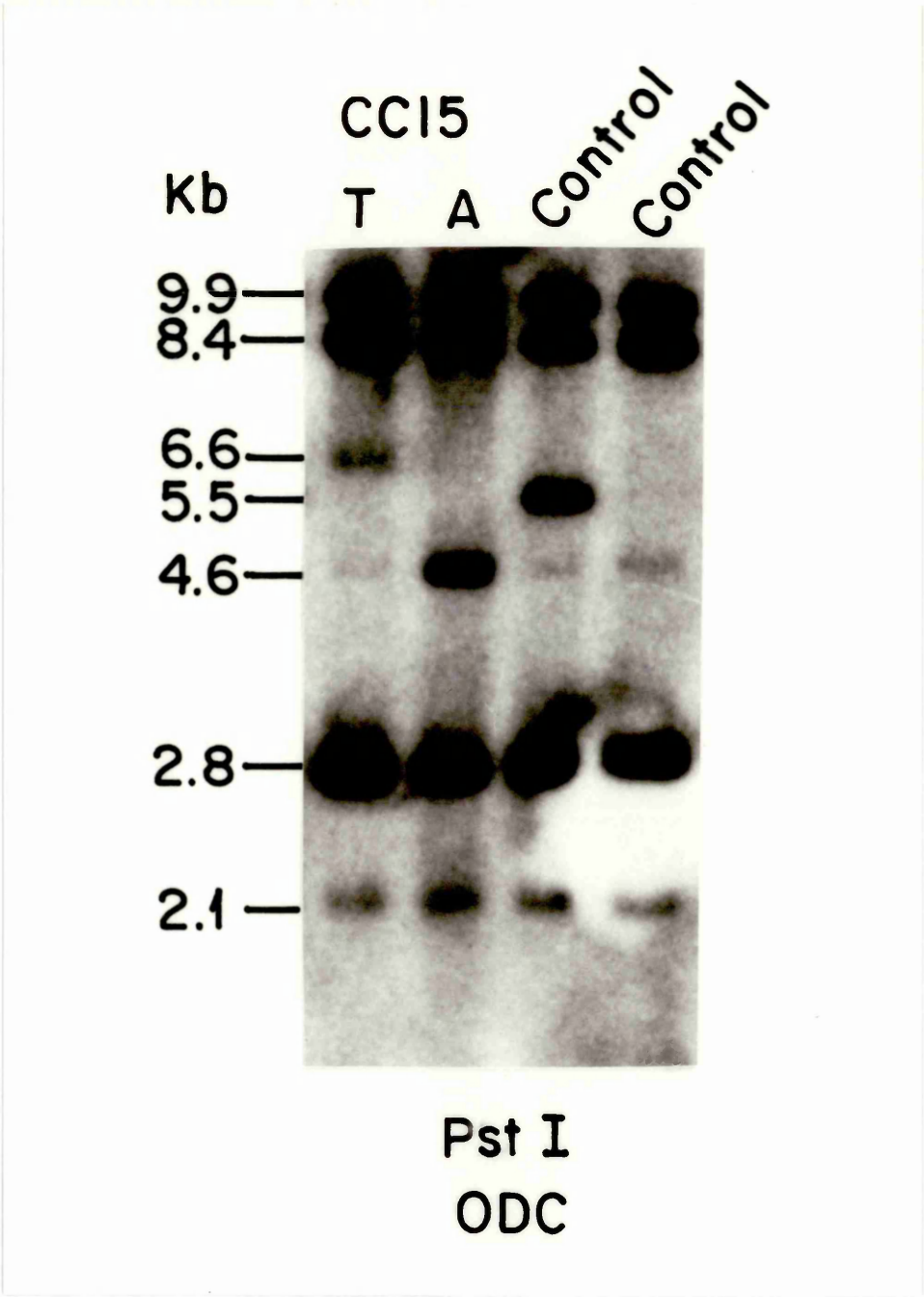

Figure 27

Southern Hybridisation of pODC 10/2H to CC 15 (colon cancer number 15)

Southern hybridisation of cDNA probe pODC 10/2H to *Pst*I digests of colorectal carcinoma CC15 (T) run alongside DNA from normal adjacent mucosa (A). A band is seen in the tumour DNA measuring 6.6 kb which is not seen in all other patients examined.

Figure 28

Southern Analysis of Three Gastric Cancer Samples Hybridised With pODC 10/2H

Southern analysis of DNA digested with *Pst*I from three patients with gastric carcinoma (lanes 1, 3, and 5) and corresponding matched adjacent mucosa as controls (lanes 2, 4, 6). In lane 3, a faint 8.4 kb band is seen which is absent in lane 4. In lane 6, the adjacent mucosa shows the 8.4 kb band suggesting the heterozygote picture but this band is absent in the tumour tissue (lane 5).

3.10 AdoMetDC DNA Analysis in Colonic Neoplasia

DNA was obtained from a total of 13 colorectal cancers and corresponding adjacent mucosa, and from five benign colonic polyps along with adjacent mucosa (Tables 10 and 11). Lymphocyte DNA was obtained from a total of nine unrelated volunteers. DNA from 26 individuals in all was examined.

Tumour or polyp DNA was run on a gel with normal mucosal DNA from the same patient. In all 26 cases, the same four human bands were found. No polymorphism in *Pst*I digested DNA was seen between individuals and no polymorphism noted between tumour or polyp and corresponding mucosa. No amplification of either the locus on 6 or on X was seen in colorectal cancer or benign colonic polyp DNA (Figure 29).

RNA analysis was attempted using the rat AdoMetDC probe; however, this was unsuccessful. Although the rat probe appeared similar enough to the human for DNA experiments, it did not hybridise well enough under the conditions used for RNA analysis.

3.11 Expression of IGF-2 in Colorectal Neoplasia

To determine whether there was any correlation between ODC and IGF-2 expression, several samples were also probed with IGF-2 cDNA.

In all, two colonic polyps, four colorectal carcinomas, and three gastric carcinomas were examined along with controls. One polyp and one colon carcinoma (CC36) revealed very enhanced expression, the normal signal being barely detectable (Figure 30). No expression was seen in the other samples. Consistently eleveated ODC expression was seen in all samples, and thus, no correlation existed between IGF-2 and ODC expression.

Table 10

Colon Carcinoma Samples and Adjacent Normal Mucosa Analysed For AdoMetDC DNA

Patient	Site	Degree of Differentation	Stage
CC14	Sigmoid	Poor	CII
CC15	Rectum	Moderate	CI
CC17	Caecum	Well	BI
CC22	Rectum	Moderate	CII
CC26	Sigmoid	Poor	BI
CC27	Caecum	Moderate	BI
CC28	Rectum	Moderate	CI
CC30	Caecum	Poor	CII
CC32	Sigmoid	Moderate	CI
CC36	Rectum	Moderate	BII
CC38	Rectum	Moderate	BII
CC39	Splenic flexure	Poor	BII
CC41	Rectum	Moderate	CI

Table 11

Colonic Polyp Samples Analysed For AdoMetDC DNA

CP 1	Rectum	Villous with mild atypia
CP 2	Ascending colon	Villous with mild atypia
CP3⁻	Caecum	Villous with mild atypia
CC36P	Sigmoid	Villous with mild atypia
FPC5	Rectum	Tubular with moderate atypia
FPC30	Sigmoid	Tubular with moderate atypia

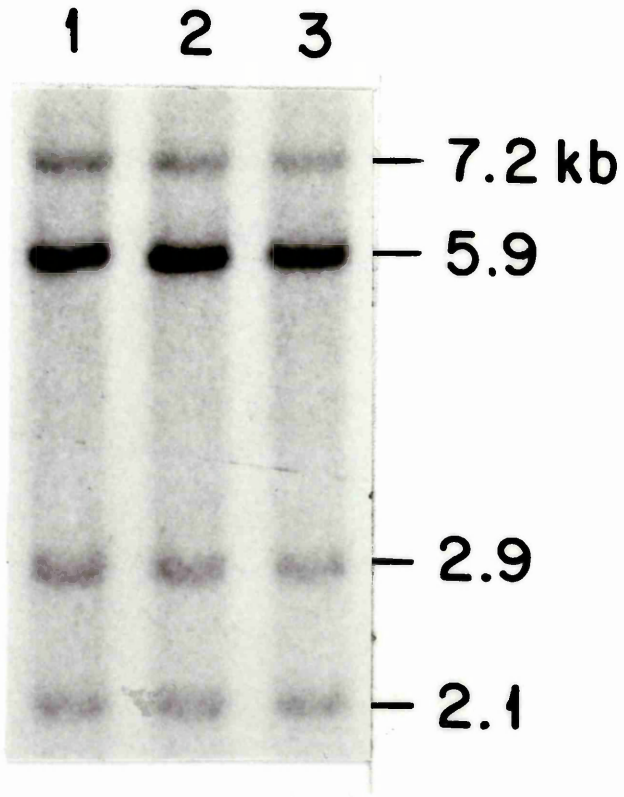

Figure 29

Southern Analysis of Colorectal Neoplasia Samples Probed With AdoMetDC

Southern analysis of tumour, polyp, and adjacent mucosal DNA from the same patient digested with *Pst*I and hybridised with the rat AdoMetDC probe. Lane 1: tumour CC36; Lane 2: CC36P - Adenomatous polyp; Lane 3: adjacent mucosa. No amplification or rearrangement is seen.

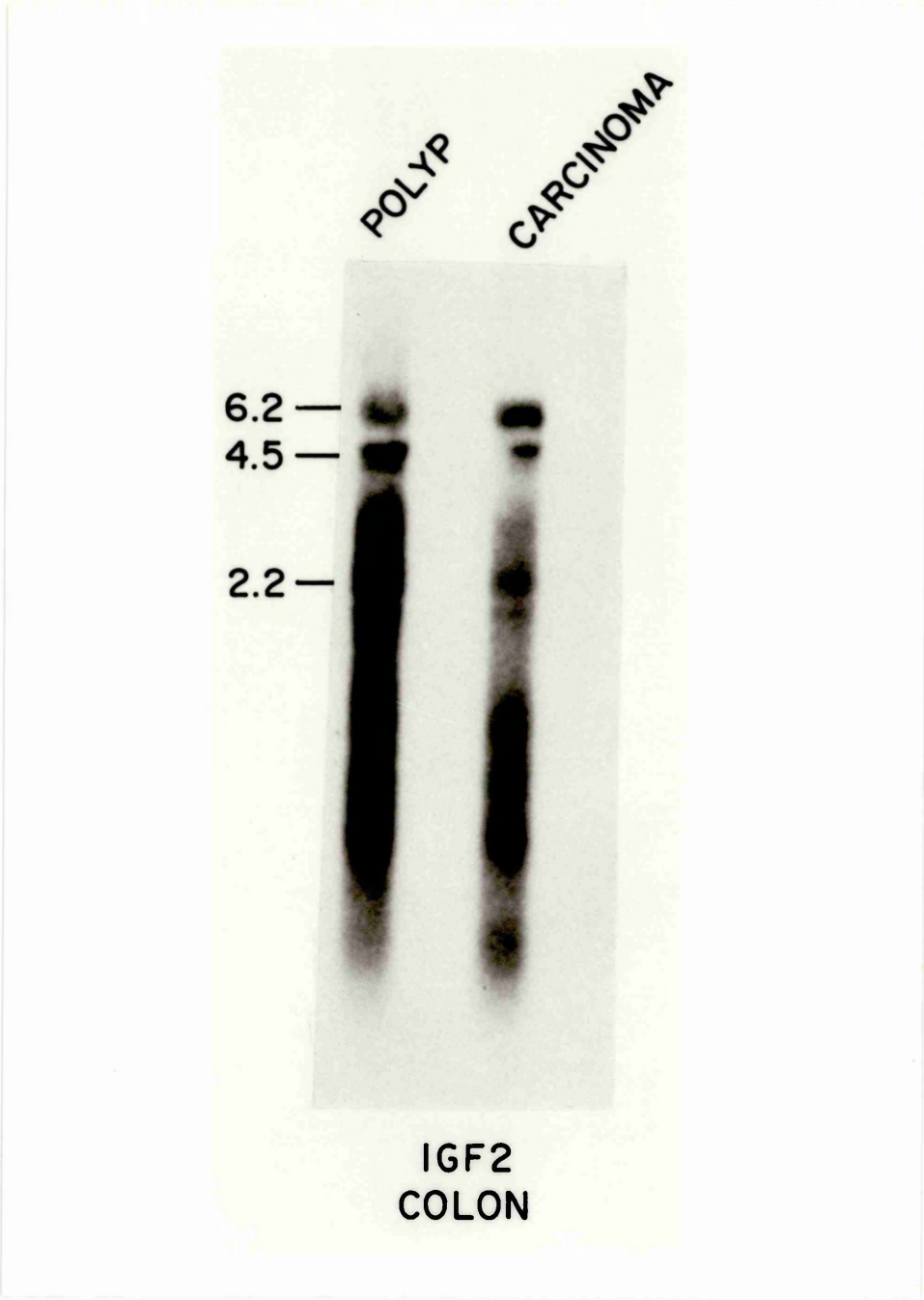

Figure 30

Northern Analysis of IGF-2 Transcripts From Colorectal Neoplasia

Northern analysis of IGF-2 transcripts from colorectal polyp and carcinoma. Normal mucosa RNA was in adjacent lanes, but the signal is barely detectable. Size of bands in kb given on the left. Elevated expression of IGF-2 is seen for one polyp and one carcinoma sample. None of the other samples examined (which all showed increased ODC expression) expressed IGF-2.

4. DISCUSSION

4.1 Mapping the ODC Gene

Knowledge of the chromosomal assignment of any gene involved in the control of growth and proliferation is a major step in elucidating possible means of understanding gene control. In this case, the ODC and AMD genes are located on different chromosomes.

In 1986, Wingvist, et al.,[222] mapped ODC gene sequences to 2pter→p23 and 7cen→qter. In the experiments described in this thesis, ODC1 was localised to 2p25 and ODC2 to 7q31→qter.

Although there was no evidence of rearrangement of the ODC gene in any of the colon neoplasias studied, rearrangement between ODC and the switch region of the gamma-1 immunoglobulin gene has been reported in mouse myeloma cells.[223] Chromosomal translocation involving the short arm of chromosome 2 have been described in various haematologic malignancies.[224,225]

It is possible that rearrangement accounts for the deregulation of ODC in certain tumour types. Thus, it is interesting to know the location of ODC in relation to other gene sequences on chromosomes 2 and 7 which are known to be involved in tumour growth and progression.

The 2p25 ODC locus is known to be close to a fragile site (2p24.2)[226] and to the avian myelocytomatosis related oncogene MVCN, formerly called NYMC[227] (2p24). A relationship between fragile sites and breakpoints of recurring chromosomal abnormalities has been hypothesised,[94] and certain carcinogens increase the frequency of chromosomal breakpoints.

Transforming growth factor α, an autocrine growth factor expressed in many solid tumours,[228] and in colon cancer cell lines,[229] is also located on the short arm of chromosome 2.[230] Glucagon, a trophic hormone, is located on 2.[231]

The proximity of the ODC gene to the ribonucleotide reductase gene is especially interesting. Ribonucleotide reductase catalyses the first unique rate limiting step in DNA synthesis, both large (RRM_1) and small (RRM_2) subunits being necessary for activity.[232] Srinivasan, et al.,[233] have shown coamplification of the genes for ribonucleotide reductase subunit M_2 (RRM_2) and ODC in hamster cell lines resistant to hydroxurea. Tonin, et al.,[234] found this

phenomenon in humans plus the amplification of p5-8, a 55,000 dalton protein. In hamsters, all three loci cosegregate with chromosome 7. The human gene for RRM_2 is found at 2p24-25[236] and is therefore closely linked with the ODC gene at 2p25.

McClarty, et al.,[237] have shown that the RRM_2, ODC and p5-8 genes exist as a single amplicon in hamster cells and that a parallel decline in hydroxyurea resistance is accompanied by a decline in copy number of all three genes.

It is interesting to speculate that this region may act as a multidrug resistance genetic unit, since DFMO resistance leads to amplification of ODC in some instances. Whether RRM_2 and p5-8 are also co-amplified in DFMO resistance has not been reported to date. The fact that two genes so involved in DNA synthesis and cell proliferation are in such proximity indicates that they may both be acted upon by the same regulatory sequences.

The relationship between the RRM_2, p5-8 and ODC coamplicon and N-myc has been expanded recently in a report by Tonin, et al.[238] Winquist,[222] et al., noted that the ODC gene was not coamplified in certain neuroblastomas which demonstrated amplified N-myc, confirming the finding described in this thesis. Tonin, et al., found, however, that coamplification of ODC and N-myc occurred in one of six human neuroblastomas. RRM_2 and p5-8 were not amplified. Since amplification of N-myc is known to correlate with advanced disease stage in neuroblastoma,[99] it is possible that coamplification of ODC may act as another prognostic indicator in this disease.

The region 7q31→qter in which ODC2 resides also contains loci for cystic fibrosis (7q31), three fragile sites (7q31.2, 7q32.3, 7q36), histones H_2A, H_2B, H_3, H_4, multiple drug resistance (7q36), the met protooncogene (7q31-q32) and the oncogene INT-1 (7q31-q32). The regulatory polypeptides protein kinase type I (p13-qter) and II (q31-qter) and two genes thought to be involved in metastasis-laminin (q31) and TTIM1 (q32-qter), also reside on 7.[239,240] The expression of epidermal growth factor receptor (EGFR), which resides on chromosome 7, correlates with structural or numerical alterations of the chromosome.[241] Genes located on 7 appear to be essential for metastasis and invasion to occur; EGFR and platelet derived growth factor A (PDGFA) have been postulated to be involved.[242]

Several new chromosomal rearrangements on chromosome 7 were reported by Trent.[224] 7q translocations were reported for colon cancer, breast cancer, and melanoma.

4.2 The Functional Gene for ODC

Having mapped two loci for ODC, the question arises, which of these represents the site of the functional gene?

In the mouse, the ornithine decarboxylase gene is represented by a family of at least 14 related loci.[243] In the human, only two loci exist. Since only one size of mRNA is present, this indicates that either both loci are functional, producing message of identical size, or one locus is functional, the other being a pseudogene.

Our preliminary data comparing ODC mRNA expression in human-mouse hybrids containing human chromosome 2, but not 7, and chromosome 7 alone, suggest that one functional gene exists and is located at 2p25.

The in situ hybridisation results also indicate that chromosome 2 may contain the major locus, since there was no hybridization signal on chromosome 7. Fowler, et al.,[208] found that human-mouse cell hybrid methodology detected loci for α-fucosidase on chromosomes 1 and 2, but in situ revealed only the functional locus on chromosome 1. In situ methodology therefore may not be as sensitive as the hybrid panel method, accounting for the lack of an ODC signal on chromosome 7. More stringent conditions may also contribute to the difference. In both the *Pst*I and *Hind*III digests of the human-mouse hybrids, the bands on 7 became less prominent after a highly stringent wash.

Alhonen-Hongisto, et al.,[244] studying a human myeloma (Sultan) cell line resistant to DFMO found the locus on chromosome 2 to be amplified, which also suggests that chromosome 2 carries the functional gene. Recently, the mouse ODC functional gene has been mapped to chromosome 12.[245] The other loci are thought to be pseudogenes. The assignment to mouse chromosome 12 has been confirmed by Berger[246] and Villani, et al.[247] Mouse chromosome 12 exhibits considerable homology with human chromosome 2. For example, the genes coding for acid phosphatase, propiomelanocortin,

ribonucleotide reductase M2 and NMYC are all located on mouse chromosome 12 and human chromosome 2. This provides further evidence that the functional ODC gene is probably located on 2.

The pseudogenes of mouse ODC appear to be very ancient, originating about 10 million years ago.[248] Pseudogenes were first described by Jacq, et al., in 1977.[249] They are closely related sequences to the functional gene but appear to be inactive. Two categories have been identified, the first being genes that have retained intervening sequences normally found in the functional gene. These are normally located adjacent to the normal functional gene. The second category, which occurs much more commonly, are the processed pseudogenes. These sequences lack introns suggesting that they are derived from processed RNA molecules after reverse transcription. Processed pseudogenes are found on different chromosomes than their functional homologous sites.[250,251]

Numerous gene families have been reported in which functional and processed pseudogenes reside on different chromosomes, including G-3-PD, immunoglobulins, metallothionein II, DHFR, Ki-ras,[250,252] ceruloplasmin[251] and aldolase.[253]

As the sequences for ODC reside on different chromosomes, it is likely that the locus on 7 is a processed pseudogene.

Another possibility is that the other sequence on 7 codes for a functionally related gene. This is unlikely, however, as no other genes have been reported to have significant homology with ODC.

4.3 Mapping the AdoMetDC (AMD) Gene

Two AMD sequences were localised to chromosomes 6 and X, the sequence on X mapping to Xq22-Xq28.

No rearrangement of the AMD loci was seen when samples were digested with *Pst*I. It is possible that there may be rearrangement which can be detected with other enzymes in colorectal neoplasia and that rearrangement may occur in other tumour types, e.g., haematologic malignancies.

On chromosome 6 where AMD1 resides, there are genes coding for five oncogenes: MYB (q22-q23), PIM (p21), ROSI (q21-q22), SYR (q21), and MAS1 (q24-q27).[224] There are also four fragile sites and the loci

PRL (prolactin) (p23-p21) and the oestrogen receptor ESR (q24-q27).[254,255] Chromosomal abnormalities involving chromosome 6 occur mainly in haematologic malignancies but are also seen in melanoma and uterine carcinoma.[224]

The short arm of chromosome 6 is the site of the gene family for the major histocompatability complex (HLA).[254] Several investigators have shown that alterations of the expression of HLA genes and thus alterations in HLA phenotype result in changes in the metastatic potential of cells.[256-259]

Chromosome X contains the locus for the oncogene MCF2 at Xq27.[260] An actively transcribed gene of the raf oncogene group, ARAF1 has been localised to Xp21-Xq11.[260] It is conceivable that AMD1 (on chromosome 6) and AMD2 (on chromosome X) may be located near one of these growth related genes or within a chromosomal rearrangement associated with abnormal growth.

It is possible that either or both of the AMD sequences which have been localised may represent functional genes, although since two bands could not be scored, a functional gene may be represented by one of these unscored bands.

In the rat, a multigene family for AMD exists,[261] at least one of which is a typical processed pseudogene lacking introns.[262]

It is possible that both loci for AdoMetDC (AMD1 and AMD2) are functional since two forms of AdoMetDC have been reported.[170]

Two forms of AdoMetDC mRNA have been reported for both rodents and humans,[261] the smaller mRNA being 2.1 kb and the larger 3.4 kb in rats and 3.6 kb in humans.[261,263,264] The size difference appears to be due to differences in the 3′ noncoding end. This may be due to two functional loci, or altered transcription of one functional locus.

4.4 Chromosome 2 Restriction Fragment Length Polymorphism of ODC

The data collected from 40 unrelated individuals revealed three consistent banding patterns. In all three, the band localising to chromosome 7 was present, the allelic diversity occurring at the chromosome 2 locus. The phenotypes represented with *Pst*I digests were the 5.5 kb homozygote, the 8.4 kb homozygote, and the 8.4/5.5 kb heterozygote.

Both Hickok[265] and colleagues and Fitzgerald and Flanagan[266] have reported RFLPs of the ODC gene seen with *PstI* digests.

In their experiments, the 5.5 kb homozygote is not identified and only two variations are seen. This discrepancy is probably accounted for by the fact that the 5.5 kb homozygote exists less frequently in the population and is only revealed when a large number of patients is examined.

The chromosome 2 polymorphism should be studied further by following its inheritance within kindreds.

4.5 ODC mRNA Expression in Colorectal Neoplasia

ODC was found to be consistently elevated in both benign and malignant forms of colorectal neoplasia, only one size of message being present. Expression was independent of site, stage, and degree of differentiation of tumour.

This finding agrees with the results of Kingsnorth, et al.,[185] who found elevated polyamine content in colorectal cancers relative to control mucosa. Similarly, elevated ODC enzyme activity in neoplasia compared to mucosa has been reported by several authors.[186-189,192,267-270] Kingsnorth and colleagues[185] found no correlation between polyamine content and tumour site, Dukes stage, histological grade and presence of liver metastases. However, LaMuraglia, et al.,[187] noted polyamine content and ODC activity to be higher in well-differentiated than poorly-differentiated tumours.

Narisawa, et al.,[270] reported a correlation of ODC activity and tumour site. They found a statistically significant increase in activity in left-sided compared to right-sided tumours. However, Porter's lab,[267] on the contrary, found the activity of colon adenocarcinomas to be slightly higher than rectal adenocarcinomas. It has been noted in recent years that the ratio of proximal to distal colon cancers has changed. An increased proportion of right-sided lesions has been reported, leading several authors to suggest that proximal cancers have a different biology and pathogenesis.[271,272]

Porter's study[267] revealed a stepwise increase in ODC activity from mucosa to polyp to carcinoma. Luk, et al.,[188] have also noted

that ODC activity increased as degree of dysplasia of polyps increased.

No clear stepwise progression of increasing expression of ODC from mucosa to polyp to cancer was found in this thesis. The mean fold increase was slightly higher in carcinoma compared to polyps (3.4 vs. 2.8).

Controversy exists concerning the levels of ODC activity in normal uninvolved mucosa. Koo, et al.,[273] found the ODC activity of rectal mucosa of patients with colonic neoplasia to be higher than in controls. A similar finding was noted by LaMuraglia and colleagues,[187] who found that the ODC activity of noninvolved colonic mucosa from tumour bearing specimens was 165% that of the colonic mucosa of nonneoplastic disease (mainly diverticulosis). Both Moorehead, et al.,[274] and Porter, et al.,[267] however, found the enzyme activity in normal colonic biopsies to be higher than in tumour-bearing patients. As Kingsnorth[275] points out, these variations may be related to variations in technique. The half-life of ODC being the shortest of any known enzyme, prompt handling of tissue samples is required to prevent degradation.

It is also possible that a gradient of ODC activity and mRNA expression exists in the normal colon. There was a suggestion that ODC mRNA expression was higher in normal rectal mucosa than in the colon, but this did not reach statistical significance.

Such a gradient could be investigated by taking multiple samples along the entire length of the colorectum from the same patient. It has been suggested that ODC activity of apparently uninvolved mucosa is a marker for increased cancer risk. Luk and Baylin[188] compared the ODC activity in normal-appearing mucosa obtained by flexible sigmoidoscopic biopsy to be higher in patients with familial polyposis than in normal controls. "At risk" members of familial polyposis families also had a higher mucosal ODC activity than their spouses who were not at risk.

Narisawa, et al., also noted that the uninvolved mucosa ODC activity was elevated in the presence of synchronous neoplasia.[270]

It has been suggested that a "mucosal field change" exists in the colon in patients with colorectal neoplasia, i.e., that the entire colon, particularly the area adjacent to the tumour, should be

regarded as abnormal. This view arose mainly from studies which demonstrated increased sialomucin production in noninvolved mucosa adjacent to the cancer[276] and from the finding of multiple colonic neoplasias (synchronous and metachronous) in about 10% of patients.[277]

In the experiments described in this thesis, adjacent mucosa was sampled approximately 5 cm from the area of neoplasia. Lawson, et al.,[278] compared the activity of ODC in transitional mucosa (defined as within 2 cm of the neoplasia) to mucosa at least 10 cm distant and found no significant difference. This finding would support the use of mucosa 5 cm distant as a suitable control.

4.6 ODC Activity and mRNA Expression: The Regulation of ODC

In general, the regulation of any enzyme's activity can be affected by changes in RNA, protein, or both. Increased RNA expression can be due to increased transcription rate, increased stability of the mRNA, or altered RNA processing. Increased numbers of protein molecules may result from increased translatability of mRNA, independent of changes in transcription, and prolonged half-life of the molecule. Altered activity, when the number of protein molecules is constant, can occur due to conformational changes of the protein or binding such as phosphorylation.

Elevations in ODC activity occur in response to a number of conditions such as addition of trophic hormones and exposure to tumour promoters such as TPA.

Early studies using cycloheximide (which inhibits protein synthesis) and actinomycin D (which inhibits DNA dependent RNA synthesis) demonstrated that both DNA and RNA synthesis caused increased ODC activity in regenerating rat liver.[279] In some instances, increased ODC levels correlated with fluctuations in cyclic AMP (cAMP).[280]

The response of mouse kidney to a single dose of androgen results in increased ODC mRNA with the amount of immunoreactive protein closely paralleling the changes in amount of mRNA.[281] Most of the data acquired to date indicates that changes in ODC activity are proportional to enzyme protein content, post-translational modifications of the protein playing a minor part.[282,283] On the other

hand, prolonged androgen stimulation results in 10- to 50-fold accumulation of mRNA but a 200- to 250-fold increase in protein content. Thus, decreased protein degradation also plays a role.[281]

Response to androgen also differs depending on the genetic background of the mice, i.e., to which inbred strain they belong.[281]

Studies with fibroblast cell lines exposed to stimulation result in increased ODC mRNA levels, but it is not clear whether increased transcription or stabilisation of existing RNA is the main mechanism. Katz and Kahana demonstrated increased transcription in BALB/c 3T3 mouse fibroblast cells exposed to serum.[284] Rose-John, et al.,[285] however, using mouse Swiss 3T3 cells to which a tumour promoter was added, demonstrated increased mRNA due mainly to prolonged mRNA half-life.

The response of the rat phaeochromocytoma cell line PC12 to nerve growth factor (NGF) was studied by Feinstein, et al.[286] Increased ODC RNA levels closely paralleled changes in ODC activity both in quantity and time-course. One-third of the ODC RNA induction was not blocked by cycloheximide treatment, but was fully inhibited by actinomysin D. This suggests that changes in ODC mRNA in this system are fully dependent on ongoing RNA transcription, not changes in stability of RNA.

White, et al.,[287] studied ODC expression in bovine lymphocytes in response to concanavalin A (Con A). A five-fold greater increase in ODC activity compared to ODC mRNA level occurred suggesting increased efficiency of translation of ODC mRNA.

mRNA levels in the cell are dependent on mRNA stability. The sequence promoting RNA decay has been identified in the 3′ noncoding region of the eukaryotic gene.[288] Stability of c-myc RNA is related to the presence of a long poly (A) tail.[218]

Whether stability of ODC RNA is related to alterations at the 3′ end is unknown.

It appears then that increased ODC activity is due to a number of different mechanisms and combinations of mechanisms in different cell systems including increased transcription, prolonged RNA stability, increased translatabality, and increased protein half-life.[289]

No studies have investigated the mechanisms of ODC regulation in solid tumours.

In the present study, ODC mRNA expression was increased a mean of four-fold in carcinomas and three-fold in polyps compared to normal tissue. Porter, et al.,[267] found ODC enzyme activity for 40 carcinomas to be a mean of approximately eight-fold greater than adjacent mucosa and for 18 polyps the activity was increased four-fold. For the 15 patients for whom ODC activity and mRNA expression data were obtained in this thesis, no correlation was found between mRNA expression and activity. It is important to note that no undue delay occurred in the handling of these tissue samples, which may have contributed to degradation of ODC. All samples were placed promptly in liquid nitrogen for later activity and RNA analysis.

Thus, the regulation in colon cancer is unlike, for example, the androgen stimulated kidney in which mRNA levels are paralleled by activity levels.

The generally increased activity compared to expression in colon neoplasia indicates that factors in addition to increased transcription or extended stability of RNA are in operation. Increased translatability of ODC mRNA may occur as described by White and colleagues for bovine lymphocytes.[287]

It is also possible that altered forms of ODC protein may occur, which have increased enzyme activity; for example, an altered form of ODC having a prolonged half-life has been found in a human neuroblastoma cell line.[290]

O'Brien, Hietala, and co-workers have found two different forms of ODC.[291-293] The ODC in mouse epidermal papillomas was activated by GTP, the ODC of normal epidermis was not. The two forms of ODC also differ with respect to heat stability, Km, and molecular weight. More recently, O'Brien's group[294,295] has found that the GTP-activated form of ODC is also present in human colorectal cancers. Hietala, et al.,[296] have reported a GTP-activatable form of ODC in 13 of 40 tumours, compared to 3 of 40 mucosa samples. Multiple size forms of ODC were found, some of which were activated by GTP. It was noted that proximally-sited tumours contained a higher proportion of these GTP-activatable forms. The fact that only one ODC message is seen in colorectal neoplasia indicates that the altered ODC must have arisen

from post-translational modifications. Such modifications may account for the lack of correlation between mRNA levels and activity in colorectal neoplasia.

Control of expression of ODC must be coupled to the transmembrane signalling pathways which are activated when a mitogen interacts with its receptor on the cell surface. Several signal transduction pathways are well-known; namely, the phosphotidylinositol/ITP_3/DAG/Ca^{++} system, protein kinase-C, the adenylate cyclase/cyclic AMP system, and the GTP binding proteins to which family the ras proteins belong. These pathways are summarised in the reviews by Arbeit[297] and Weinstein.[298]

Growth factor receptors (GFRs) reside in the cell membrane and are composed of an extracellular domain, a transmembrane segment, and an intracellular domain having tyrosine kinase activity.

The binding of ligand to the GFR results in a cascade of proteins phosphorylated on tyrosine, resulting in the release of second messengers into the cell. Phospholipase C is one of the key second messengers which becomes phosphorylated in this manner. Phospholipase C splits phosphoinositol-bis-phosphate (PIP_2) to inosine triphosphate (ITP_3) and diacylglycerol (DAG). DAG then activates protein kinase C (PKC) in conjunction with calcium released from cytoplasmic vesicles by ITP_3.

Protein kinase C[299] is one of the key messengers within the cell. The tumour promotor TPA has structural similarity to DAG and can activate PKC in the absence of ITP_3 and calcium. PKC undergoes a conformational change which results in phosphorylation of a protein substrate.[298]

G proteins are a class of proteins which bind GTP. The products of the ras proto-oncogene family are related to G proteins. The binding of a ligand to GFR causes alterations in the ras protein product which causes subsequent binding of GTP. PLC and PKC are then activated and thereby nuclear expression is affected. Mutations of the ras proteins occur characteristically at codons 12, 13, or 61, which cause continuous activation of PKC. G proteins are also important in the β-adrenergic transduction pathway. The binding of a molecule to the β-adrenergic receptor via a G protein activates adenylate cylase, rises cAMP levels and results in increased protein

kinase A levels, which then produces protein phosphorylation in the nucleus.[298]

It is well-known that the application of TPA to skin results in an increase in ODC activity,[127,164,165] mediated via protein kinase C.[300] In NIH-3T3 fibroblast cell lines, elevations of ODC activity and mRNA in response to growth-promoting factors occurred by two mechanisms: one involving protein kinase C and the other independent of PKC.[301]

Rose-John, et al.,[285] noted that TPA can also affect ODC expression by increasing the stability of the mRNA.[285] In bovine T lymphocytes exposed to mitogens, ODC expression is also mediated via PKC.[302] Hsieh and Verma,[303] studying T24 human bladder cancer cells exposed to TPA noted that the ODC increase was proportional to increased mRNA activity. No change in mRNA half-life occurred. The effect of TPA on transcription was abolished in PKC-deficient cells, implying that TPA causes an increase in rate of transcription mediated via PKC. The transient induction of ODC mRNA by TPA in rat hepatoma cells was inhibited by actinomysin D, which again suggests a direct effect on new transcription rather than prolonged half-life of mRNA.[304]

Mustelin and collegues[305] have discovered a method of ODC activation in T lymphocytes which is independent of PKC. In these cells, exposure to mitogens results in increased ODC within one minute, reaching a plateau in 5-10 minutes. Such early activation is independent of new protein synthesis. Their experiments revealed that ODC is bound to phosphotidylinositol at the cell membrane. The binding of mitogen to a cell receptor activated PLC, mediated via a G protein. The PLC enzyme then activated the phosphotidylinositol-ODC complex apparently by cleaving ODC from the diacylglycerol part of the molecule.

Thus, it appears that in both T lymphocytes and nonlymphoid cells, PKC dependent and independent pathways exist for the regulation of ODC expression.[306]

Both TPA and bile acids administered intrarectally result in a rise in ODC activity.[191] Bile acids have been shown to enhance PKC activity in colonic mucosa, which may account for their tumour promotor effect.[307]

Studies on PKC content of colon cancers have revealed a decrease in tumours compared to normal mucosa of about three-fold. PKC is found in both the cytosolic and membrane compartments of cells. In those cancers which contained an admixture of benign adenomatous tissue, there was an apparent shift of PKC activity to the membrane fraction. It has been proposed that early transformation of the mucosa is associated with this translocation of PKC activity, and in later stages of the disease, total PKC activity is down regulated.[308-310]

More insight into the regulation of the ODC gene in humans has been provided by the cloning and sequencing of the whole length human ODC DNA. The pODC 10/2H cDNA used in the experiments in this thesis contained 1,825 nucleotides, with an open reading frame of 1,383 nucleotides, yielding a polypeptide chain of 461 amino acid residues. The 5′ noncoding sequence was 87 nucleotides long, whereas it was determined that the full length of the 5′ end should be 335 nucleotides long. The 3′ noncoding region comprised 346 nucleotides.[265] Fitzgerald and Flanagan[266] went on to clone the complete length of the human gene, using the pODC 10/2H partial length clone. The gene is 8 kb in length and divided into 12 exons. Van Steeg, et al.,[311] have also reported the full length ODC sequence. There is considerable conservation of homology between species as would be expected for a gene essential in cell proliferation. Eighty percent to 90% homology has been reported for mouse, hamster, and rat ODC genes.[133,312-318] The sequence of a rat ODC pseudogene revealed over 80% homology with the cDNA.[319]

The regulation of eukaryotic genes is influenced by cis-acting DNA sequences termed promoters and enhancers.[320] The promoter is essential for the accurate and efficient initiation of transcription and is usually located immediately upstream from the transcription initiation site. Enhancers, on the other hand, increase the rate of transcription from promoters and act in an orientation-independent manner. Typical promoters comprise an AT-rich region termed the TATA box and several 8 to 12 base pair elements termed upstream promoter elements (UPEs). The TATA box influences accuracy of transcription, the UPEs, the rate of transcription. The protein which binds to the

TATA box, TFIID, and which thus plays a central role in mRNA synthesis, has recently been identified.[321,322]

The ODC promoter has been identified in the mouse using a transient expression assay which assesses chloramphenicol acetyltransferase activity (CAT). These studies revealed the promoter to be located within a region several hundred base pairs long 5′ to the initiation start site.[323]

Study of the 5′ region of the rat, mouse, and human genes reveal conserved transcription elements. The human gene contains a TATA box, CAAT box, GC rich region, and a cyclic AMP responsive element.[311] Fitzgerald and Flanagan[266] also noted an AP-2 binding site whereas Van Steeg, et al., did not.[311] The mouse 5′ region also contains a cAMP-responsive element,[312] three AP-1 binding sites and two oestrogen-responsive elements.[313] The rat and mouse 5′ regions have over 90% homology in some areas.[313] The rat and mouse genes differ in intron length. Nine GC boxes are present in the rat, as are two AP-1 binding sites, and one cAMP responsive element.[318]

The cAMP responsive element is coded by an 8-base palindrome 5′-TGACGTCA-3′.[324]

The discovery of AP-1 and AP-2 transcription factors within the ODC gene explains the induction of the gene by TPA. Several genes which are regulated by phorbol esters contain inducible transcriptional enhancers which respond to TPA. The TPA responsive element (TRE) binds to the transcription factor AP-1.[325,326] Whereas AP-1 is activated only by phorbol ester, AP-2 is controlled both by the DAG and phorbol ester-stimulated protein kinase C signal transduction system and by signalling systems involving cAMP.[327]

The GC rich areas of the 5′ region, for example, exon 1 of the mouse gene,[314] encode secondary structures which may be involved in the regulation of translation of the RNA. Polyamines are known to negatively regulate ODC primarily at the level of translation.[328-330] It is possible that polyamines may bind to these GC rich areas and stabilise potential secondary structures thereby decreasing translatability.[314,317,331]

Van Daalen Wetters, et al.,[332] found ODC mRNA and enzymatic activity to be induced by serum and TPA with similar kinetics even when constructs were used which contained only the protein-coding

region. Thus, without the flanking sequences, regulation of expression and activity can still occur. This suggests that the protein coding region may contain regulatory elements which can alter the conversion of transcripts to RNA or stability of the mRNA.

The ODC clone used in this thesis, pODC 10/2H, contains only a small part of the 5′ end of the gene. It is likely, therefore, that any alterations of the UPEs, which are responsible for enhanced expression, will not be detected by these experiments.

4.7 ODC Expression in Other Tumours

ODC mRNA expression was higher in colorectal neoplasia than in gastric cancer and sarcomas, although the number of gastric and sarcoma samples examined was small. This may indicate tissue specific changes in ODC regulation which will require further study.

4.8 ODC DNA Analysis

It is well-known that amplification of the ODC gene can occur under certain circumstances, particularly polyamine deprivation and exposure to DFMO.[138,140]

A human myeloma (Sultan) cell line was also induced to amplify ODC in the presence of DFMO. The amplified signal which corresponded to 5.8 kb band on *Hind*III digests, and a 9 kb band in *Eco*RI digests was localised to chromosome 2.[246,333] Another human myeloma cell line (Fravel), when exposed to DFMO, did not develop gene amplification, but was found to overproduce arginine.[334]

Although in the solid tumour experiments ODC mRNA expression was generally increased, no amplification or rearrangement of either ODC or either AdoMetDC locus was found implying that gene activation does not occur due to increased gene dosage or to translocation of the genes resulting in inappropriate expression.

Interestingly, using the restriction enzyme *Pst*I, a restriction fragment length polymorphism was detected on chromosome 2. Two common alleles represented by 8.4 and 5.5 kb bands represent markers that will be useful in gene mapping family studies and for determining the association and expression of specific ODC alleles

with tumours and for possibly predicting a tumour phenotype in families. One patient was observed with an ODC *PstI* digest phenotype that suggested that alteration of the chromosome 2 polymorphic site occurred. It is not implied that this alteration caused the tumour but suggests that these polymorphic markers can be used to study colorectal neoplasia.

The two hit theory of neoplasia developed by Knudson[95] states that disease occurs due to two mutations at a single disease locus. In the case of familial tumours a constitutional or germ line mutation is unmasked by a later somatic mutation or deletion. This hypothesis was subsequently shown to be true for both retinoblastoma[96,97] and Wilms tumour.[98] Loss of heterozygosity has also been seen in acoustic neuroma,[335] breast cancer,[336] small cell lung cancer,[337] ovarian cancer,[338] and neuroblastoma.[339]

No loss of heterozygosity of the ODC loci was seen in colorectal neoplasia, indicating that removal of a tentative suppressor gene does not allow increased ODC expression in this system.

In two out of six patients with gastric tumours abnormalities of one of the chromosome 2 alleles was present (Figure 28). In lanes 3 and 4 the tumour and mucosal DNA are depicted for one patient. The 8.4 kb allele is faint in the tumour and absent in the adjacent mucosa. This may represent possible contamination from another patient sample. In lines 5 and 6 the adjacent mucosa shows a faint 8.4 kb band which is absent in the tumour, the tumour tissue being homozygous. Since the 8.4 kb band is so faint in the normal tissue this may not represent a true loss of heterozygosity. Loss of heterozygosity has been reported to occur infrequently in gastric cancers,[340] although chromosome 2 was not examined in that study.

4.9 AdoMetDC DNA Analysis

No amplification, rearrangement, or allelic loss was found in any of the tumours examined.

During stimulated growth, increases in AdoMetDC activity occur which are mainly due to increases in the amount of enzyme protein.

There is both an increase in expression of AdoMetDC RNA and increased rate of translation.[263]

The human and rat genes have been cloned and show a high degree of homology, over 90%.[261] Both contain a long untranslated 5′ leader sequence. Very few genes have leader sequences greater than 200 nucleotides, ODC being one of these. This has important implications for the methods of regulation of AdoMetDC.

Depletion of polyamines by DFMO leads to an increase in AdoMetDC activity due entirely to increases in amount of protein.[261,341,342] A five-fold increase in synthesis rate was accompanied by a two-fold increase in mRNA implying alteration of both transcription and translation.[343]

Both AdoMetDC and ODC contain a strong "PEST" sequence, one or more regions rich in proline (P), glutamic acid (E), serine (S), and threonine (T). This region is believed to influence the fast rate of enzyme breakdown.[344]

AdoMetDC RNA is expressed as two RNA species (1.7-2.4 kb and 3.0-3.6 kb). It is still unknown whether these are the results of two genes, or one gene subject to multiple polyadenylation signals.[331]

The regulation of the coordinate expression of ODC and AdoMetDC is still unknown. An increase in AdoMetDC activity usually follows ODC activity temporally. It is possible that trans-acting factors stimulated by increased ODC expression affect AdoMetDC transcription.

4.10 Correlations With Other Genes Expressed in Colon Cancer

Tricoli, et al.,[86] reported a moderately high elevation of IGF-2 mRNA levels in 40% of colorectal tumours examined. There was an obvious correlation with site of origin of the tumour: levels being much higher for those of rectosigmoid origin than for cancers from the proximal colon. Dukes C tumours were noted to have a higher level of expression than Dukes B.

In this study, no correlation was found between IGF-2 expression and ODC expression. Of the six colorectal neoplasia samples examined, only one polyp and one carcinoma (a rectal BII lesion) showed enhanced IGF-2 expression whereas all samples demonstrated increased ODC expression.

This may indicate that IGF-2 expression occurs at a later stage of tumour progression than ODC expression, and may be more specific for metastatic potential of the cancer.

It is possible that enhanced IGF-2 expression is one of the variables accounting for differences in tumour biology between proximal and distal cancers.

4.11 The Chronology of Genetic Alterations in Colorectal Neoplasia

Over the past few years, there has been an exponential increase in knowledge concerning the molecular events in colon carcinogenesis. This was prompted by the recognition of the familial adenomatous polyposis (FAP) gene on chromosome 5. Subsequent work from the laboratories of Ray White, Bert Vogelstein, and Johannes Bos have helped to elucidate our current understanding of genetic alterations in colorectal neoplasia, particularly in relation to allelic loss on chromosomes 5, 17, and 18.

It is believed that cancer results due to a series of genetic alterations leading to a progressive disorder of the normal mechanisms which control cell growth.[345-347] A neoplastic clone of cells develops enhanced genetic instability which increases the probability of further genetic alterations and subsequent progression.[347] Such biologic characteristics of progression would be, for example, loss of differentiation, invasion, metastasis, and resistance to therapy.[346]

Analysis of age-incidence curves have suggested that three to four mutation-like changes are necessary for the development of leukemia and six to seven for carcinomas.[348,349] One of the difficulties encountered in cancer research is determining which genetic changes are tumourigenic and which are random, occurring later on in tumour development.

Fearon, et al.,[350] have demonstrated that colorectal tumours are clonal in origin. Studies of colorectal polyps have helped greatly in defining the chronology of subsequent genetic changes leading to progression. Since polyps are the premalignant precursors of colorectal cancer, any genetic alterations seen in polyps are likely to be involved in the early stages of oncogenesis.

4.11.1 Hypomethylation

DNA hypomethylation can result in increased gene activity.[202] Feinberg and Vogelstein have shown that numerous genes (e.g., growth hormone, gamma globulin, alpha globulin, Harvey Ras, and c-myc) are hypomethylated in 100% of colon cancers.[89,351,352] Hypomethylation also occurred to an equal degree in benign polyps thus hypomethylation precedes the development of malignancy,[353,354] and may be one of the earliest changes in the oncogenic cascade.

The expression of ODC is also altered by changes in methylation.

Alhonen-Hongisto, et al., found that the ODC of the cells of a human lymphatic B-cell leukaemia were significantly hypomethylated, compared to mononuclear leucocytes or a human myeloma (Sultan) cell line.[244] Lipsanen, et al.,[355] have shown hypomethylation of ODC from blood samples of patients with chronic lymphatic leukaemia (CLL). Since an increase in ODC activity was noted in lymphocytes from patients with CLL compared to normal lymphocytes,[356] it is possible that altered methylation is one mechanism for gene deregulation in this disease. Such hypomethylation of ODC is not seen in chronic or acute myeloid leukemia or polycythemia rubra vera.[357] Other work has shown that ODC hypomethylation in mouse L1210 leukemia cells may account for increased gene expression in the absence of gene amplification.[358,359]

The essential nature of methylation state on expression of the ODC gene was demonstrated in experiments by Halmekyto, et al. Methylation of ODC prior to transfection in Chinese hamster ovary cells was found to abolish transient expression of the gene.[360]

Since it is well-known that ODC levels increase in response to TPA, Hsieh and Verma[361] studied the relationship between hypomethylation and the response to TPA in a human bladder carcinoma cell line. They found that application of TPA produces no change in methylation of the gene, although a consistent increase in ODC levels results. Changes in

methylation status of other genes usually occur in the 5′ flanking end containing regulatory sequences.[361,362] The pODC 10/2H clone used by Hsieh and Verma contains only 87 bp of the 5′ end. Different results may be obtained with a full-length clone.

Insight into how hypomethylation may result in a sequence of further genetic events was provided by Schmid, et al.[363] Hypomethylation was shown to inhibit chromosome condensation - this may lead to mitotic nondysjunction resulting in the loss or gain of chromosomes.

Further study of methylation status of the ODC gene in colon cancers and other solid tumours should now be performed using the full-length ODC clones containing all of the 5′ end.

4.11.2 Ras Gene Mutations

Alterations in the ras genes in colon neoplasia has been noted by a number of authors. Spandidos and Kerr[84] found enhanced expression of both Ki-ras and Ha-ras in polyps and carcinoma. Increased Ha-ras protein in colon cancers was also seen by Hand, et al.[364] Amplification and rearrangement of ras genes has not been found.[365] Although hypomethylation of ras genes does occur in colon neoplasia,[89] other mechanisms of activation also occur.

The most frequently observed ras mutation is of the Kirsten-ras gene which is altered in about 50% of colon tumours and premalignant lesions,[366,367] the predominant mutation being at position 12.

Bert Vogelstein, in collaboration with Roy White and Johannes Bos, has elucidated further the involvement of ras mutations in the transition from benign to malignant neoplasia. Ninety-two carcinomas and 80 adenomas were studied.[368] The adenomas were divided into three classes: Class I - small tubular adenomas with low grade dysplasia from patients with FAP; Class II - polyps without evidence of carcinoma from patients without FAP; Class III - polyps containing areas of carcinoma from patients without FAP.

Eighty-eight percent of all the ras mutations found were of the K-ras gene, predominantly codons 12, 13, and 61. There was no correlation between site of tumour, Dukes stage, degree of differentiation, or age or sex of the patient.

The Class II and III adenomas contained ras mutations as frequently as carcinomas, however, the small low grade adenomas of Class I contained mutations much less frequently. A much higher proportion of polyps with high grade dysplasia contained mutations. Of note, only 9% of polyps less than 1 cm in diameter contained mutations compared to 70% greater than 2 cm. This suggests that mutations of the ras gene are involved in the progression of polyps from low to high grade dysplasia. Ras mutations are not the initiating factor in colon neoplasia since they are not seen frequently in low grade adenomas. Burmer and Loeb[369] demonstrated that Ki-ras mutations preceded changes in ploidy in colon carcinomas.

Mutations of the ras genes, resulting in an altered protein product (p21) can alter signal transduction by at least two mechanisms: 1) When a growth factor binds to its receptor, a cytoplasmic G protein (such as the ras product) binds GTP and attaches to the membrane by a fatty acid tail. PLC is then stimulated resulting in cleavage of PIP_2 to ITP_3 and DAG. DAG activates PKC in conjunction with calcium. PKC results in protein phosphorylation on threonine or serine residues and enhances expression of c-myc and c-fos; and, 2) G proteins are necessary for signal transduction via the adenylate cyclase pathway. Binding of molecules to the β-adrenergic receptor in the presence of G protein activates adenylate cyclase, increases levels of cAMP, which produces activated PKA. PKA then produces protein phosphorylation and enhanced expression of nuclear oncogenes.

Mutation of the ras gene results in enhanced ODC expression. Holtta, et al.,[370] studied ODC expression in NIH 3T3 cells transformed with human C-Ha-ras. They found much higher levels of ODC in the transformed compared to normal cells. Furthermore, ODC was maintained at a constituitively high level, with loss of normal cell cycle control. Increased

ODC expression was due to altered mRNA processing or turnover and not to enhanced transcription. Sistonen, et al., noted that levels of ODC expression were dependent on mutant ras gene copy number.[371]

Further evidence of the transforming activity of mutated ras protein was provided by work by Wasylyk and Imler,[372,373] on the transcription factor which binds to ras. A ras responsive element (RRE) has been identified in the polyoma virus. This sequence also mediates activation by TPA and serum and is a binding site for the mouse transcription factor PEA 1. PEA 1 is a homologue of AP-1 and c-jun. It is thought that PEA 1 belongs to a closely related family of transcription factors which includes human AP-1 , the v-jun oncogene and yeast GCN4. Thus, it appears that altered ras product p21 may bind to the AP-1 site of the ODC gene and result in increased expression. Mutated p21 may also activate ODC via its cAMP responsive element. Sistonen, et al.,[374] studying NIH 3T3 cells transfected with mutated c-Ha-ras have provided further support for this idea. ODC mRNA was enhanced following induction of p21 c-Ha-ras protein. Two other serum and tumour-promoter regulator genes were also increased-transin and the glucose transporter. The high level of expression of ODC was insensitive to tumour-promoter stimulation suggesting that the TPA responsive site was already maximally stimulated by ras.

It is now accepted that the AP-1 site is the product of the nuclear protooncogene jun.[375] The product of the fos protooncogene (p55 fos) is also related to AP-1. It appears that cooperation between fos and jun is required for full activation of transcription via the TPA-responsive elements.[376]

4.11.3 Chromosome 5

A major breakthrough in the study of colorectal cancer was the mapping of the familial adenomatous polyposis (FAP) gene to chromosome 5. In 1986, A. A. Sandberg's lab at Roswell Park Cancer Institute published a report of a patient with Gardner's syndrome who had a constitutional deletion of the long arm of

chromosome 5.[377] Following this report, two laboratories working independently mapped the FAP gene to the long arm of chromosome 5.[378,379] More recently, the locus has been defined to reside at 5q21-22.[380,381] Approximately 20% of familial and sporadic colon carcinomas showed loss of heterozygosity of the FAP locus.[382-384] Vogelstein, et al.,[368] found allelic loss on chromosome 5 in 36% of carcinomas and 29% of both Class II and III adenomas. However, the tubular low grade adenomas of patients with FAP (Class I adenomas) revealed no losses on chromosome 5. This finding is not compatible with Knudson's model of suppressor genes. Vogelstein hypothesises that the FAP locus is involved in epithelial hyperproliferation of the colonic mucosa and that a mutation of only one of the alleles is necessary for activation to occur. He suggests that further genetic alterations, for example, deletions of 17 and 18 are necessary to complete the transformation to malignancy. Law, et al.,[385] also believe that the FAP locus acts in a dominant, not a recessive, manner. Two synchronous colon cancers from a patient with FAP did not lose the chromosome 5 allele.

4.11.4 Chromosomes 17 and 18

In comparison with allelic loss of chromosome 5, deletions of chromosomes 17 and 18 occur much more frequently, in the range of 40-75% of carcinomas.[368,385-387] In contrast, deletions in adenomas occur in 6-47% of cases.[368] Deletions of 17p and 18q were uncommon in Class I and II adenomas, though allelic deletions of 18q occurred in about 50% of class III adenomas. Thus, it appeared that deletions of chromosome 5 and ras gene mutations precede deletions of 18q, which in turn precede allelic losses of chromosome 17. This is confirmed by looking at the accumulation of genetic changes in adenomas compared to carcinomas. Only 9% of Class I adenomas had more than one genetic change, however, over 90% of carcinomas had two or more genetic alterations.

The chronology of genetic changes was studied by Vogelstein using an elegant microdissection technique. The

benign areas of Class III adenomas were separated from the carcinomatous elements and the DNA extracted separately. In general, alterations of ras and chromosome 5 occurred before chromosome 18q deletions in the same patient. There were a few exceptions, however, suggesting that it is the accumulation of genetic changes over time rather than their exact sequence, which is important in colon neoplasia.[368]

Vogelstein's group has also coined the term fractional allelic loss (FAL)[388] to describe the number of chromosomal arms on which allelic loss was observed divided by the number of chromosomal arms for which allelic markers were informative in the patients' normal cells. The median FAL of 56 colorectal tumours was 0.20. A significant finding was that a higher FAL was associated with a poorer prognosis. Thirty percent of patients with FAL less than 0.2 had recurrence of tumour compared to 68% with FAL greater than 0.2. Similarly, the figures for death from disease were 26% versus 64%. These figures were independent of Dukes stage. A follow-up study[389] confirmed the above and found that distant metastases were significantly associated with a high FAL. Ras mutations and chromosome 5 deletions did not have prognostic significance, but FAL was associated with 17p and 18q deletions. Allelic deletions of 17p were more common in left-sided than right-sided tumours. It appears that 17p and 18q deletions are markers of tumour aggressiveness and indicate metastatic potential. Delattre, et al.,[390] also noted a higher frequency of 17p and 18q deletions in distal compared to proximal colon cancers.

The site of chromosome 17 deletion has been localised to 17p12-17p13.3. This region contains the gene for the transformation associated protein p53. Baker, et al.,[387] confirmed that mutations of this locus result in enhanced p53 expression in colorectal carcinoma. Enhanced p53 expression has been noted in 55% of colon cancers compared to 8% of tubular adenomas. Enhanced expression correlated with areas of dysplasia.[391] Mutations of the p53 gene occur in many other

tumour types, e.g., lung, breast, and brain and are not exclusive to colon cancer.[392-394]

For a gene to be a tue suppressor gene, the presence of the normal wild type allele should prevent transformation. This has been shown to be true for the Wilms tumour locus on chromosome 11.[395] The transformation of primary rat embryo fibroblasts is prevented by addition of the wild type p53 gene.[396] Colon adenoma cell lines containing the wild type p53 do not have tumourigenic activity.[397] Thus, p53 acts as a true suppressor gene.

The second most common region of allelic loss in colorectal tumours is 18q, which is lost in 44-73% of colorectal carcinomas.[368,388,390,398,399] Loss of this region in Class I and II adenomas is much less frequent, 13 and 11%, respectively, but occurs in 47% of late stage adenomas.[368] Vogelstein's studies using microdissection of Class III adenomas reveal that allelic deletions of 18q generally occur at a later stage than deletions of 5q and precede changes of 17p. The DNA for the consistently deleted region on 18q has now been cloned[400] and identified as a candidate tumour suppressor gene termed DCC (deleted in colorectal carcinoma).

The predicted amino acid sequence of DCC suggests that the protein is related to molecules involved in cell adhesion. Thus, the gene may play a role in normal cell to cell interactions or interactions with the extracellular matrix which may be necessary for metastasis.

The region on chromosome 18 is particularly interesting in that Lynch syndrome II, hereditary nonpolyposis colorectal cancer (HNPCC) is linked to the Kidd blood group. Both genes have been mapped to 18q.[401,402] It will be important to know how DCC and HNPCC genes relate and if they are in fact the same locus.

The recognition of the accumulation of genetic changes necessary for colorectal cancer to occur has led Vogelstein's group to propose a model of tumourigenesis[403] (Figure 31). Mutations of the FAP gene on chromosome 5 may give rise to the general hyperproliferation which precedes adenoma formation.

Hypomethylation then contributes to the general instability of the genome at an early stage of adenoma formation. Alterations of the ras genes, particularly K ras, are responsible for changes in signal transduction which result in progression to a later stage of adenoma. The deletions of 18q and 17p with concomitant loss of suppressor gene function then result in carcinoma formation, 18q deletion generally occurring before 17p deletions. Other mutations are believed to account for the development of the metastatic phenotype.

The tumourigenic phenotype is distinct from the metastatic phenotype and "metastasis suppressor genes" have been proposed which function in the same manner as the recessive genes involved in tumour formation.[404] Metastasis is a multistep process involving invasion of intercellular matrices, penetration of the basement membrane, extravasation, and induction of angiogenesis in the target organ. Metastasis also involves the coordinated expression of gene products. One of the first steps is attachment via cell-surface receptors to components of the basement membrane, e.g., laminin.[404,405] Laminin expression is increased in colorectal carcinoma.[406] Laminin increases the release of type IV collagenase which would thus permit invasion through the matrix.[407] Metastatic potential is known to be related to the MHC genes, which in turn appear to be regulated by the nuclear oncogene c-fos.[408,409]

In addition to the allelic deletions in Vogelstein's model, other gene products are consistently altered in colorectal carcinomas. An example would be c-myc. The product of c-myc is a transcriptional activator which can act upon cellular genes and thereby alter growth control.[410] Elevated c-myc expression occurs in approximately 70-80% of colorectal cancers,[411,412] in the absence of rearrangement or amplification. Amplification has been reported in colon cancer cell lines, however.[100]

Higher c-myc mRNA levels were associated with cancers of the distal rather than proximal colon.[413] Deregulation of c-myc appears to be associated with deletion of the FAP locus. Forty-seven percent of tumours with elevated expression of c-

myc exhibited allelic loss on 5q, compared to 0/8 tumours with normal c-myc expression,[414] thus, cooperation between gene loci may exist.

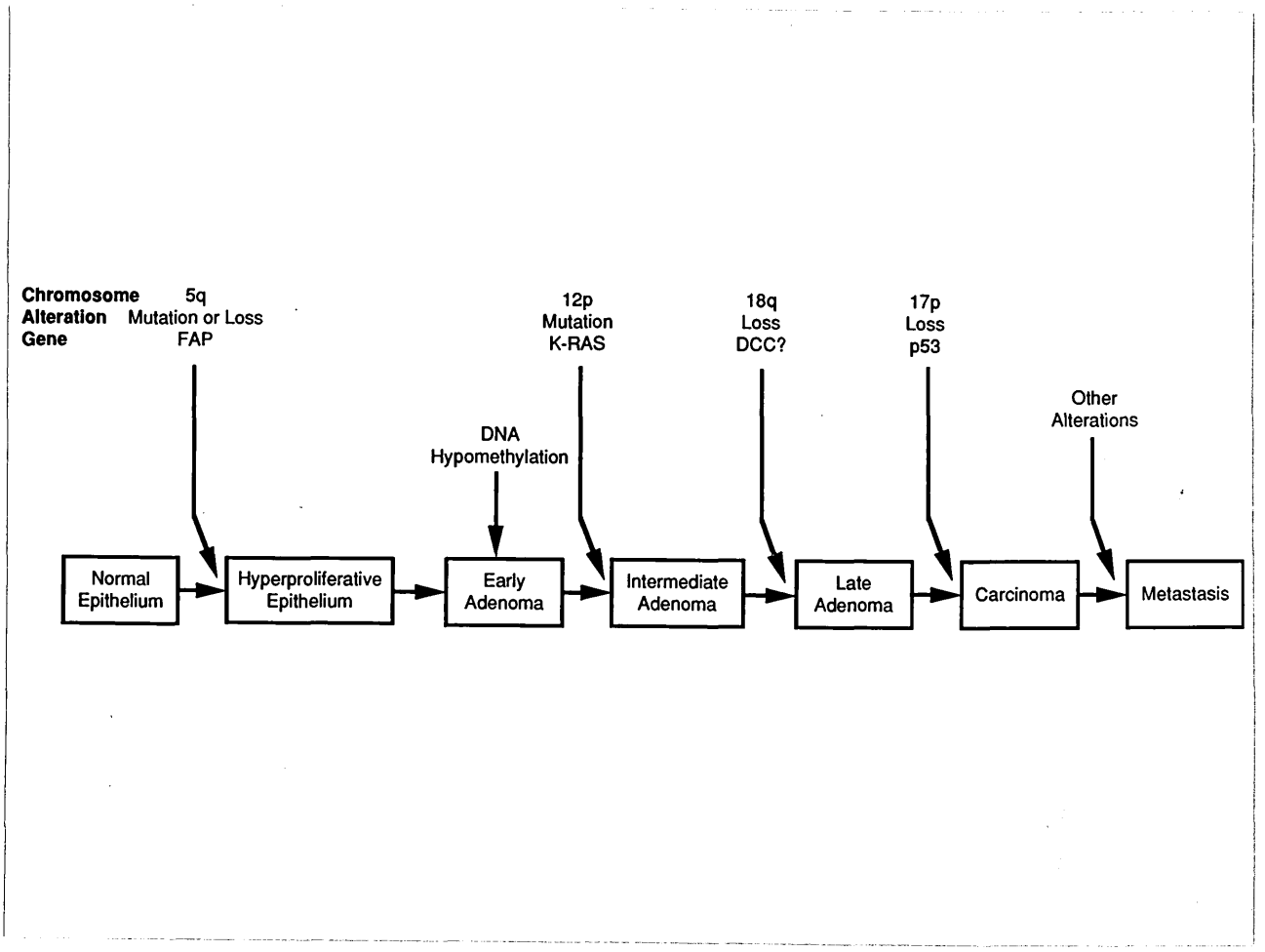

Figure 31

The Model of Colorectal Tumourigenesis

Colorectal cancer develops through a series of genetic alterations involving tumour suppressor genes (particularly on chromosomes 5, 17, and 18) and oncogenes (RAS). Accumulation of these changes gives rise to increasingly large polyps which eventually become malignant.

4.11.5 ODC in the Model of Colorectal Tumourigenesis

A normal resting population of animal cells exists in the G_0 state. Activation by mitogens induces the cells to enter the G_1 phase and then progress through DNA synthesis to cell division. Genes which are involved in the transition from G_0 to G_1 are said to be under "growth control" whereas those which operate in G_1 to M are under "cell cycle control".[306] Clearly, one of the key questions to be answered in cancer research is to identify the growth control genes which activate the cell from its resting to transformed state.[415] Evidence points to ODC and AdoMetDC as being growth controlled genes, whereas histone mRNAs, for example, are under cell cycle control.[306] This contradicts earlier reports that ODC was elevated mainly in late G_1 and S.[111] Elevation of ODC mRNA is one of the earliest changes seen upon ligand binding to cell surface receptors.

Sistonen, et al.,[416] studying the neu tyrosine kinase receptor found that one of the early changes induced by receptor activation was induction of the fos/jun transcription factor complex, closely followed by an increase in ODC mRNA. The elevation of ODC expression is thought to be mediated by the AP-1/PEA 1 transcription factor which is known to exist among the 5′ regulatory sequences of the ODC gene. The elevated expression of ODC mRNA after the induction of mutated p21 c-Ha-ras oncoprotein,[376] which may be mediated via the fos/jun transcription factor complex, also lends support to the idea that ODC is involved in the early stages of the colon carcinogenesis model. ODC is likely to be involved in the general hyperproliferation of cells as they move from a resting to an active state, and in the progression of adenomas from early to intermediate stage associated with mutations of the ras genes. Mutated ras genes may influence ODC in both AP-1 and cAMP regulatory sequences. Ras and ODC may cooperate in the early stages of tumourigenesis, since ras mutations enhance ODC expression.[370,371]

In this thesis, ODC expression was elevated in both tubular, tubulovillous, and villous adenomata, i.e., early, intermediate, and late stages. This also promotes ODC as an integral part in early tumourigenesis.

The global hypomethylation of genes which occurs in polyps, as well as carcinomas, is known to inhibit chromosome condensation and may lead to mitotic nondysjunction. Such genetic instability may lead to further loss of regulation of a number of genes, including ODC. It has been demonstrated that altered methylation of ODC is essential for its full expression in some systems.[360] Alterations in DNA methylation of the promoter regions is known to alter gene transcription.[417] The possible cooperation of ODC with other genetic changes known to operate in colorectal oncogenesis is depicted in Figure 32.

The integral part played by ODC and AdoMetDC in the carcinogenesis cascade is emphasized by the effect of the polyamines on gene transcription. Celano, et al.,[418] demonstrated that depletion of ODC by DFMO resulted in a 90% decrease in c-myc expression. A subsequent study[419] using a colon carcinoma cell line revealed that polyamines can also regulate the expression of the c-fos and histone 2-A genes. The mechanism of polyamine regulation of gene transcription is by several mechanisms: alterations of chromatin condensation, increased DNA stability, and a conformational change from β- to Z-DNA.[419] Structural changes in chromatin and nucleosomes induced by polyamines may result in increased availability of DNA for transcription.[420] This may be mediated by altered binding of transcriptional regulatory factors.[421]

Binding of spermine to β-DNA (a right-handed helix) results in a conformational change to Z-DNA (a left-handed helix).[422,423] Spermine increases stability of β-DNA by a decrease in the size of the major groove of DNA and an increase in the minor groove, with subsequent change in the tertiary structure.[121] Polyamines stabilise Z-DNA better than β-DNA. Such conformational changes at the 5′ regulatory end of the gene can be predicted to enhance gene transcription.[424]

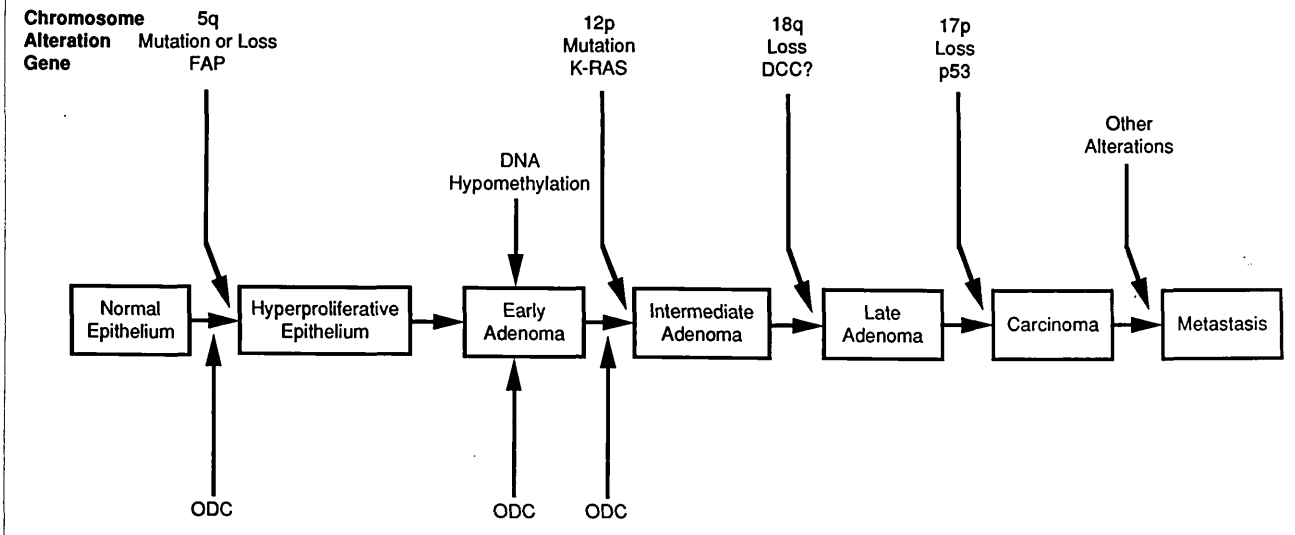

Figure 32

ODC in the Model of Colorectal Tumourigenesis

ODC is involved early in colorectal tumourigenesis, an elevation in expression being seen soon after ligand binding to receptor on the cell membrane. Elevated ODC is essential for cell growth and proliferation. Altered methylation is known to activate the ODC gene. Mutation of the p21 as protein also activates the ODC gene. The effect of polyamines on gene transcription may also be involved in further genetic changes as cancer progresses to metastasis.

Polyamines can alter transcription then by acting as chromatin openers and by altering the conformation of promoters and enhancers.[425] Changes in transcription, due to alterations in RNA elongation, may also account for the effect on c-myc expression.[426]

ODC and AdoMetDC exhibit coordinate expression in general. The fact that their loci exist on different chromosomes may indicate that trans-acting factors stimulated by ODC affect transcription of the AdoMetDC gene. The other polyamines then produced by the activity of AdoMetDC may then go on to promote expression by other cancer related genes.

It is also possible that the regulatory sequences at the 5′ end of the AdoMetDC gene have some features in common with ODC and respond to the same stimuli. Examples would be a glucocorticoid recognition site and AP-1 and AP-2 transcription factors. It is known that the AdoMetDC gene contains a long 5′ noncoding region,[261] but the exact nature of the regulatory sequences will not be known until the full-length genomic DNA is cloned.

ODC expression was noted to be higher in colorectal cancer than other malignancies, e.g., breast and sarcoma. The nature of tissue specific gene regulation is largely unknown. Recent works on the albumin gene indicates the presence of particular transcription binding sites (CHNF-1 and C/EBP) which act in a tissue specific manner.[427] It is likely that such variation in transcription binding sites also accounts for the differences seen with ODC expression. Cis-acting regulatory sequences also account for globin expression only in erythroid cells.[428]

4.11.6 Clinical Applications of Polyamine Research

Because ODC appears to be so essential for tumour growth and maintenance, it is not surprising major developments are taking place in the research and development of ODC inhibitors in cancer chemotherapy. Since the discovery of DFMO by Metcalf,[116] numerous inhibitors of the polyamine pathway[429] have been devised including ornithine analogues,[430,431] putrescine

analogues,[432] spermine and spermidine synthases inhibitors,[433] diamine oxidase and polyamine oxidase inhibitors,[434] AdoMetDC inhibitors,[137,435,436] and spermidine derivatives.[437]

The irreversible inhibitor of ODC, DFMO, has been shown to act in a synergistic fashion with several other antineoplastic drugs. The combination of DFMO with the S-phase specific drug ara-C, an inhibitor of DNA polymerase, resulted in preferential tumour cell kill in vivo and in vitro.[438] DFMO will also act synergistically with hydroxyurea (an inhibitor of ribonucleotide reductase).[439]

The activity of the alkylating agent BCNU is enhanced by polyamine depletion via DFMO.[440] Such synergism is thought to be due to changes in DNA conformation brought about by lack of polyamines.

The combination of DFMO with 5FU[441] and with mitomycin C[442] are also synergistic in action against colon cancer cell lines. In theory, the combination of an ODC inhibitor with an AdoMetDC inhibitor (Figure 2) should result in greater polyamine depletion and hence increased efficacy - this was found to be the case. The use of DFMO with MGBG (an inhibitor of AdoMetDC) resulted in four-fold increase in toxicity to Hela cells.[438] Similar results are obtained with other AdoMetDC inhibitors.[443]

The first clinical trial using DFMO with MGBG was on five children with advanced leukemia. An impressive response occurred with mild toxicity. Responses have also been seen with this regimen in Hodgkin's disease, lymphoma, sarcoma, and thyroid carcinoma. Ten out of 16 patients with recurrent astrocytoma responded to this drug combination.[444]

Our knowledge of the polyamine biology of certain tumours may help to predict the response to single therapy with DFMO. Casero, et al.,[445] have shown that human lung tumour sensitivity to DFMO is related to ODC mRNA steady state levels - the lower the ODC mRNA, the more likely the response to DFMO.

The finding that ODC mRNA levels were higher in colon neoplasia than gastric carcinoma and sarcoma may indicate that ODC inhibitors will be more effective in therapy of the latter two tumour types.

Since toxicity from DFMO is low, the prophylactic use of this agent in patients at high risk of colorectal cancer or patients with multiple polyps may be considered. Luk and Baylin also suggested its use in the relatives at risk of familial adenomatous polyposis or patients with FAP who have undergone colectomy without proctectomy.[188]

Kingsnorth, et al.,[446] noted that polyamine levels in breast cancer were correlated with poor prognostic factors such as histologic grade and oestrogen receptor negative status. Thomas[447] has shown that ODC mRNA levels in hormone unresponsive breast cancer were two- to four-fold higher than in hormone responsive cancer. This finding may again be related to the DNA interactions of the polyamines - Thomas and Kiang[448] showed that polyamines are capable of modulating the binding of progesterone receptor to DNA, thus playing a role in gene regulation in breast cancer.

Antioestrogen therapy with tamoxifen and 4-hydroxy-amoxifen reduced ODC mRNA expression in the hormone responsive breast cancer cell line MCF-7.[449] This is likely to be due to down regulation of oestrogen responsive elements of the ODC gene.

4.11.7 Closing Remarks

Despite the great inroads into the molecular biology of colon cancer seen in the past few years, many questions are still to be answered. For example, what is the relationship between the familial aspects of colon cancer and carcinogens such as bile acids? This is complicated even more by the discovery by Watne, et al.[450] that FAP patients had higher concentrations of cholesterol and bile acids in their stools. How do dietary factors influence gene regulation? Llor et al.[451] have shown that increased dietary calcium can reduce the incidence of K-ras mutations in carcinogen-induced colon cancer.

Cannon-Albright, et al.,[452] have determined that inherited susceptibility to colon cancer is much more frequent than

previously thought. Susceptibility to polyps and cancer probably account for the majority of colon cancers, with a gene frequency of 19%. Leppert, et al.,[453] have discovered that the susceptibility gene in patients with a family history of colon cancer is located on chromosome 5 at or very near the FAP locus. Recently, Kinzler and colleagues[454] in Vogelstein's laboratory have identified the putative suppressor gene located at 5q21 in sporadic colon cancers. The gene, which they term MCC (mutated in colon cancer) has homology with the G protein-activating region of the muscarinic acetylcholine receptor. This discovery is fascinating, bearing in mind the role of the ras proteins, which are members of the G protein family, in signal transduction and colon tumourigenesis.

It is likely that Hill[15] was correct in that an environmental carcinogen can only cause progression to the neoplastic state when genetic predisposition is present.

Knowledge of the molecular cascade involved in colon cancer opens many new approaches for chemotherapy. For example, drugs which inhibit binding of fatty acids to the tails of ras protein, such as compactin or lovastatin, would prevent activation of second messengers. Monoclonal antibodies to growth factor receptors are known to decrease tumour growth. Tyrophostins are a new class of drugs which competitively inhibit the tyrosine kinase domain of GFRs.[297] Molecular antineoplastic therapy is now at hand. These drugs could be used alone or in combination with ODC and AdoMetDC inhibitors for a synergistic effect.

Further research into the specific molecular steps involved in colorectal cancer tumourigenesis can only increase the specificity of therapy we have for the disease.

4.11.8 Conclusions

1. The ODC gene maps to 2p25 and 7q31→qter. Preliminary data suggests the locus on chromosome 2 to be functional.
2. The AMD gene maps to a locus on chromosome 6 and to Xq22-Xq28.
3. Polymorphism of the ODC gene between individuals was seen with *Pst*I digests. Allele frequencies conform to the Hardy Weinberg law with p = 0.28 and q = 0.72.
4. Increased expression of the ODC gene is seen in both benign and malignant colorectal neoplasia when compared to adjacent mucosa. In polyps, the mean increase is 3.2-fold and in carcinomas, 4.2-fold.
5. When ODC activity and mRNA expression are compared for the same tissue sample, no correlation is seen. As ODC activity and amount of protein present are generally parallel, this implies that post-transcriptional factors account for altered activity. Possible examples would be altered translatability of the RNA or posttranslational modifications of the protein.
6. ODC expression was higher in colorectal neoplasia than in gastric carcinoma or sarcomas. This may indicate that tissue-specific mechanisms of gene regulation are in operation.
7. No amplification or rearrangement of either ODC locus or either AMD locus was seen consistently in colorectal neoplasia. No amplification or rearrangement of ODC was seen in sarcomas, breast carcinoma, lung carcinoma, or gastric carcinoma.
8. No correlation was found between ODC expression and IGF-2 expression.

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APPENDIX

Solutions and Buffers

SSC - Standard sodium citrate; 20 x SSC is 3MNaCl, 0.3 MNa_3 citrate pH 7.

Denhardt's solution; 50x: Ficoll 5g
polyvinylpyrrolidone 5g
BSA (Pentax fraction V) 5g
H_2O to 500 ml

Nick translation buffer 10x: 0.5 M Tris HCl pH 7.8
0.05 M $MgCl_2$
0.1 M α-mercaptoethanol

DNase I activation buffer: 10 mM Tris HCl pH 7.6
5 mM $MgCl_2$
1 mg/ml nuclease free BSA

Random priming buffer: RPRB 2.5 x comprises
Hepes pH 6.6 0.5 M
$MgCl_2$ 12.5 mM
β-mercaptoethanol 0.025 M
Tris pH 8.0 0.125 M
3x d NTP 50 mM

DNase I: 1 mg/nl in 0.1 N HCl

Buffer A: BSA 0.5%
Na_2 EDTA 1 mM
SDS 5%
$NaHPO_4$ pH 6.8 40 mM

Buffer B: Na_2 EDTA 1 mM
SDS 1%