# Expression of Cloned Sequences in Biopsies of Human Colonic Tissue and in Colonic Carcinoma Cells Induced to Differentiate *in Vitro*<sup>1</sup>

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#### ABSTRACT

A computer-based scanning and image-processing system has been developed to quantitate the relative level of expression of each of 4000 cloned complementary DNA sequences in small biopsies routinely removed from the mucosa of normal and neoplastic human large intestine. Individuals have been studied from well-defined population groups in which colonic epithelial cells have progressed to increasingly advanced stages of neoplastic transformation. Comparison of normal colonic mucosa to colonic carcinomas demonstrated alterations in expression of approximately 7% of the cloned sequences; fewer changes were found between benign colonic adenomas and either normal colonic mucosa or carcinomas. A subset of the sequences which change in expression during progression from normal mucosa, to adenoma, to carcinoma showed complementary changes when colon carcinoma cells were induced to differentiate *in vitro* with sodium butyrate; quantitative correlations between *in vivo* and *in vitro* results were highly significant.

Comparison of normal colonic mucosa with mucosa from patients with the autosomal dominant disease familial polyposis revealed more extensive alterations in gene expression involving approximately 25% of the clones screened. Flat colonic mucosa in familial polyposis is therefore markedly aberrant and may be highly dedifferentiated, suggesting several possible mechanisms for the very high incidence of cancer that develops in this epithelium.

## **INTRODUCTION**

The generation of the fully transformed phenotype involves extensive and complex changes in gene expression, as documented by the wide variety of biochemical, enzymatic, and antigenic differences between normal cells and their transformed counterparts (1). Such complexity of change has been observed a number of times in analyzing the kinetics of mRNA hybridization to DNA and to cDNA<sup>3</sup> (e.g., Refs. 2–4). In one important example, Groudine and Weintraub (5) demonstrated that infection of normal chick embryo fibroblasts by the Rous sarcoma virus resulted in changes in expression of approximately 1000 host cell genes. Hence, even when the etiology of transformation is well defined, in this case the introduction of the viral *onc* gene *src*, the cell rapidly exhibits a complex alteration in the pattern of gene expression which ultimately determines the transformed phenotype.

We previously studied the complexity of change in gene expression in a chemically induced mouse colon tumor compared with the mouse normal colonic mucosa (6). Investigation of the relative levels of expression of each of 400 cDNA sequences cloned from the colon tumor revealed that approximately 15% of the sequences exhibited reproducible changes in the level of expression in the tumor compared to the normal mucosa (6). This involved principally modest increases and decreases in the level of abundance, rather than large changes on the order of 10-fold or greater. Further, relationships were noted between the extent of change in expression of individual sequences and their expression in other normal and malignant tissue (6-9).

Since mucosal biopsies of human subjects at varying cancer risk can readily be obtained during sigmoidoscopy, the tissue provides a unique opportunity to extend our investigations into human colon cancer progression and risk. However, in approaching the identification of sequences which change in expression in human colon cancer and characterization of human tissue phenotype by patterns of gene expression, one faces additional difficulties. First, the extremely small size (less than 1 mm<sup>3</sup>) of the biopsies is not often appreciated. The tissue generally yields less than 50 ng of polyadenylated RNA, amounts which preclude techniques such as Northern blot analysis. In addition, the colon is a complex tissue made up of many cell types, and hence the biopsies are heterogeneous on a cellular level. Finally, genetic and environmental differences between different individuals, as well as regional and sampling variation within the same individual, may compound the variability of the starting material.

Recognizing these difficulties in design and interpretation of the experiments, we have begun to study the patterns of gene expression which distinguish among normal, benign adenomatous, and carcinoma biopsies and which characterize the tissue at risk for development of colon cancer. To do this, we have developed a computer-based scanning and image-processing system which has permitted us to quantitate the relative level of expression of each of 4000 cloned cDNA sequences in such human colonic biopsies or in small tissue culture samples. We have found that there are indeed changes in expression of cloned sequences which characterize the progression to colon cancer and that a subset of these sequences are altered in a complementary manner when colon carcinoma cells in culture are induced to differentiate with sodium butyrate. Most surprising has been the observation that the pattern of gene expression is remarkably different in colonic mucosa of high risk individuals with familial polyposis compared to low risk normal mucosa. This contrasts markedly with more limited changes in gene expression that we found in the progression from normal mucosa to adenoma to carcinoma.

## MATERIALS AND METHODS

A reference cDNA library was prepared from polyadenylated RNA of the human colon carcinoma cell line HT-29; this cell line is relatively undifferentiated and does not contain a gene capable of inducing morphological transformation upon transfection into 3T3 cells (10).<sup>4</sup> These cells do express both the c-myc and N-myc genes at high levels (11). The cDNA was inserted into the PST site of pBR322 using homopolymeric tails and transfected into *Escherichia coli* LE 392. Each of 4032 individual clones was picked and inoculated into a separate well of standard 96-well microtiter plates (12). This number of randomly selected clones provides an 80% probability of representing every abundant and middle abundant sequence, assuming there are  $5 \times 10^2$ 

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<sup>&</sup>lt;sup>3</sup> The abbreviation used is: cDNA, complementary DNA.

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Fig. 1. Examples of the bacterial colonies (A), autoradiograph (B), and format (C) which generated the data on gene expression. Each of 42 microtiter plates was replica plated onto nitrocellulose and lysed. Following hybridization and washing, the filters were affixed in groups of 4 to the format. Exposure to X-ray film then generated the autoradiograph (B). Each autoradiograph was processed using components of the TYCHO analysis system. The fiducial markings in A and C (P) were copied on the autoradiograph (A) along with a reference number (in B, 4AS) and used to align the image with a computer-stored copy of the format (C). Attached to the top and bottom of the format (A) are strips of nitrocellulose which contain standard dilutions of the radioactive probe used. These generate the spots of decreasing intensity along the top and bottom of the autoradiograph and are an alternate method of normalizing the data between experiments (see "Materials and Methods").

different abundant and middle abundant sequences per cell, each present at a concentration of  $2 \times 10^2$  copies in a total population of  $5 \times$ 10<sup>5</sup> mRNA molecules/cell [data assumptions based on the work of Hastie and Bishop (13)]. The library, consisting of 42 such 96-well plates (including controls for antibiotic selection and hybridization background in each plate), was stored at -80°C and replicated onto nitrocellulose filters using a 96-pin stainless steel device which preserves the relative positions of the clones from each plate. Each replica of the library, consisting of 42 filters, was hybridized to a [32P]cDNA probe synthesized with the polyadenylated RNA from a human biopsy sample, as described previously (6). Following hybridization and washing, the filters were affixed in groups of four (Fig. 1A) on copies of the format shown in Fig. 1C. Exposure to X-OMAT film (Kodak) in the presence of an intensifying screen (Dupont) was at -80°C for a period equal to the product of the input cpm for each probe  $\times$  minutes = 5.8  $\times$  10<sup>10</sup> (4  $\times$  10<sup>9</sup> when only the four filters of the sublibrary were hybridized; see "Results"). Fig. 1B shows an example of the autoradiographic exposure of the filters in Fig. 1A. Each replica set of the library was used only once and generated 11 films, each from 4 filters.

Each film was processed using components of the TYCHO analysis system (14). Hardware included a Vax 11/780 computer (Digital Equipment), a model 785 image scanner (Eikonix), an AP120B array processor (Floating Point systems), and an IP 8500 image-processing system (Gould). Absorbance was first measured for each film in a 2048 x 2048 grid with 100-µm spacing. Each of these measurements (pixels) represented an 8-bit absorbance value obtained from a 12-bit transmission value. Processing of the resultant image with a 5 x 5 median filter replaced each pixel with the median value of the pixels in the 5 x 5 neighborhood surrounding it and suppressed grain and other high frequency noise. Film background (in contrast to hybridization background, below) was subtracted in two steps. An intermediate image was produced from the median filtered image by selecting the minimum value of a 161-pixel horizontal line centered at the element being generated. A second intermediate image was produced from the first in the same manner except that a maximum value was used. This image was then subtracted on a pixel by pixel basis to produce the processed image.

Fiducial points (Fig. 1*C*, *triangles*) were interactively marked for each processed image so that the clone regions (intersections of the grids in Fig. 1*C*) could be aligned with a previously stored template image. The computer then recorded the maximum absorbance value in a circular region with a 0.5-cm radius for each clone on each of the films. Maximum rather than integrated values were used since the clones vary in size.

The hybridization background (in contrast to noise and film background, above) was corrected for each of the 96 clones in a quadrant of the format by using the value at the position of the host LE392 harboring nonrecombinant plasmid pBR322. To normalize the data between experiments, the hybridization value for each clone was expressed as its ratio to the average of all clones for the same probe. This normalization procedure is similar to the practice of rehybridizing

Northern blots to a sequence not expected to change in abundance (e.g., actin) in order to correct for differences in amount of RNA on a filter. In this case, we are normalizing for probe-specific activity between experiments, since each clone is present in great excess over its corresponding sequence in the probe, and when all sequences are considered (i.e., 4000, not just a single sequence such as actin) it is a good assumption that the overall level of hybridization between probes should be comparable. Using these normalized hybridization values for each of the clones, the mean hybridization of each clone was calculated for each of four tissue groups (described below): low risk normal mucosa (n = 6); high risk familial polyposis flat mucosa (n = 7); adenomas (n = 6); high risk familial polyposis flat mucosa (n = 6); high risk familial polyposis flat muco = 6); carcinomas (n = 7). For each clone, the six possible two-way comparisons of these four groups were then analyzed by calculating the ratio of mean hybridization (expression) for clone N in one group to the mean hybridization for clone N in the second group and the distribution of these ratios plotted as histograms (Figs. 2 and 5).

Patients. The data reported are based on probes prepared from biopsies of colonic mucosa from patients in four groups: Group 1, colonic mucosa from six subjects (mean age, 31 years; range, 25-63 years) at low risk for colon cancer, no family history of colon or other cancer for two generations, and a normal pattern of thymidine labeling of cells in the mucosa, with no expansion of the proliferative compartment as previously found in individuals at increased risk for colon cancer and carcinogen-treated rodents (15); Group 2, colonic mucosa from seven individuals (mean age, 36 years; range, 27-70 years) with the autosomal dominant disease familial polyposis who were at markedly increased risk for colon cancer. The colonic mucosa that was biopsied in this group was flat mucosa, and the cells had not accumulated into adenomas; Group 3, benign adenomas from six individuals (mean age, 41 years; range, 19-69 years) with familial polyposis; Group 4, colonic carcinomas from six patients (mean age, 60 years; range, 37-79 years) in the general population whose malignancies were believed to arise from adenomas. Biopsies were taken through a flexible sigmoidoscope and immediately placed into and stored in liquid nitrogen.

Butyrate Experiments. HT-29 or SW-480 colon carcinoma cells were grown in T-75 flasks in minimum Eagle's medium supplemented with 10% fetal calf serum and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer. The cells were refed daily for 3 days to avoid glucose depletion and subsequent differentiation (16). The medium was then changed to include 5 mM sodium butyrate, which induces a more differentiated phenotype of HT-29 and other colon carcinoma cells in culture (11, 17–23). Daily refeeding was continued with and without butyrate; the cells were harvested at various times thereafter; and RNA, polyadenylated RNA, and probes were prepared and used as described for the biopsy samples.

#### RESULTS

Initial Screening. Initial screening of the 4000-member cDNA library from HT-29 cells in patterned arrays was done with 6



Fig. 2. Comparison of tissues. Using the normalized hybridization values for each of the 379 clones in the sublibrary, the mean hybridization of each clone was calculated for each of the four tissue groups: low risk normal mucosa (n = 6); high risk familial polyposis flat mucosa (n = 7): adenomas (n = 6): carcinomas (n = 7). For each clone, the 6 possible two-way comparisons of these 4 groups were then analyzed by calculating the ratio of the mean hybridization (expression) for clone N in one group to the mean hybridization for clone N in the second group and the distribution of these ratios plotted as histograms. Ordinate, number of clones (of the 379); abscissa, ratio of expression in one tissue group as compared to another. Ratio of expression in A, adenomas/low risk mucosa; B, carcinomas/low risk mucosa; C, carcinomas/adenomas.

separate probes from 6 different biopsies. Two probes were made from the RNA of biopsies of colonic mucosa from individuals at low risk for colon cancer; 2 probes were made from biopsies of adenomas of subjects with familial polyposis; and the final 2 probes were from biopsies of 2 colon cancers (see "Materials and Methods").

We selected 379 clones (the number which can be scanned, along with positive and negative controls, in a single computer run) for further analysis by eliminating all sequences which were of low abundance in all tissues and an additional 111 which were modestly above background and showed no change in abundance in these original 6 biopsies.

Each of the 379 clones was physically picked from the original library and inoculated into a new position in one of four 96-well microtiter plates to assemble a sublibrary of these clones. Each plate contained a position with LE-392 harboring the plasmid pBR-322, which served as a hybridization background spot for each filter in subsequent analysis. In addition, the first plate contained no bacteria in one position. This served as a control for antibiotic selection during growth of each replica of the sublibrary.

Further Screening. Replicas of this sublibrary were hybridized against probes made from biopsies of tissue from the four groups described in "Materials and Methods."

Data Analysis. The mean  $\pm$  SD of the normalized hybridization value for each sequence was calculated for each of the four tissue groups. In Fig. 2 and Table 1, the mean value for each clone was compared in several of six possible two-way pairs of the four tissue groups. The data are presented as the distribution of number of sequences (of the 379) which were at a particular ratio in one tissue group compared to another. Thus, Fig. 2*A* illustrates that in comparing the average expression of each sequence in the adenomas with its average expression in the low risk flat mucosa biopsies, most sequences showed little difference in expression, exhibiting ratios of expression in the two tissues between 1 and 2. Some sequences did, however, show greater differences in expression. Eight sequences were increased in expression 3- to 4-fold in the

Table 1 Summary of alterations in gene expression

| Comparison                                     | Increases<br>(>3-fold) | Decreases<br>(>3-fold) |
|--|------------------------|------------------------|
| Adenoma/low risk mucosa                        | 8 (2) <sup>a</sup>     | 9 (2)                  |
| Carcinoma/low risk mucosa                      | 20 (5)                 | 7 (3)                  |
| Carcinoma/adenoma                              | 10 (3)                 | 1 (<1)                 |
| Familial polyposis flat mucosa/low risk mucosa | 76 (20)                | 18 (5)                 |

<sup>4</sup> Numbers in parentheses, percentage.

adenomas compared to the low risk flat mucosa (ratio on *abscissa* of Fig. 2A of 3-4) and nine were decreased in expression by 60 to 90% (ratio on *abscissa* of 0.1-0.4). A similar picture emerges from comparing each sequence in carcinomas to the low risk flat mucosa (Fig. 2B) and each sequence in the adenomas to the carcinomas (Fig. 2C), although in each of these comparisons one sequence was elevated in expression between 6- and 8-fold. Interestingly, the greatest number of differences was found in comparing the two extremes of carcinoma to low risk flat mucosa (Fig. 3B). Here, 20 sequences were elevated greater than 3-fold and 11 sequences decreased more than 60%.

Because the biopsies represent heterogeneous cell populations and only a single biopsy could be analyzed from each individual, it is possible that differences in expression of any sequence may be under- or overestimated in the comparisons presented. In order to investigate further which sequences may be of particular interest in the progression from normal mucosa to adenoma to carcinoma, we turned to a more simple in vitro model system. HT-29 colon carcinoma cells were induced to differentiate by treatment with sodium butyrate for 24, 48, or 96 h. RNA was prepared from these cells and control cells not treated with butyrate and used to make probes for screening of the HT-29 cDNA library. The data bases for the in vivo and in vitro results were compared. Sequences were selected which showed increases or decreases in expression during the progression from normal mucosa, to adenoma, to carcinoma and which also showed a complementary change when the HT-29 colon carcinoma cells were induced to differentiate in vitro. Fig. 3 shows the data for one sequence, 52B1. The mean level of expression of this sequence increased in adenomas compared to normal mucosa and increased further in carcinomas, shown in Fig. 3 (top). Fig. 3 (bottom) illustrates that when the HT-29 cells colon carcinoma cells were induced to differentiate in culture, the level of expression of this sequence decreased, back to a level more characteristic of normal mucosa. Sixteen sequences which showed such complementary changes in vivo and in vitro were found. Of these 16, 8 showed the same results when the in vitro experiment was repeated with SW-480 colon carcinoma cells (Fig. 3, bottom). When the quantitative extent of change in expression for each of these sequences in vitro was compared to the extent of change in vivo, the in vitro and in vivo results were linearly correlated. As shown in Fig. 4, the correlation coefficient was 0.94 when the in vitro data from HT-29 cells treated for 96 h with butyrate were compared to the difference in expression for these sequences between the



Fig. 3. Expression of clone 52B1 in biopsies and tissue culture. The normalized hybridization value for clone 52B1 was determined as outlined in Figs. 1 and 2. *Top*, hybridization of 52B1 in biopsies taken of normal mucosa (M), adenomas (A), and colon carcinomas (C). *Column*, mean hybridization for each tissue group;  $\Phi$ , data points for each biopsy. *Bottom*, normalized hybridization for clone 52B1 determined in untreated HT-29 and SW-480 colon carcinoma cells and in cells treated with 5 mM sodium butyrate for 24, 48, or 96 h. The 24-h time points were done in duplicate for each cell line. Data are plotted as percentage of the untreated control cells.



Fig. 4. Comparison between *in vitro* and *in vivo* results. Eight clones were chosen which showed complementary alterations in expression *in vivo* and *in vitro*, as illustrated in Fig. 3. The percentage of change in expression of each of these clones between untreated HT-29 cells and those treated with sodium butyrate for 96 h (*abscissa*) was then plotted against the percentage of change between the mean normalized hybridization value for the clone in normal mucosa as compared to the mean normalized hybridization value for the adenomas (*top*) or the carcinomas (*bottom*). *r*, correlation coefficient.

normal mucosa and adenoma. The correlation coefficient was 0.86 when the same *in vitro* data were compared to the change in expression between normal mucosa and carcinoma. Both of these figures are significant at P < 0.01. It is important to note that the clones were selected from the data bases because they showed complementary changes *in vitro* and *in vivo*, without regard to the quantitative extent of change. This emphasizes the significance of the high degree of correlation between the extent of change for each of these sequences *in vivo* and *in vitro*. Regulation of the relative levels of expression of these 8 sequences may therefore be part of a genetic program which generates at least one lineage of normal colonic cell differentia-

tion, this regulation being altered in at least one pathway by which colonic epithelial cells progress towards increasing degrees of transformation.

The final analysis of the data is a comparison of the expression of each of the 379 sequences in our sublibrary in normal mucosa to their expression in the flat mucosa of high risk familial polyposis patients. In this case, the histogram of ratios of expression (Fig. 5) was dramatically skewed. Twenty (76 of 379) of the sequences were elevated in expression between 3and 10-fold in high risk familial polyposis mucosa and 5% (18 of 379) were decreased in expression 60 to 90% (Fig. 5; Table 1). These extensive changes in gene expression in low risk mucosa compared with high risk familial polyposis mucosa, adenomas, and carcinomas, in which changes in expression were far fewer and quantitatively smaller (Fig. 2; Table 1).

#### DISCUSSION

We have demonstrated that alterations in expression of cloned genes can be detected in human biopsy specimens during the progression from normal colonic mucosa, to adenoma, to carcinoma and that the flat mucosa of genetically high risk familial polyposis individuals exhibits a pronounced change in the pattern of gene expression when compared to the normal mucosa of very low risk individuals. In addition, we have been able to identify a subset of 8 sequences in which the relative levels of expression characterize colonic epithelial cells as differentiated or transformed.

While it is clear that differences in gene expression within biopsies may be accompanied by additional variations due to anatomical position within the colon, dietary habits of the individual, changes in colonic microflora which influence cell physiology, inflammation of the mucosa, or other reasons, the ability to quantitate the expression of each cloned sequence and hence average the results from many individuals allows us to reach certain overall conclusions. Chief among these are that the pattern of gene expression in the tissue as a whole is very different in the familial polyposis flat mucosa compared to the normal mucosa (Fig. 5), especially in relation to the far fewer alterations seen among the normal mucosa, adenoma, and carcinoma (Fig. 2). This latter point also reflects on the question of cell heterogeneity. While the flat mucosa of high risk familial polyposis individuals is not identical to normal mucosa, these tissues are far more similar than are the normal mucosa, adenoma, and carcinoma. It is unlikely, therefore, that all or even most of the alterations in the high risk flat mucosa are due to a shift in representation of cell types. The flat mucosa in familial



Fig. 5. Comparison of familial polyposis flat mucosa to normal mucosa. Using the normalized hybridization values for each of the 379 clones in the sublibrary, the mean hybridization of each clone in the familial polyposis flat mucosa (n = 7) was compared to its hybridization in the normal mucosa (n = 6) by calculating the ratio of the values for each clone N. The distribution of these ratios is plotted as a histogram. Ordinate, number of clones (of the 379); abscissa, ratio of expression in familial polyposis flat mucosa.

polyposis which is at high risk for colon cancer development therefore exhibits a very different pattern of gene expression than the adenomas which have been regarded as benign premalignant neoplasms in progression to cancer and the carcinomas themselves.

These extensive changes in gene expression may be associated with risk in several ways: lineages of differentiation may be altered or arrested; cell interactions may be affected which can be permissive for or accelerate tumor development (24); or multiple pathways which converge on the transformed phenotype (25, 26) may be initiated by the extensive changes, any one of which can progress under the effects of other carcinogens or promotors.

Although the differences in gene expression that we have observed in each stage of progression from low risk colonic mucosa, to adenoma, to carcinoma are less extensive than between the low risk mucosa and the mucosa of familial polyposis, they are substantial. In comparing carcinoma to normal colonic mucosa, 27 of the 379 sequences (7%) show either increases or decreases in average level of expression greater than 3-fold. In the case of platelet-derived growth factor stimulation of 3T3 cell proliferation, it has been suggested that the number of alterations in expression of genes in the abundant and middle abundant class reflect a similar extent of alteration in rare sequences (27). Therefore, on the order of 700 sequences (7% of 10,000 different cellular sequences) in the colonic mucosa may be altered in expression in progressing through the stages of neoplastic cell transformation. In analyzing the data on progression from normal mucosa, to adenoma, to carcinoma we have presented data on complementary changes in expression of a subset of these sequences upon induction of differentiation of HT-29 colon carcinoma cells in culture to a more normal phenotype. These data provide direct evidence that the patterns of gene expression seen are part of a differentiation/ transformation program in colonic epithelial cells, rather than reflecting shifts in cell type within the biopsies. Further, this overall extent in the number of mRNA sequences which change between the normal colonic mucosa and the carcinoma, on the order of 10%, is consistent with other estimates of the extent of alteration in gene expression in transformation in other systems. These include a chemically induced mouse colon tumor (6), rat hepatomas (2, 3), human lymphoid neoplasia (4), and most important, even the relatively simple transformation of normal chick embryo fibroblasts with the Rous sarcoma virus, in which approximately 1000 host cell sequences are altered in expression (5).

We do not yet know the function of any of these sequences for which the expression is altered in the tissue at risk or during progression. Some may be related to lineages of colonic cell differentiation, some to the mechanism or regulation of cell cycle progression. Distinctions such as these are difficult to make even with well-studied sequences. For example, expression of the c-Ha-ras-1 gene is related to colonic mucin secretion (11) and also is a cell cycle-linked gene in colon carcinoma cells (28), and further, the wild type gene can be considered to provide a "housekeeping" function since it is expressed in so many cell types. However, Klein (29) has argued that normal cells must be finely tuned with regard to growth control, and hence even alterations in expression of a gene of about 50% (much less than the 3- to 10-fold seen here) may be highly significant in determining cell phenotype. Baserga (30) also has suggested that alterations in expression of any of a large number of cell cycle linked genes could alter cell growth as a first step in the progression to full cell transformation.

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