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c-*myc* Protein Distribution

Neoplastic Tissues of the Human Colon

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There is an extensive literature documenting the increased or deregulated expression of the* c-myc *oncogene in human malignancies. The authors have recently devised a sensitive immunocytochemical method for studying the tissue localization of* c-myc *protein in tissue sections of human colon. We have compared nuclear* c-myc *staining using a polyclonal rabbit anti-c-*myc *antibody and a mouse monoclonal* myc *antibody NCM II 274. Microscopic observation of the tissue specific pattern of* c-myc *protein distribution shows that nuclear staining intensity varies in normal and neoplastic crypt cell nuclei in parallel with morphologic criteria of neoplasia. These studies yield further information on the usefulness of* c-myc *protein as a prognostic indicator. (Am J Pathol 1992, 140:719–729)

There is extensive literature linking the deregulation and overexpression of the c-*myc* proto-oncogene with tumorigenesis.[1–3] The c-*myc* gene, the cellular homolog of the avian myelocytomatosis virus, encodes three exons; the first exon is not translated whereas the second and third exons encode a protein product.[4] The DNA sequence of c-*myc* predicts a protein of 439 amino acids with a calculated molecular weight of 49,000 daltons (49 Kd)[5]; however, the range of molecular weights actually determined on isolated proteins have varied between 58 and 64 Kd.[6–10] Various studies have shown that the c-*myc* protein is a nuclear phosphoprotein that binds nonspecifically to DNA.[5,11–13] However, Blackwell et al[14] and Prendergast and Ziff[15] have reported that the carboxyl-terminal fragment of the c-*myc* protein mediates sequence-specific binding to DNA. Sequence-specific DNA binding may also be dependent on complex formation with another protein, *max*.[16]

Control of transcription resides in two promoters, located at the 5′ end of exon I and at the 3′ boundary of the first exon and intron; the latter is subject to negative regulation.[17,18] Alteration of normal c-*myc* expression by chromosomal translocation, retroviral insertion, or DNA amplification is believed to represent an important step in the development of certain malignancies.[19] Transgenic mice, harboring a construct in which the c-*myc* coding sequence is linked to a mammary specific regulatory element, develop clonal tumors of the breast at a high frequency.[20]

The induction of c-*myc* mRNA is regulated by agents that stimulate growth responses in lymphocytes and fibroblasts, and is associated with the G1 phase of the cell cycle.[21] Buchler and coworkers[22] have demonstrated that actively proliferating cells express c-*myc* at a constant level in all phases of the cell cycle, and propose that this temporal deregulation of c-*myc* expression may be more important than absolute quantitative levels.

Although the precise function of the c-*myc* protein remains enigmatic,[23] there is substantial evidence showing a correlation between c-*myc* gene overexpression and transforming properties of the protein.[24–26] *myc* proteins have been implicated in diverse cellular processes including transcription, DNA replication, and RNA processing.[27,28]

Despite evidence that there are no gross rearrangements or amplification of c-*myc* genes in cell lines derived from many human colon adenocarcinomas,[29] or in surgical samples of primary human colon adenocarcinomas,[30] various investigators have noted increases in c-*myc* mRNA concentrations during colon tumorigenesis in both humans and in experimental animal models.[2,30–32] Using an *in situ* hybridization technique to measure c-*myc* RNA concentrations, Mariani-Constantini

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et al[33] found a progressive increase when comparing normal human colonic epithelium, villous adenoma, and carcinoma samples. Conversely, Mulder and Brattain[34] have shown that reduced c-*myc* expression is associated with diminished cellular proliferation and a more benign phenotype in human colon carcinoma cells. Similarly, Erisman and coworkers[29] have found that c-*myc* protein levels are significantly elevated in cell lines derived from human colorectal tumors.

The immunocytochemical localization of c-*myc* protein was first determined in quail cells (Q8) nonproductively transformed with the avian myelocytomatosis virus MC 29, and was shown to be nuclear.[28,35] The distinctive extranucleolar distribution observed within the nucleus is maintained after paraformaldehyde, methanol, glutaraldehyde or acetone fixation.[28] Electron microscopy studies have characterized c-*myc* protein to be represented by electron-dense bodies 0.5 to 3 μm in diameter found in the nucleus, which are distinct from nucleoli but often associated with them.[28,36]

Although the biochemical and immunohistochemical localization of the c-*myc* protein in cultured cells has been shown to be predominantly nuclear,[7,8,11–13,17,28,29,35,36] its localization in formalin-fixed, paraffin-embedded tissue sections of human lymphoid and colonic tissues has been claimed to be predominantly cytoplasmic.[37–42] We have described a method using polyclonal antibody for studying the tissue localization of the c-*myc* protein in frozen tissue sections of rat and human colon,[43–45] and this method reveals a predominantly nuclear localization. We have also tested some monoclonal *myc* antibodies[46] in our system to attempt to further explore the specificity and sensitivity of our technique. We report the tissue localization of c-*myc* protein using both a polyclonal rabbit anti-c-*myc* antibody[5] and a mouse monoclonal anti-*myc* antibody[46] and have with these assessed the distribution of the c-*myc* protein within a series of colonic tissues that include carcinomas, epithelial dysplasias arising in idiopathic inflammatory bowel disease (IBD), and normal mucosa.

Materials and Methods

Antibodies

The rabbit polyclonal c-*myc* antibody used in these studies recognizes a 64KD c-*myc* protein[5] and was provided by R. Watt (SmithKline, and Beecham Labs, King of Prussia, PA) The mouse monoclonal *myc* antibodies directed against N-*myc*/ c-*myc* fusion proteins[46] were provided by N. Ikegaki (Dept. of Human Genetics, University of Pennsylvania Medical School, Philadelphia, PA).

Immunohistochemistry

Colonic tissue was obtained from material submitted to the surgical pathology division of the Department of Pathology, The Mount Sinai School of Medicine. The methods have been previously described.[43] Briefly, 1- to 2-cm pieces of grossly normal colon and colonic tumors from the same patient were snapfrozen in liquid nitrogen for processing with Cryostat Frozen Sectioning Aid (CFSA) (Instrumedics, Teaneck, NJ).[47] The frozen tissue was transferred from liquid nitrogen in a container in the cryostat to an embedding oil (an approximately eutectic mixture of aliphatic esters) which is held liquid in a thermostatted bath at −8°C, but freezes at about −10°C. The tissue was mounted in frozen oil, and sectioned using a special adhesive tape to capture the section while it was being cut and a microscope slide coated with a UV-polymerizable adhesive to adhere the section (Instrumedics). An 8-msec UV-flash was used to permanently bind the section to the slide, and the tape was peeled away. This methodology better maintains the adhesion of the sections to the slides during long and/or multiple incubations and extensive washing than other methods.

The slide-mounted 6-micron frozen sections were melted at room temperature and airdried for 1 hour before being dipped in −20°C methanol, and then dried for 5 minutes before being used. The antibody was diluted in phosphate-buffered saline (PBS) (Sigma) that was prepared in a 1:3 dilution with human serum used to block nonspecific immunoglobulin binding. The sections were incubated in a moist chamber with the primary rabbit polyclonal c-*myc* antibody diluted 1:250. It is difficult to estimate the specific antibody protein concentration from the dilution of immunized rabbit serum (affinity purified rabbit polyclonal c-*myc* antibody (gift from R. Watt) produced visibly discrete nuclear staining at 0.1 μg/μl). The primary mouse monoclonal *myc* antibody NCM II 274 was diluted 1:1 from the mouse hybridoma supernatant of unknown antibody concentration and produced the clearest nuclear signal of the monoclonals tested. Immunoperoxidase staining was performed by the unlabelled antibody method of Sternberger et al[48] After an overnight incubation at 4°C, detection was achieved with the peroxidase-anti-peroxidase technique (Dako) or with the avidin-biotin-peroxidase complex technique (Dako).

The stain was developed by a final incubation with 0.4% 3′3′-diaminobenzidine tetrahydrochloride (Sigma) plus 0.02% hydrogen peroxide in a 0.1 M pH 6.5 cacodylate buffer solution. To determine the specificity of the antibody binding, control sections were incubated with either the primary antibody, preabsorbed for ½ hour with the c-*myc* protein at I μg/μl of rabbit serum (gift from R. Watt), or with the appropriate dilution of normal rabbit

serum or mouse immunoglobulins. The preabsorption of the rabbit polyclonal c-*myc* antibody with c-*myc* protein or the incubation of routine sections with preimmune serum did not show nuclear staining in sections of human colon cancer. We compared the sensitivity and specificity of the polyclonal c-*myc* antibody and the mouse monoclonal *myc* antibodies in successive sections from the same block. This was especially informative when there were both negative and positive nuclei in the same section. The endogenous peroxide present in the eosinophils in tissue sections was not completely blocked by the preincubation of the slides with solutions such as hydrogen peroxide in methanol. We omitted such procedures in our technique to not diminish the intensity of nuclear staining; the eosinophils were soon easily recognized and differentiated from the positive nuclear staining.

Results

Correlations of c-*myc* protein distribution and tissue morphology were derived from successive H and E and immunostained sections. We studied abnormal regions in 10 neoplastic and 4 preneoplastic specimens and apparently uninvolved surrounding mucosa in 7 of those cases (Table 1). We also studied the normal mucosa from a patient whose colon was removed in treating complications of previous surgery.

At a dilution of 1:250 of the rabbit polyclonal anti-c-*myc* antibody, samples of normal mucosa showed variable staining of individual epithelial nuclei (Table 1, Figures 1, 2). In this context, tissue sections in which there were both positively and negatively stained nuclei were useful for comparing the range of c-*myc* staining with reference to the morphology of the tissue. In the normal mucosa from a case of ulcerative colitis, the range of nuclear staining varied from the crypt cell nuclei in the basal region to the luminal surface. The basal crypt cell nuclei were darkly stained, whereas the nuclei at the luminal surface were unstained (Figure 1B). The normal mucosa from the patient whose colon was removed due to complications of previous surgery had regions of positive basal crypt cell nuclei, as well as other regions in which even basal crypt nuclei were unstained (Table 1).

The range of nuclear staining from negative (−) to moderately positive (+ +) was found in colonic tissue samples characterized as dysplastic (Table 1). With increased c-*myc* staining, there were more nuclei in each crypt that was stained. In addition, more crypts were stained further from the basal region until the epithelial nuclei of the entire mucosa were stained including those at the luminal surface.

In a dysplastic crypt (Figure 2B) from a case of ulcerative colitis, although all of the nuclei were stained, the size of the intranuclear granules was small in comparison to the size of the nucleus, and this was considered moderately positive staining (+ +). In an invasive carcinoma (specimen E, Table I, Figure 2D) the stained granules in the nucleus were larger, and this was scored as moderately intense staining (+ + +).

The most intense staining of the c-*myc* protein revealed with a rabbit polyclonal anti-c-*myc* antibody in frozen sections of human colon was confined primarily to the nuclei of epithelial cells of neoplastic glands in invasive carcinomas (Figures 3, 4, 5). In the well-differentiated and moderately well-differentiated invasive carcinomas, dark diffuse c-*myc* staining filled the whole nucleus, and this was scored as intense staining (+ + + +), (Table 1, Figure 3C, 5B, 5C). In a poorly differentiated carcinoma

Table 1. *c*-myc *Protein in Colonic Neoplasia*

Specimen	Histologic findings	Antibody staining		Clinical history
		Abnormal	Normal	
A	Architecture deranged with mild dysplasia	+ +	±	Uc
B	Focal dysplasia	+ +	−	Uc
C	Adenomatous population with atypia; dysplasia	+ +	na	Uc
D1	Dysplasia	+ +	na	Fp
D2	*In situ* carcinoma	+ + +	na	Fp
E	Invasive carcinoma	+ + +	±	NH
F	Well-differentiated invasive carcinoma	+ + + +	na	Fp
G	Well-differentiated invasive carcinoma	+ + + +	±	NH
H	Well-differentiated invasive carcinoma	+ + + +	±	NH
I	Well-differentiated invasive carcinoma	+ + + +	na	NH
J	Moderately differentiated invasive carcinoma	+ + + +	na	NH
K	Moderately differentiated invasive carcinoma	+ + + +	−	NH
L	Poorly differentiated invasive carcinoma	+ + + +	na	Uc
M	Poorly differentiated invasive carcinoma	+ + + +	+	NH
N	Normal tissue		±	NH

na = not available, Uc = ulcerative colitis, Fp = familial polyposis, NH = no history.

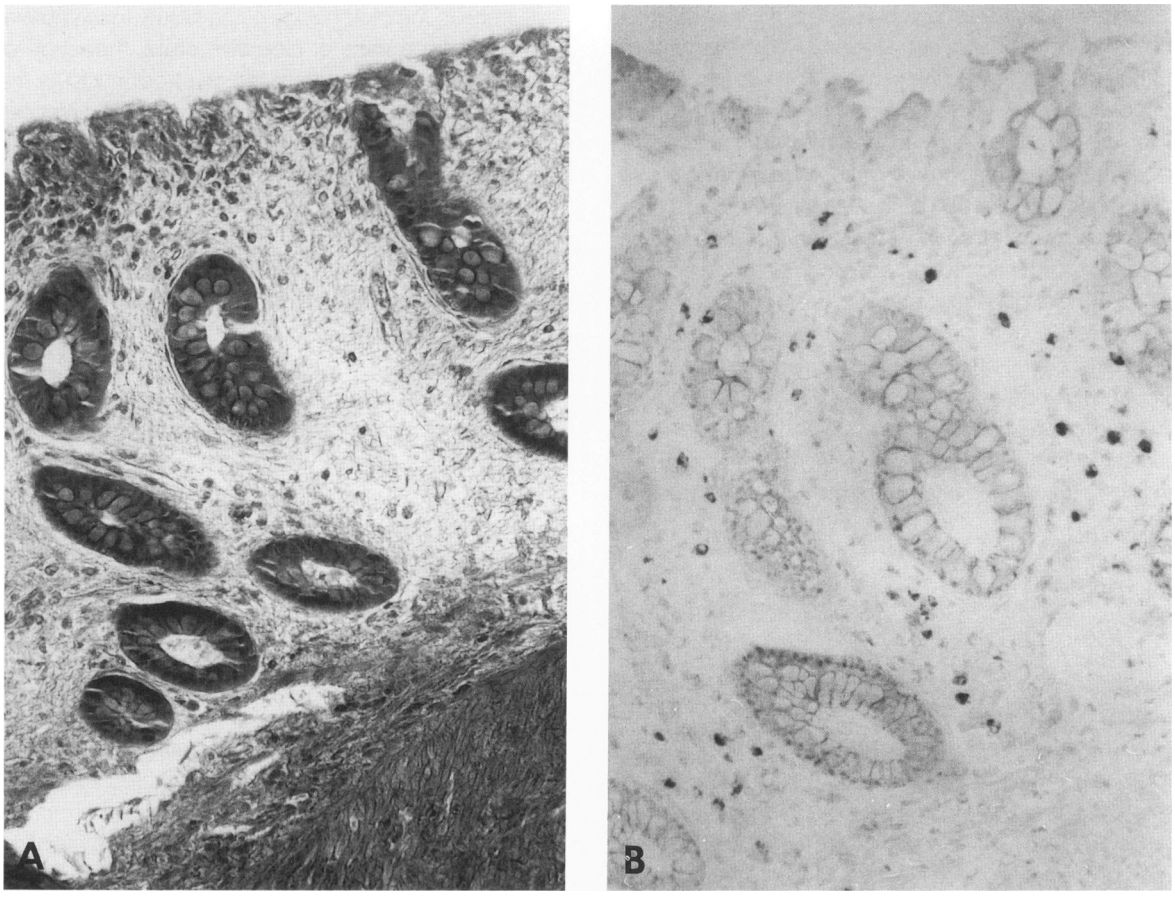


Figure 1. *Immunohistochemistry of apparently uninvolved normal mucosa from specimen A using rabbit polyclonal anti-c-*myc *antibody and the Sternberger anti rabbit IgG peroxidase-anti peroxidase method.* **A**: *H and E stained section ×175.* **B**: *Immunostained section of similar region as* **(A)**; *note darkly stained crypt cell nuclei in the basal region of the mucosa, with no counterstain. Also note staining of the eosinophils in the lamina propria, ×175.*

from a case of ulcerative colitis (Figure 4 A,B,C) the c-*myc* staining was dark and discretely granular (intense staining + + + +).

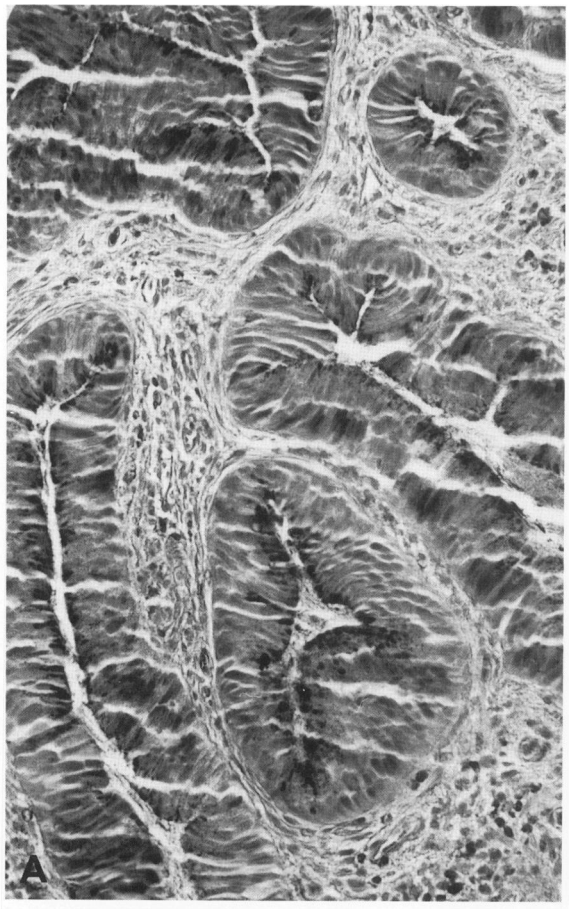
When compared with the rabbit polyclonal anti-c-*myc* antibody, mouse monoclonal *myc* antibodies especially NCM II, 274 produced a less granular, more homogeneous, staining pattern in the nuclei of neoplastic glands and this is demonstrated in the case of a well-differentiated invasive carcinoma (Figure 5C). The staining of nonepithelial interstitial cell nuclei between the neoplastic crypts was also less clearly discernible in the sections stained with the anti-*myc* monoclonal antibody (Figure 5C).

Our results show that there was not a simple quantitative correlation between tissue morphology and the amount of c-*myc* protein detected in cell nuclei. However, the pattern of distribution within tissues and nuclei was characteristic for the different neoplastic tissues. We should emphasize that it is necessary to preserve the morphology of the tissue to preserve c-*myc* staining; sections of tumors which were necrotic lost cellular definition and c-*myc* staining.

Discussion

Based on microscopic observations of immunostained frozen CFSA sections, there was a strong correlation between morphologic immaturity and c-*myc* staining intensity in epithelial cell nuclei of the human colon. We have

Figure 2. *Immunohistochemistry of c-*myc *staining in an adenoma from specimen C and an invasive carcinoma from specimen E using polyclonal anti-c-*myc *antibody and Sternberger anti rabbit IgG peroxidase-anti-peroxidase method.* **A**: *H and E stained section of an adenomatous population of cells with atypia:dysplasia, ×175.* **B**: *Immunostained section of similar region as* **(A)**; *Focal, nuclear c-*myc *staining is found in virtually every cell, with no counterstain ×175. Note staining of the eosinophils in the lamina propria.* **C**: *H and E stained section of invasive carcinoma, ×175.* **D**: *Immunostained section of similar region as* **(C)** *reveals diffuse coarse nuclear c-*myc *staining, with no counterstain, ×175.*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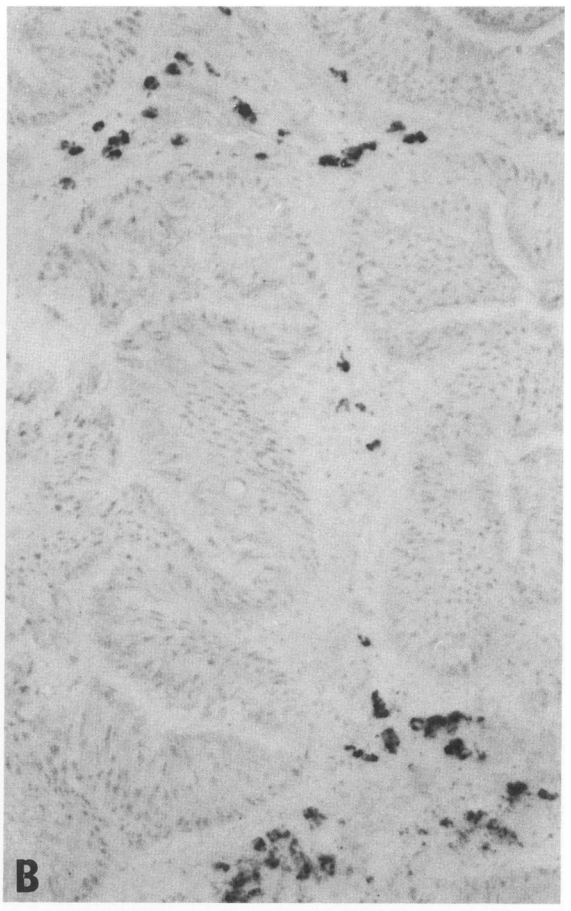
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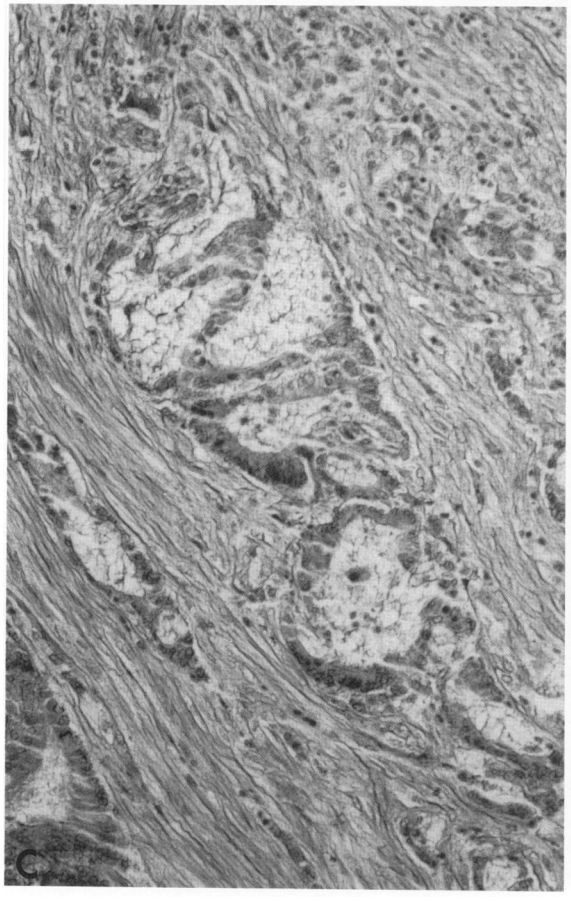
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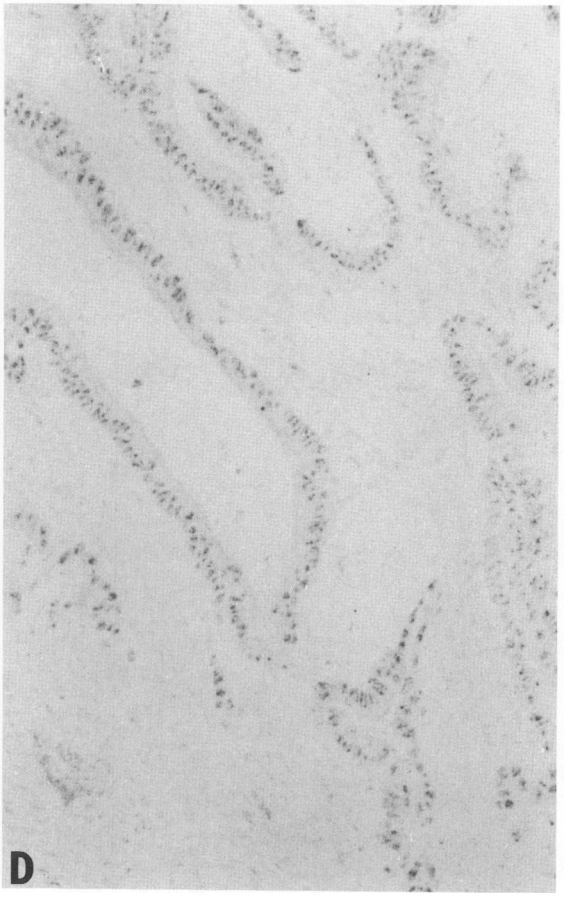
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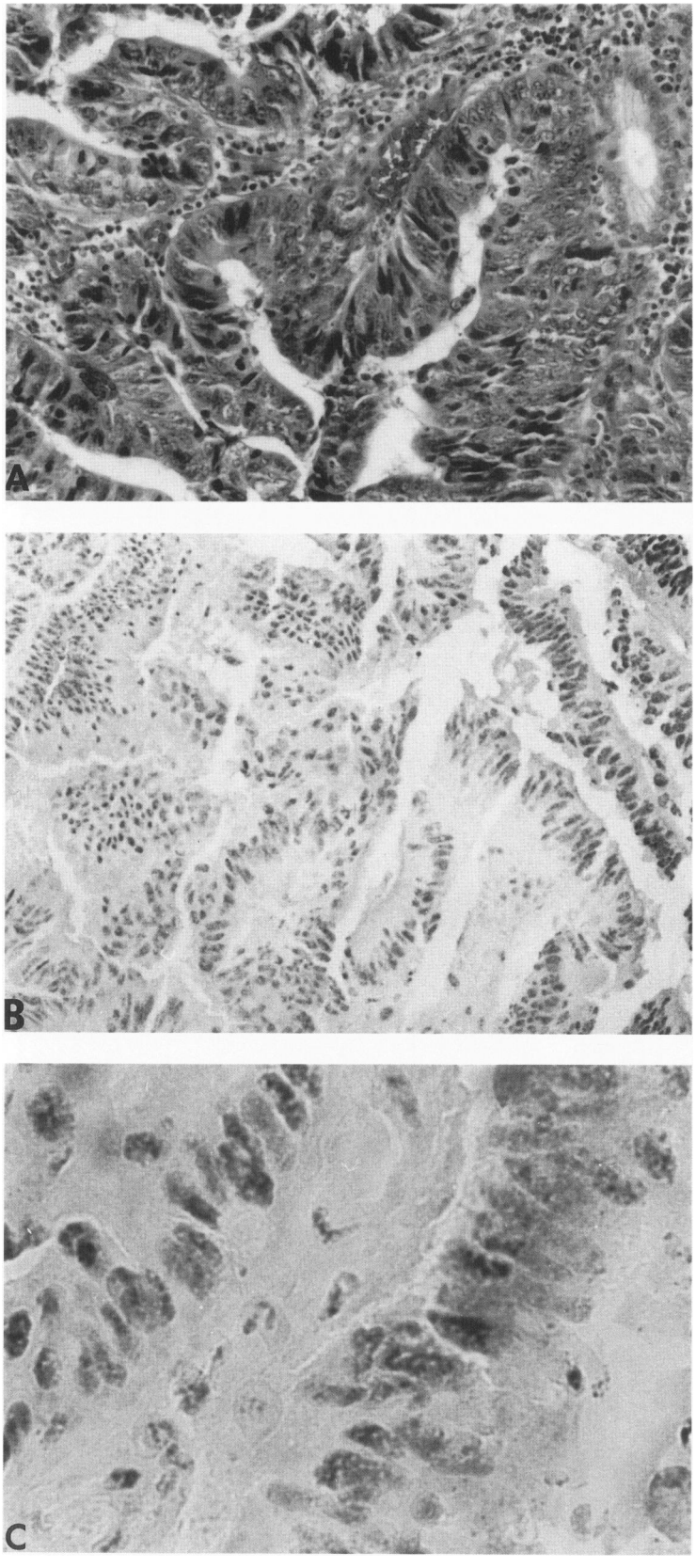

Figure 3. *Immunohistochemistry of well-differentiated invasive carcinoma from specimen G using rabbit polyclonal anti-c-*myc *antibody and Sternberger anti-rabbit IgG peroxidase-anti-peroxidase method.* **A**: *H and E stained section of well-differentiated invasive carcinoma, ×200.* **B**: *Immunostained section of similar region as* **(A)**, *with no counterstain, ×200.* **C**: *Higher magnification of immunostained section, neoplastic nuclei are filled with dense, diffuse, darkly staining c-*myc *material, ×800.*

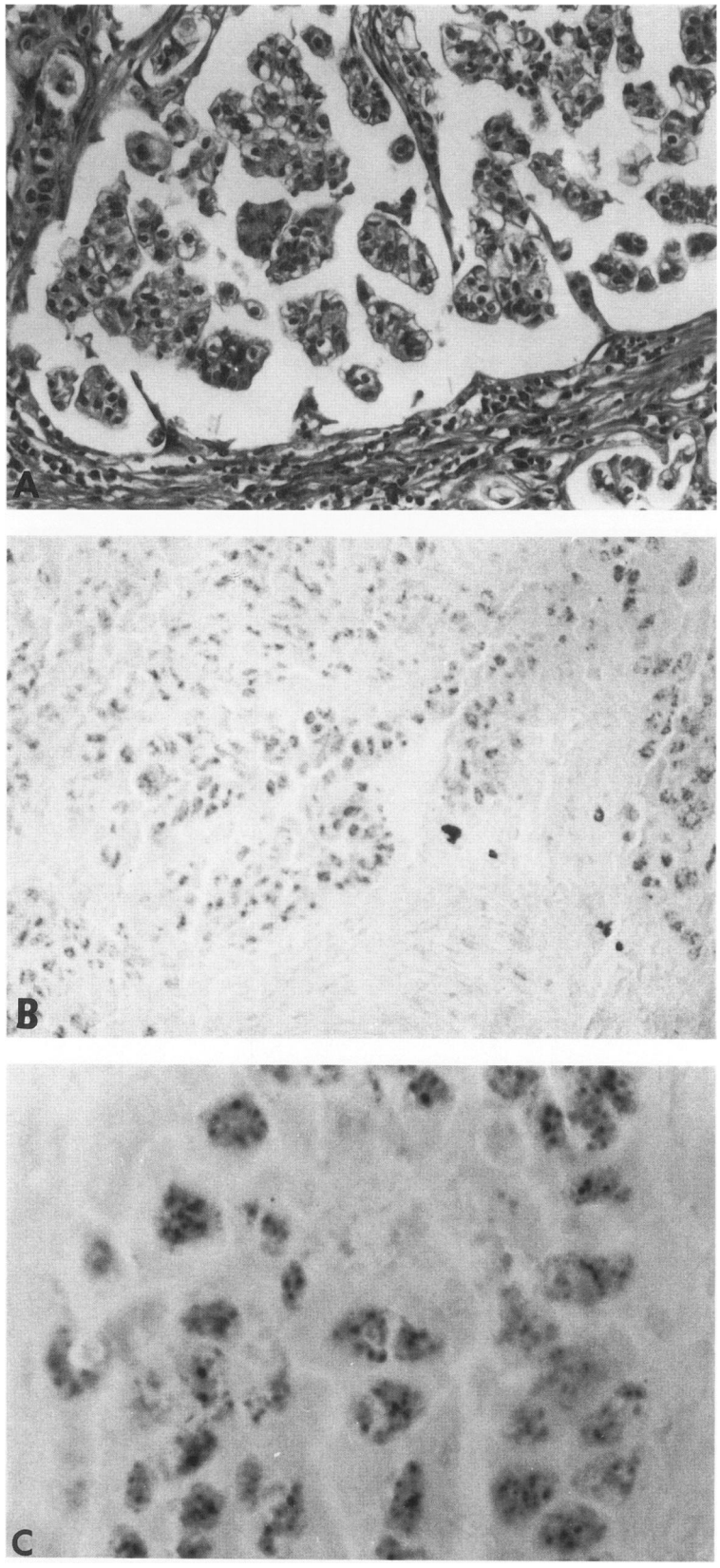

Figure 4. *Immunohistochemistry of a poorly differentiated invasive carcinoma from specimen L using rabbit polyclonal anti-c-*myc *antibody and Sternberger anti-rabbit IgG peroxidase anti-peroxidase method.* **A**: *H and E stained section of poorly differentiated invasive carcinoma, ×200.* **B**: *Immunostained section of similar region as* **(A)**, *with no counterstain, ×200.* **C**: *Higher magnification of immunostained section, neoplastic gland nuclei appear to be filled with 7–14 coarse granules of c-*myc *stained material, ×800.*

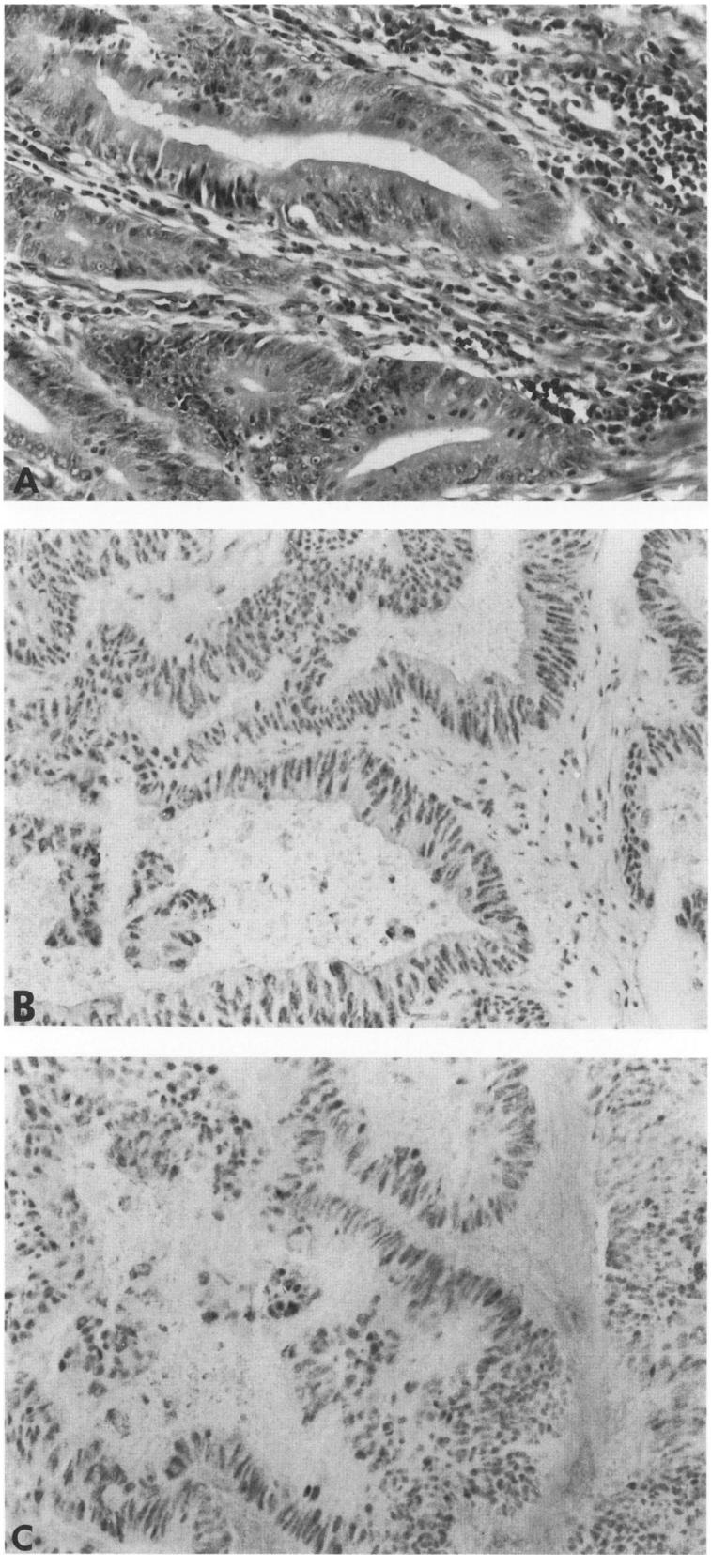

Figure 5. *Immunohistochemistry of well-differentiated invasive carcinoma from specimen H.* **A:** *H and E stained section of well-differentiated invasive carcinoma, ×200.* **B:** *Immunostained section of similar region as* **(A)**, *using rabbit polyclonal anti-c-*myc *antibody and biotinylated anti-rabbit IgG and avidin-biotin-peroxidase detection system; neoplastic nuclei are filled with dense, coarse, c-*myc *stained material, with no counterstain. Note also that interstitial cell nuclei also reveal positive c-*myc *material, ×200.* **C:** *Immunostained section of similar region as* **(A)**; *using mouse monoclonal* myc *antibody NCM II 274 and biotinylated anti-mouse IgG and avidin-biotin-peroxidase detection system; neoplastic nuclei show darkly staining c-*myc *material, with no counterstain. Note the absence of differential c-*myc *staining of interstitial cell nuclei, ×200.*

focused on the c-*myc* staining of the glandular epithelial cell nuclei of the neoplastic tissues because they are stained at higher dilutions of the antibodies, and therefore, presumably have more *myc* protein. We have also observed c-*myc* staining of nonepithelial interstitial cell nuclei in some of the immunostained sections. This requires further analysis. We previously demonstrated that there was an increase in c-*myc* staining with tumorigenesis in a rat model of 5-azoxymethane induced colon cancer.[43] The nuclear localization and increased quantity of c-*myc* protein found in neoplastic glandular epithelial cell nuclei of human colonic tumors further support a relationship of this protein with the malignant process in human colorectal cancer.

These results are in contrast to the work of others,[38–42] who detected cytoplasmic staining in midcrypt and epithelial surface cells in formalin-fixed paraffin-embedded tissues of human colonic polyps and carcinomas, using a monoclonal antibody to $p62^{c\text{-}myc}$, Myc-1-6E10. The problem of localization and retention of the c-*myc* protein in nuclei has been extensively explored,[6–13,25,26,35,36] and some investigators have concluded that the predominantly nuclear localization found in fresh tissue becomes cytoplasmic after fixation.[38] This is inconsistent with other work that indicates that c-*myc* protein retains its nuclear localization after fixation[28,36] and therefore it is possible that the monoclonal antibody studies that detect a cytoplasmic localization are detecting a crossreacting epitope.[42]

The specificity of the antibody reaction described in our studies is supported by data from the comparison of staining with polyclonal and monoclonal *myc* antibodies that produce similar nuclear staining patterns on the same set of tissues. Absorption of the c-*myc* rabbit polyclonal antibody with purified c-*myc* protein gave no appreciable nuclear staining in tissue sections of human colorectal cancer. We have preliminary data on c-*myc* staining of (Tulchin et al, manuscript in preparation) reveals a transgenic mouse model of breast cancer that reveal nuclear localization of the c-*myc* protein.

These immunohistochemical studies show the regional variability in c-*myc* protein distribution among cell nuclei of colonic tumors, which would be masked in biochemical assays. It is not clear why the apparently uninvolved normal mucosal regions also display variable c-*myc* staining in closely adjacent crypts. This may reflect variations in cell proliferation, or biochemical events in the G1 phase of the cell cycle. Further work is required to extend these results to larger numbers of colonic tumors and to develop methods that would permit the analysis of fixed sections from paraffin-embedded material. This would permit us to perform retrospective studies that could further elucidate the prognostic significance of c-*myc* protein expression in diseases of the human colon.

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