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c-myc *Protein in Normal Tissue*

Effects of Fixation on Its Apparent Subcellular Distribution

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The c-*myc* protein is thought to be a DNA-associated nuclear protein. However, immunohistochemical studies on normal or tumor tissues have shown conflicting findings on its subcellular distribution. By using various fixation procedures on cytospin preparations of HL60 cells, the authors found the subcellular distribution of the c-*myc* protein to be dependent on the method of fixation. When studying mouse tissues in frozen sections using a biotinylated monoclonal antibody against the c-*myc* protein, they found the protein to be widely distributed in various normal adult mouse tissues, in most cases localized to the nucleus. However, when these tissues were studied after formalin fixation and paraffin embedding, a loss of nuclear staining was observed concurrent with the appearance of c-*myc* protein immunoreactivity in the cytoplasm. It is concluded that immunohistochemical studies on the expression of this oncogene should take into consideration the effects of fixation when its subcellular distribution is being examined. (Am J Pathol 1988; 131: 29–37)

THE v-*myc* GENE was first detected as the transforming gene of the avian myelocytomatosis virus MC29.[1] Expression of the homologous cellular c-*myc* protooncogene has been shown to occur in various cell lines of different vertebrate species, indicating a high degree of evolutionary conservation.[2] Abnormalities involving the protooncogene have been described, including amplification,[3] translocation,[4] and deregulation.[5] Certainly, expression of the gene *per se* is not sufficient for oncogenesis, because the c-*myc* gene is transcribed in proliferating normal cells.[6,7] On the other hand, its level of expression is subject to control with respect to stages of the cell cycle,[8] the tissue type,[9] and the stage of differentiation[10] or maturation.[11,12] Deregulation of its expression, usually implying an increased steady-state mRNA level, has often been cited as a possible step in oncogenesis.[13]

With the availability of monoclonal and polyclonal anti-*myc* antibodies, several studies have been undertaken to determine the subcellular distribution and possible functions of the c-*myc* protein. Human c-*myc* protein has been found to be unstable (half-life of about 20–30 minutes).[14] The protein is predominantly found in the nucleus,[15,16] possesses DNA binding capacity,[17] and has been implicated in DNA synthesis.[18]

Less has been reported on the tissue distribution of the c-*myc* protein. It is well known that c-*myc* is normally absent or present at a very low level in resting peripheral blood lymphocytes.[7] When peripheral blood lymphocytes are stimulated to proliferate by phytohemagglutinin, nuclear c-*myc* protein content increases[2] and becomes demonstrable by immunostaining. Stewart et al[19] found that the protein is mainly cytoplasmic in distribution in normal human colonic epithelium, while in normal testicular tissue, Sikora et al[20] found the protein in both the cytoplasm and the nucleus. Using formalin-fixed, paraffin-embedded tissues, Jack et al[21] found that c-*myc* protein is widely distributed in different tissues, but marked differences are apparent in its intracellular distribu-

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Table 1—Effects of Fixation on the Distribution of Immunoreactivity of c-*myc* Protein as Demonstrated by 3C7 on Cytospin Preparations of HL60

	Fixation time (minutes)						
Fixative	5	10	20	30	60	120	240
paraf	n*	n	n	n	n	n	n/c
paraf/Triton	n	n	n	n	n	n	n/c
paraf/Triton/heat	n	n	n	n	n	n	n
acetone	n	n	n/c	n/c	c	c	c
acetone/heat	n	n	n	n	n/c	n/c	n/c
formalin	n	n/c	n/c	n/c	c	c	c
formalin/heat	n	n	n	n/c	n/c	n/c	n/c
B-5	–	–	–	–	–/n	–/n	c
B-5/heat	–	–	–	–	–/n	–/n	c

Paraf, paraformaldehyde; n, nuclear staining; c, cytoplasmic staining; – no staining.

* The relative intensity of staining is not shown.

tion. Except in hemopoietic tissues, they found the protein to be mostly intracytoplasmic. Similar conclusions were made by Iwanaga et al[22] when using B5-fixed paraffin-embedded tissues.

Although this is not in direct contradiction to studies performed with cell lines, these findings are in conflict with the notion that the c-*myc* protein is a nuclear protein having close association with DNA. Evan et al[15] have shown that the protein is elutable from nuclei by a high salt buffer. Whether the processing and/or fixation of the tissue will elute the protein or affect the intracellular localization of the protein is unknown.

We undertook our study to determine effects of fixation on the immunostaining of c-*myc* protein on cytospin preparations and tissue sections processed in various ways. We conclude that the c-*myc* protein is generally localized to the nucleus in optimally processed tissue sections but that variations in processing and fixation can alter its subcellular distribution.

Materials and Methods

Anti-c-*myc* Antibody

The monoclonal anti-c-*myc* protein antibody (3C7) was raised and described by Evan et al[23] (kindly supplied as a gift by Dr. G. Evan, Cambridge, England). It is a mouse IgG1 raised against a synthetic peptide corresponding to residues 171 through 188 of human $p62^{c\text{-}myc}$, and the antibody binds to human $p62^{c\text{-}myc}$ as well as mouse $p64,p66^{c\text{-}myc}$.

For staining mouse tissues, this antibody was biotinylated with biotin-*N*-hydroxysuccinimide ester (Calbiochem, La Jolla, Calif) and dialyzed against PBS overnight before use. This step of labeling the antibody enabled us to apply a murine monoclonal antibody onto mouse tissue without requiring a further step of adding a biotinylated anti-mouse second antibody. No steps were taken to block endogenous peroxidase in both frozen and paraffin sections. Three clones (3C7, 8F9, and 6E10) were initially tested for immunoreactivity with HL60 cells. Although all three antibodies have been shown to immunoprecipitate c-*myc*,[23] the 3C7 clone demonstrated optimal staining by immunocytochemistry. This antibody was utilized for all subsequent studies on the tissue distribution of the c-*myc* protein.

Cell Lines and Tissues

To assess the effects of various fixation procedures, the human promyelocytic leukemic cell line HL60 was used, while normal peripheral blood lymphocytes served as negative controls. In some experiments, HL60 cells induced to differentiate by phorbol ester or dibutyryl cyclic AMP[24] were used as negative controls. Exponentially growing HL60 cells were washed twice in cold PBS and centrifuged (Cytospin II, Shandon Scientific Co., Sewickley, Pa) at 900 rpm for 4 minutes onto clear glass slides (10^5 cells/slide). After brief air drying, the slides were fixed by various methods (see below).

To assess the effects of tissue processing, adult female BALB/c mice were sacrificed, and frozen blocks and corresponding paraffin blocks were taken from kidneys, liver, spleen, intestine, salivary gland, heart, lung, lymph node, ovary, and brain.

Fixation and Processing

Cytospin preparations were fixed with one of the following methods: 1) 4% neutral buffered formalin at room temperature; 2) B-5 fixative (4% formaldehyde with 6% wt/vol mercuric chloride and 1.25% wt/vol sodium acetate) at room temperature; 3) 1% paraformaldehyde in phosphate-buffered saline (PBS), pH 7, at room temperature; 4) 1% paraformaldehyde in PBS, pH 7, then 0.1% Triton X-100 (Research Products International Corp., Elk Grove Village, Ill) in PBS at room temperature; and 5) 95% acetone at room temperature. For each method of fixation, eight different durations of fixation were evaluated: 5, 10, 20, or 30 minutes and 1, 2, 3, or 4 hours. The effects of preheating to 41 C for 5 minutes to immobilize c-*myc* protein within the nuclear matrix (see below and Evan and Hancock[15]) were also assessed.

For staining mouse tissues, immediately after sacrificing the animal, one block of each organ was put in O.C.T. embedding medium (Miles Laboratories Inc., Naperville, Ill), snap-frozen in liquid nitrogen, and

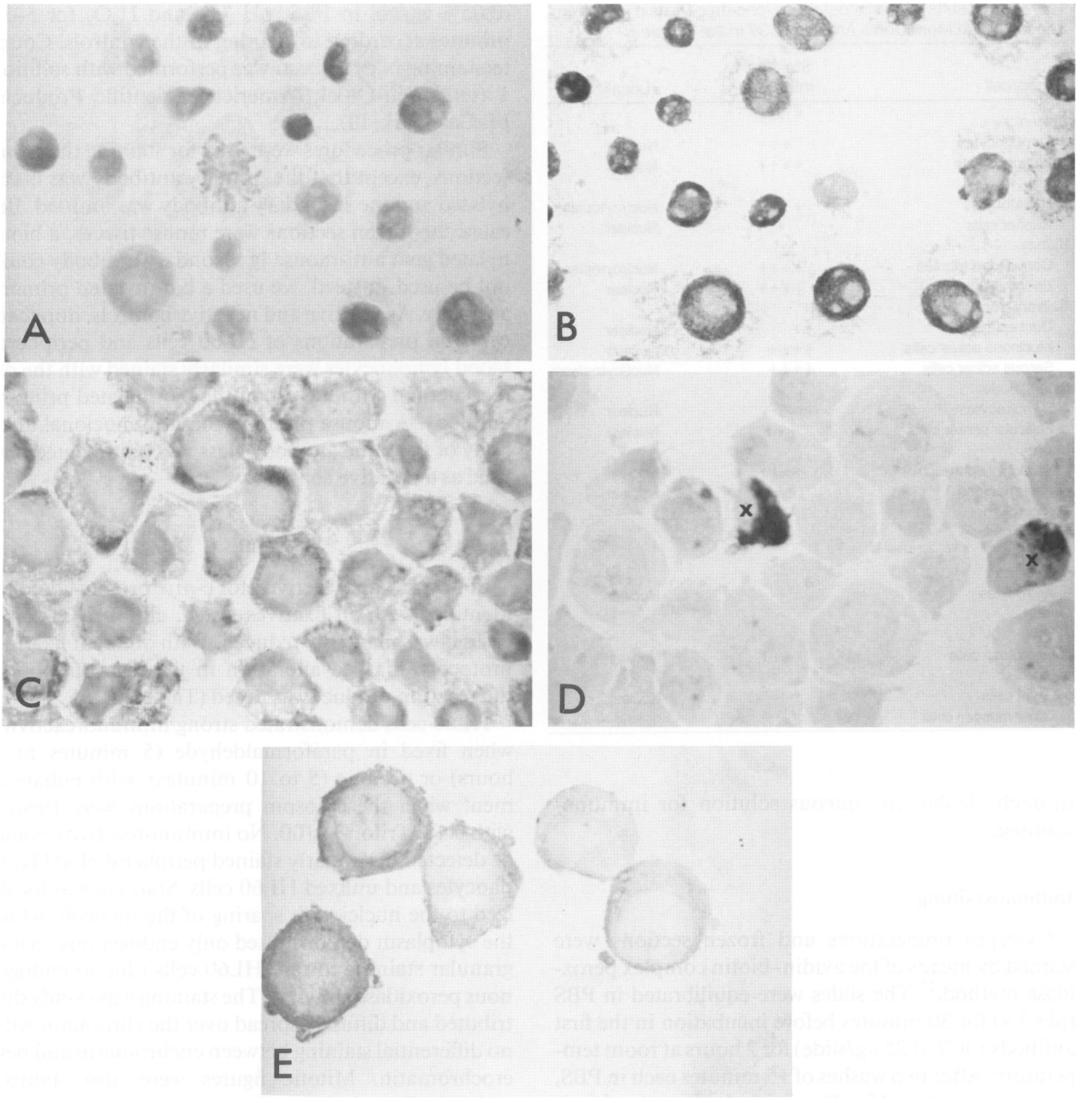


Figure 1—Photomicrograph showing cytospin preparations of HL60 cells stained with anti-c-*myc* monoclonal antibody using various fixative procedures: **A,** 1% paraformaldehyde and 0.1% Triton X-100 for 30 minutes; **B,** 4% (wt/vol) neutral buffered formalin for 5 minutes; **C,** 4% (wt/vol) neutral buffered formalin for 30 minutes; **D,** B-5 fixative for 5 minutes; and **E,** B-5 fixative for 4 hours. (*x*, endogenous peroxidase activity of HL60 cells.) Note variations in staining intensity and pattern with different fixatives and time. One percent paraformaldehyde with Triton X-100 demonstrates optimal nuclear staining (see Results). (×1000)

stored at −70 C until sectioned. The method of fixation that optimally demonstrated c-*myc* protein in the HL60 cells was chosen for staining frozen sections. Sections 4–6 μ thick were cut within 12 hours of immunostaining. The sections were air-dried at room temperature overnight, preheated to 41 C, fixed in 1% paraformaldehyde for 30 minutes, and incubated in 0.1% Triton X-100 in PBS, pH 7, for 5 minutes before immunostaining. Triton X-100 permeabilizes the nuclear membrane to allow immunostaining of the nuclear antigens. To simulate routine processing of tissues in clinical situations, a corresponding block from each organ was fixed at the same time in neutral buffered formalin for 4 hours and processed for paraffin blocks. Sections 6 μ thick were cut, deparaffinized without heating, and rehydrated stepwise

Table 2—Results of Immunostaining for c-*myc* Protein in Frozen Sections With Monoclonal Antibody 3C7 in the Mouse

Organ/cell	Staining intensity	Localization
Spleen		
Lymphocytes	++	Nuclear
Immunoblasts	++++	Nuclear
Liver		
Hepatocytes	++++	Nucl/cytoplasm
Kupffer cells	+	Nuclear
Kidney		
Convoluted tubules	++++	Nucl/cytoplasm
Endothelium	++++	Nuclear
Salivary gland		
Duct epithelium	++	Nuclear
Mucinous acinar cells	++++	Nuclear
Serous acinar cells	++++	Nucl/cytoplasm
Lymph node		
Lymphocytes	+/−	Nuclear
Follicular center cells	+++	Nuclear
Lung		
Bronchial epithelium	++++	Nuclear
Pneumocytes	+/−	Nuclear
Myocardium	−	—
Striated muscle	−	—
Brain		
Neurons	−	—
Astrocytes	+	Nuclear
Ovary		
Ova	−	—
Granulosa cells	++++	Nuclear
Ileum		
Crypt cells	+	Nuclear
Other mucosa cells	−	—

through alcohol to aqueous solution for immunostaining.

Immunostaining

Cytospin preparations and frozen sections were stained by means of the avidin–biotin complex peroxidase method.[25] The slides were equilibrated in PBS (pH 7.4) for 30 minutes before incubation in the first antibody (3C7, 0.25 μg/slide) for 2 hours at room temperature. After two washes of 15 minutes each in PBS, they were incubated for 30 minutes in the second antibody, biotinylated goat anti-mouse IgG (Tago, Burlingame, Calif) diluted 1:40 with 1% normal goat serum in PBS. After being washed twice with PBS, the slides were incubated in biotinylated horse peroxidase and avidin (Vectastain, Vector Laboratories, Inc., Burlingame, Calif) for 30 minutes before final washing and color development with diaminobenzidine (0.05% wt/vol in PBS, pH 7.4) and H_2O_2 for 5–10 minutes according to staining in the controls. Counterstaining of cytoplasm was performed with solution 1 (red), Diff-Quick (American Scientific Products, MaGaw Park, Ill).

Similar procedures were used for staining the tissue sections, except that the primary antibody was biotinylated and the secondary antibody was omitted. Because the frozen sections were mouse tissues, a biotinylated goat anti-mouse Ig secondary antibody could not be used. Instead, we used a biotinylated primary antibody. As positive and negative controls, duplicate cytospin preparations of HL60 cells and peripheral blood lymphocytes were similarly stained with the direct method with the use of the biotinylated primary antibody. A mouse plasmacytoma monoclonal antibody of the same antibody class was biotinylated and used as a negative control.

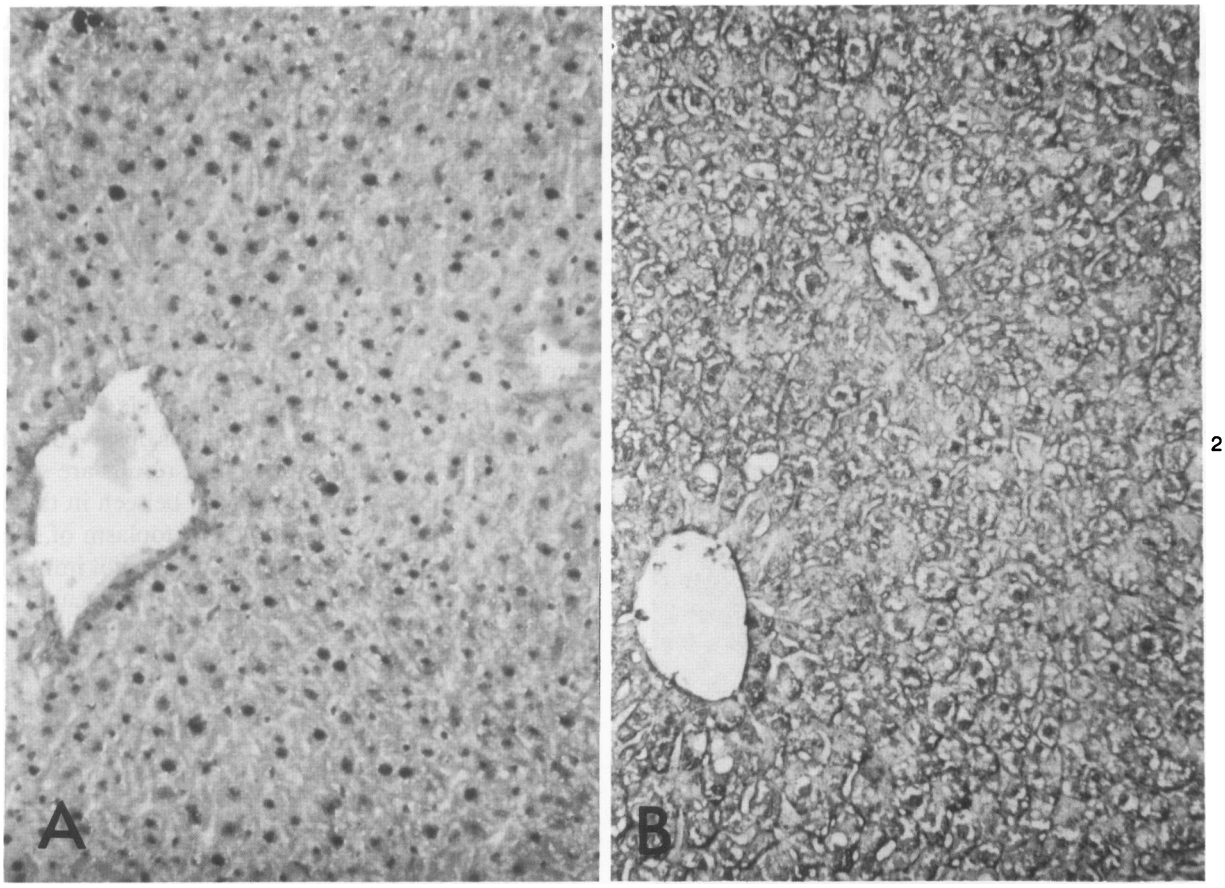
Results

Some immunoreactivity for c-*myc* protein could be identified with all fixatives tested, although in some cases it was markedly reduced. With different fixation protocols, marked alteration in the distributions of the reaction product was noted (Table 1).

HL60 cells demonstrated strong immunoreactivity when fixed in paraformaldehyde (5 minutes to 2 hours) or acetone (5 to 10 minutes), with enhancement when the cytospin preparations were treated with 0.1% Triton X-100. No immunoreactivity could be detected in similarly stained peripheral blood lymphocytes and unfixed HL60 cells. Staining was localized to the nuclei with sparing of the nucleoli, while the cytoplasm demonstrated only endogenous coarse granular staining in rare HL60 cells (due to endogenous peroxidase activity). The staining was evenly distributed and diffusely spread over the chromatin with no differential staining between euchromatin and heterochromatin. Mitotic figures were also stained, which indicated that c-*myc* protein was associated with the chromosomes during the metaphase of mitosis (Figure 1A).

The cytospin preparations fixed in formalin for 5 minutes also demonstrated strong positive immunoreactivity. The staining was diffusely localized to the nuclei as after paraformaldehyde fixation. Again, nucleoli were not stained (Figure 1B). When the cytos-

Figure 2—Photomicrograph showing murine liver tissue sections stained with anti-c-*myc* monoclonal antibody on **A**, frozen sections fixed in 1% paraformaldehyde and **B**, paraffin-embedded formalin-fixed sections. Note weaker staining in formalin-fixed paraffin-embedded sections and strong nuclear immunoreactivity on the frozen sections. (×400) **Figure 3**—Photomicrograph showing murine renal tissue sections stained with anti-c-*myc* monoclonal antibody on: **A**, frozen sections fixed in 1% paraformaldehyde and **B**, formalin-fixed paraffin-embedded sections. Note granular cytoplasmic staining in the renal tubular cells (*arrow*) in the paraffin-embedded sections. *g*, glomeruli. (×400)



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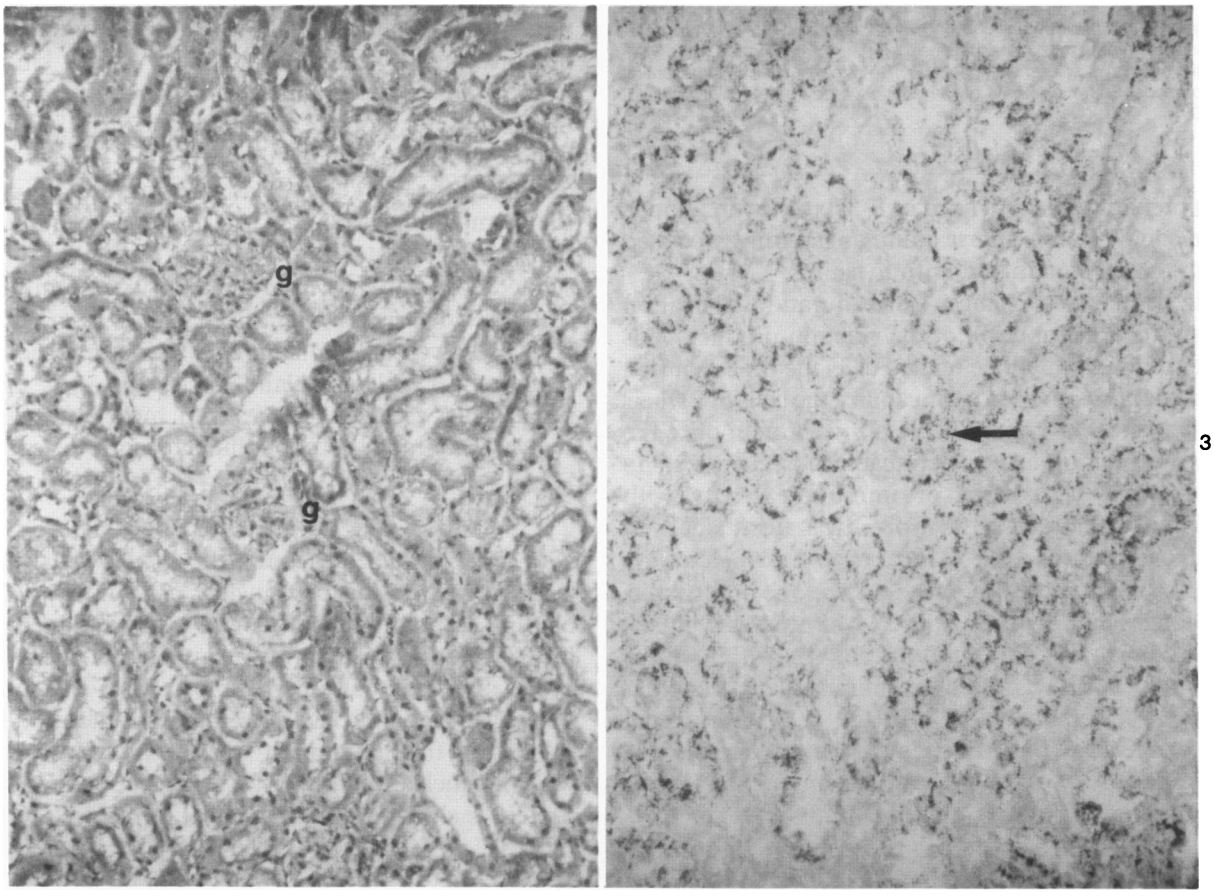

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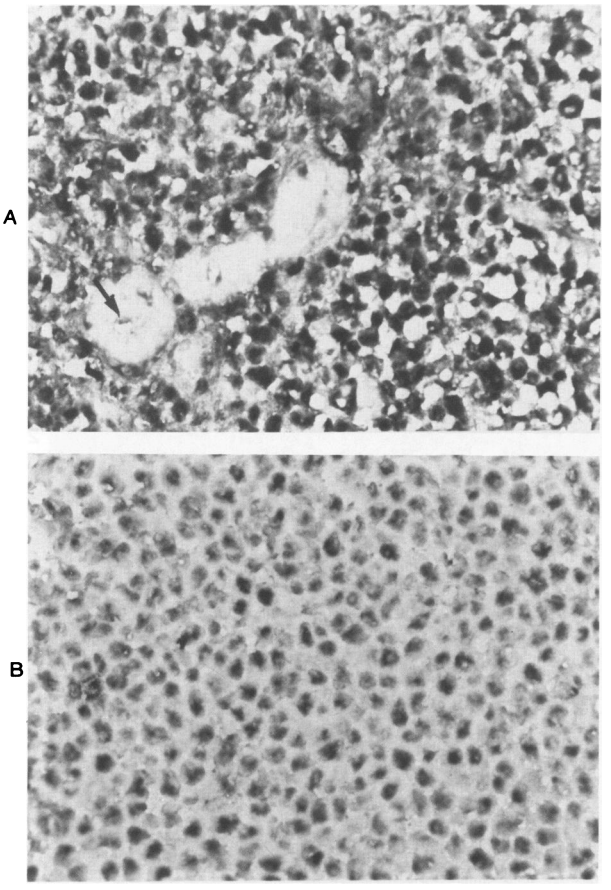

Figure 4—Photomicrograph showing variation in the degree of immunoreactivity in lymphoid cells when stained with anti-c-*myc* antibody. **A**—Strong staining in splenic white pulp. **B**—Weaker staining in peripheral lymph node. Endothelial cells of the arteriole (*arrow*) are weakly stained. (×800)

pin preparations were fixed in B-5 fixative for 2 hours, one could observe sparse granular staining associated with the chromatin and mitotic figures.

In some cases, changes in the duration of fixation altered the distribution of the reaction product. This phenomenon might be related to 1) the time required by various fixatives to permeabilize the cells or 2) their effectiveness in immobilizing c-*myc* protein once the nuclear membrane was permeabilized. There was no change in the pattern of immunoreactivity with different durations of fixation in paraformaldehyde with or without Triton X-100. However, when the cytospin preparations were fixed in acetone or formalin for longer than 5 minutes, there was progressively increased cytoplasmic immunoreactivity with a corresponding decrease of nuclear staining and a widening of the nucleolar silhouette (Figure 1C). With over 1 hour of fixation, nuclear staining was lost and the immunoreactivity was purely cytoplasmic. When the cytospin preparations were fixed in B-5 for less than 30 minutes, there was no observable staining (Figure 1D). After about 2 hours of fixation, one could observe sparse granular staining associated with the chromatin and mitotic figures. When the fixation time was lengthened to 4 hours, there was faint but completely cytoplasmic staining with no nuclear immunoreactivity (Figure 1E).

The application of brief heat treatment before the application of chemical fixatives appeared to enhance the degree of immunoreactivity and delayed, although not totally abolished, the process of elution of c-*myc* protein into the cytoplasm.

These results are summarized in Table 1.

Widespread immunoreactivity was observed in frozen sections of mouse tissues. The results are shown in Table 2. Positive staining could be seen in the nuclei of most cell types and in the cytoplasm of some. Neurons, ova, and striated muscle were not immunoreactive. Staining intensity varied but appeared homogeneous within a tissue cell type. On the whole, the observed staining intensity in various tissues corresponded well with the expected rate of proliferation of the cells in that particular tissue (Figures 2A, 3A, 4, and 5).

Significant cytoplasmic staining was observed in kidney, liver, and salivary glands. In the latter, this was seen only in the serous glands, while the mucinous glands showed only nuclear staining. In the liver, there was a suggestion that only the perivenular zone demonstrated cytoplasmic staining. In the kidney, where cytoplasmic granular staining could be seen, the morphology of the nuclei was less distinct and poorly visualized by hematoxylin.

There was a marked decrease in immunostaining in the formalin-fixed blocks. The pattern of predominantly nuclear staining was lost. Many cells that stained positively in frozen sections were negative in sections cut from formalin-fixed tissues (Figures 2B and 3B). Cytoplasmic staining was observed in the kidney, liver, salivary gland ducts, and some lymphoid cells, especially those with more cytoplasm, such as immunoblasts and follicular center cells.

Discussion

In tissue culture cell lines, the c-*myc* protein appears to be a nonhistone nuclear protein with DNA binding capacity that may be involved indirectly in DNA synthesis.[18] The protein can be localized to the nuclei of cells in culture.[23] The protein is also found to localize in the nucleus after microinjection.[14] After subcellular fractionation, it is found to be associated with the nuclear matrix. Evan et al[15] showed that the protein is elutable from the nuclei in high salt buffer

and that its localization to the nuclear matrix may be an artifact due to mild heat exposure.

Two studies on the localization of c-*myc* protein in normal tissues reported a predominantly cytoplasmic intracellular distribution.[21,22] Both studies used paraffin-embedded formalin- or B-5-fixed material. Such fixation procedures are known to affect the results of immunostaining, mainly by denaturing immunoreactive epitopes of the target antigen. In the case of c-*myc* protein, its short half-life and elutibility from nuclei might lead to altered distribution after such fixation procedures.

Clevenger et al[26] have shown that the sequential use of paraformaldehyde and 0.1% Triton X-100 results in optimal permeabilization of cells for the demonstration of nuclear antigens. We compared a modified version of this fixation sequence with various commonly used fixatives for processing of clinical materials and have studied their effects on the distribution and immunoreactivity of c-*myc* protein. Immunoreactivity as recognized by the monoclonal antibody 3C7 is preserved to some extent in all fixatives tested. However, the present study shows that even in cultured cells, the fixation procedure may affect the apparent intracellular localization of the c-*myc* protein. With formalin and B-5 fixatives, there is apparent difficulty in immobilizing the denatured c-*myc* protein in the nuclei, and the protein is eluted out of the nuclei if the sample is left in the fixative too long. Thus, cytoplasmic immunoreactivity increases while nuclear immunoreactivity decreases with time. In formalin-fixed tissues, the elution problem is prevented by the use of a shorter fixation time. On the other hand, with shorter periods of B-5 fixation, immunoreactivity is undetectable or weak, perhaps because of decreased cellular permeabilization, resulting in poor antibody penetration. The addition of a brief heat treatment tends to decrease the degree of nuclear elution, presumably by locking the protein into the nuclear matrix,[15] while the use of detergents in fixation enhances immunoreactivity, presumably by further permeabilizing the cell membrane.

Because previous studies[19,21,22] on the normal tissue distribution of c-*myc* protein used fixed, paraffin-embedded material, we thought other approaches might yield a better representation of the tissue distribution of the protein. We studied the protein in frozen sections to avoid alterations associated with paraffin embedding. To have full control of the time elapsed after excision of the tissues, murine tissues, rather than clinical human material, were used. This was feasible because 3C7 is known to cross-react with the murine c-*myc* protein product. To overcome the problem of applying a goat anti-mouse second antibody onto murine tissues, we biotinylated the primary monoclonal antibody 3C7. Both the biotinylated antibody and the unconjugated antibody demonstrated comparable immunoreactivity when tested on HL60 cells and appropriate controls.

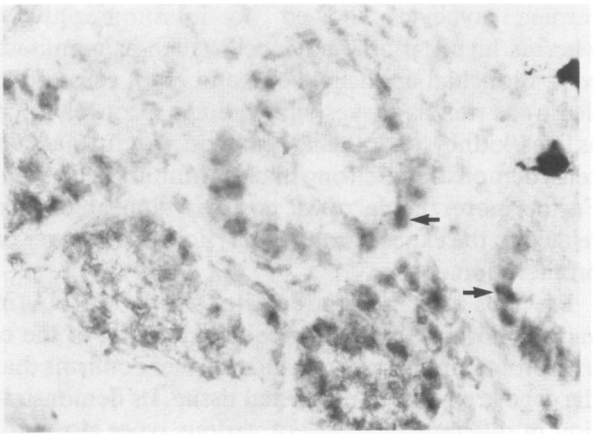

Figure 5—Photomicrograph showing immunoreactivity for c-*myc* protein in the nuclei (*arrows*) of crypt cells in the ileum. (×800)

Our results demonstrate a predominantly nuclear reactivity in various tissues. Cytoplasmic staining was restricted to the renal convoluted tubules, the hepatocytes, and some salivary acinar cells. Differences between our results and those of others[21,22] are probably due to the different fixation procedures employed. Although further studies in frozen sections of human tissues will be necessary to confirm our findings, a predominantly nuclear distribution of the c-*myc* protein is probably more representative of the *in vivo* situation.

Despite a varity of fixation methods, some cell types still exhibit cytoplasmic staining for c-*myc* protein. These tissues are either rich in hydrolytic enzymes or metabolically very active, suggesting that there may be a high rate of paramortem protein degradation or turnover in these tissues and, consequently, a poorer localization of the c-*myc* protein. Interestingly, the nuclei of the convoluted tubular cells showing granular intracytoplasmic staining are difficult to visualize with usual counterstaining. Perhaps occurrence of autolysis, which is inevitable even under such controlled experimental conditions, makes the nuclear membrane porous and results in diffusion of the protein into the cytoplasm.

Our study also demonstrates that c-*myc* protein is widely but not universally distributed in tissues of the mouse. If the protein is normally associated with cellular proliferation, it should be found in tissues with proliferative activity and should be absent in quiescent tissues. Such an hypothesis is supported by the present study. Neurons, ova, skeletal muscles, and

cardiac myocytes showed no immunoreactivity, whereas highly proliferative cells (such as granulosa cells, bronchial epithelial cells, and crypt cells of the ileum) as well as tissues with cellular turnover (such as liver, kidney, endothelial cells, and glandular acinar cells) demonstrated strong nuclear immunoreactivity. Furthermore, the degree of positivity appeared to be related to the expected rate of physiologic turnover or proliferation.

Previous studies have detected c-*myc* mRNA in various normal tissues. The demonstration of the c-*myc* protein in the present study further confirms that the gene is expressed in normal tissue. Its demonstration in differentiated cells of various types also indicates that repression of the c-*myc* gene is not required for full differentiation in many tissues. In fact, it seems from our studies that repression is the exception, rather than the rule, in differentiated cells.

Although the present study does not clarify the function of the c-*myc* protein, its wide distribution suggests that the temporal control of its expression with respect to the cell cycle is not stringent. Winqvist et al[27] found fairly even cytoplasmic distribution of c-*myc* protein in mitotic cells, as demonstrated by a rabbit anti-sera raised against a fragment of v-*myc* from transfected bacteria. In the present study, we found c-*myc* immunoreactivity associated with chromatin during mitosis. The reason for this discrepancy is uncertain at present and certainly needs future clarification. However, a difference in fixation procedure, such as the use of brief heat treatment, or a difference in antibody/antisera avidity might be the underlying cause. Both our study and the data of Winqvist et al are in keeping with the findings of Thompson et al and Rabbitts et al, who both concluded the c-*myc* protein and mRNA are detectable with invariant levels throughout the cell cycle.[28,29]

In conclusion, we have demonstrated that the c-*myc* protein is predominantly localized to the nuclei of various mouse tissues. However, certain fixation procedures allow the protein to diffuse into the cytoplasm. Care must be taken when interpreting data on subcellular distribution of the protein in paraffin-embedded tissues. Furthermore, our data suggest that repression of the c-*myc* gene is not a prerequisite for cellular differentiation, because we have observed c-*myc* protein immunoreactivity in many normal, fully differentiated mouse tissues.

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