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Enhanced c-*myc* gene expression during forelimb regenerative outgrowth in the young *Xenopus laevis*

(limb regeneration/protooncogene/proliferation/differentiation/*in situ* hybridization)

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ABSTRACT **Analysis of the expression of the c-*myc* protooncogene has been carried out in the forelimb regenerate of the *Xenopus laevis* froglet. Northern blot hybridization analysis revealed the presence of a 2.5-kilobase c-*myc* transcript in the regenerate forelimb at a level at least 7-fold more than the one found in nonregenerating forelimbs or stumps of regenerating forelimbs. *In situ* hybridization analyses confirmed the relative abundance of c-*myc* RNA in the regenerate forelimb and provided evidence of spatial localization of high levels of c-*myc* RNA in specific cell layers. The deepest layers of the wound epithelium of epidermal origin showed a strong signal, whereas virtually no c-*myc* RNA was detected in the outermost layers. Labeling was also observed in mesenchymal cells of the blastema where it was relatively evenly distributed. This pattern of c-*myc* RNA in the regenerate might indicate that the expression of c-*myc* plays a role in the regulation of the continued proliferation of specific cells of the regenerate, whereas repression of this gene in the epidermis correlates with terminal differentiation of keratinocytes.**

Among Amphibia, limb regeneration is a well-documented biological phenomenon, especially in Urodeles (1, 2). In Anurans, froglets of some species will partially regenerate their amputated limbs during early postmetamorphic stages. This system provides an interesting model to study the pattern of cell proliferation as it produces the formation of a fibroblast-like cell accumulation at the tip of the stump after epithelial wound healing and the formation of an elongated cartilaginous spike lacking normal musculature and digits (3–5).

In an effort to understand the molecular mechanisms involved in the regulation of the cell cycle and differentiation during the regenerative process, we have analyzed the expression of the protooncogene c-*myc* during this phenomenon. Regulated expression of c-*myc* has been described during cell proliferation and differentiation in different models (6–9). In the studies presented here, limb regeneration was examined in *Xenopus laevis*; our laboratory and others have characterized and sequenced c-*myc* from *Xenopus* (10–12). Its developmental expression shows that it is involved in nuclear function(s) necessary for the early cleavage stage of development (10–14). We have also observed strong c-*myc* expression during eye formation, which might prevent terminal differentiation in this tissue during early organogenesis (13). The role of c-*myc* in the equilibrium between growth and differentiation was examined in limb regeneration. Here we report that c-*myc* is strongly expressed during limb regeneration in distinct epithelial cell layers of the regenerate, in correlation with cell proliferation. In contrast, low c-*myc* RNA levels are observed in cell layers committed to differentiation. The data are compared with those obtained from the stump and with previous observations made for the whole embryo (10–14).

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MATERIALS AND METHODS

Animals and Cell Cultures. All experiments were carried out on *X. laevis* froglets 5 months postmetamorphosis. Animals were accommodated and fed as described by Gurdon (15). *Xenopus* A6 cells were cultured at 20–22°C in 75% L15 Leibovitz medium (GIBCO) supplemented with gentamycin and 10% heat-inactivated fetal calf serum. Cells were harvested in their exponential phase of division.

Amputations. Animals were anesthetized with 0.3% MS 222 (3-aminobenzoic acid ethyl ester methanesulfonate salt) and then bilaterally amputated with fine scissors through the mid-zeugopodial region (radius-ulna) of the forelimb under sterile conditions (see Fig. 1*A*). When necessary, soft tissues and skin of the stump were then trimmed to provide as flat an amputation surface as possible. Experimental animals were raised individually at room temperature (18–20°C) in small plastic containers. Harvest of the regenerates and stumps were performed under sterile conditions 4 weeks after amputation. For RNA extraction procedures, regenerates as well as equal amounts of stump tissue minus bone were pooled. Specimens were stored at −80°C before RNA extraction.

RNA Extraction and Northern Blot Hybridization. Frozen samples were rapidly homogenized in a solution of 3 M LiCl/6 M urea at 4°C using a Ultra-turrax homogenizer. The homogenates were processed for RNA extraction and agarose gel electrophoresis as described (10). Northern blot hybridization was accomplished using a single-stranded antisense ^{32}P-labeled 630-base-pair *Xenopus Hin*cII–*Pvu* II c-*myc* coding region sequence (10) and washed using standard procedures (14). Autoradiography and scanning densitometry of different exposures were performed to quantitate the c-*myc* RNA level in each sample (10).

***In Situ* Hybridization of Cut Sections from Regenerate with c-*myc* RNA Probes.** Albino *Xenopus* were used to avoid confusion between pigment granules and silver grains. One-month-old regenerates and stumps were fixed into freshly prepared 4% paraformaldehyde in 0.5× phosphate-buffered saline for 1 hr at 4°C. Samples were processed exactly as described (13). Hybridization was done with a ^{35}S-labeled antisense c-*myc* RNA probe (4 to 6 × 10^8 cpm/μg) prepared as described (13). Sense probes were run in parallel as a negative control for background and nonspecific interactions. Sections of *Xenopus* oocytes were also hybridized in parallel on the same slides as positive controls, as c-*myc* is known to be highly expressed in these cells (10, 13).

Abbreviation: TEM, transmission electron microscopy.

Transmission Electron Microscopy (TEM). To examine the structure of the wound epithelium cells of the regenerate and epidermis of the stump, the tissues of regenerating limbs were fixed by immersion for 2 hr in 1.5% glutaraldehyde/1.5% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). The samples were routinely processed for TEM according to the procedure of Géraudie (16), whereupon they were embedded in Epon for longitudinal sectioning.

RESULTS

At the time of harvest, the limb regenerate is composed of a wound epithelium of epidermal origin covering a mound of relatively undifferentiated cells, the pseudoblastema cells as described by Skowron and Komala (17). It arises through cell migration and proliferation, which is the causal event allowing the construction and enlargement of the limb blastema cellular accumulation capping the limb stump.

Northern Blot Hybridization Analysis of c-*myc* RNA in Limb Regenerates and Control Limbs. The expression of c-*myc* was followed by RNA extraction of limb tissues, as illustrated in Fig. 1. c-*myc* transcripts were observed in total RNA from limb regenerates and stumps of *Xenopus* froglets (Fig. 1, lanes 2 and 3). A prominent c-*myc* transcript of 2.5 kb in size was detected. This c-*myc* transcript was also present in nonregenerating control limbs (Fig. 1, lane 1). Expression of the c-*myc* in nonregenerating control limb is not surprising in that the *Xenopus* froglets are in an active stage of growth. The 2.5-kb c-*myc* transcript is also expressed in *Xenopus* A6 proliferating cells (Fig. 1, lanes 4 and 5) and stage II *Xenopus*

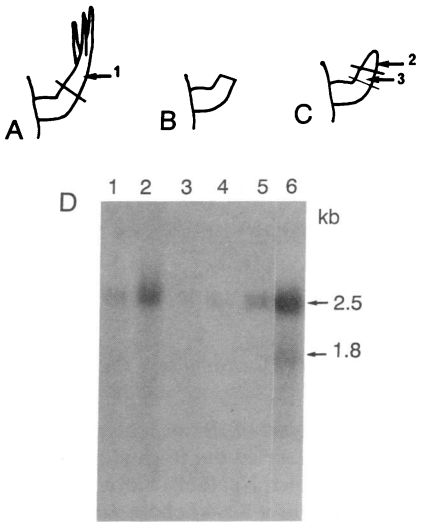

FIG. 1. Northern blot analysis of c-*myc* expression in *Xenopus* limb regenerates and stumps. Limbs were amputated at the level of the radius-ulna (*A* and *B*), and the distal regions (severed limbs) were kept as control limbs (sample 1). Four weeks after amputation (*C*), the regenerates, considered as the outgrowth about the amputation site, were harvested (sample 2) as were stump tissues located above the elbow (sample 3). (*D*) Northern blot analysis of total mRNA extracted from the different tissues. Lane 1, control limbs (8 μg of RNA); lane 2, regenerates (12 μg); lane 3, stumps (13 μg); lane 4, 3×10^4 *Xenopus* proliferative cells; lane 5, 10^5 *Xenopus* proliferative cells; lane 6, stage II oocytes (5 μg). kb, Kilobases.

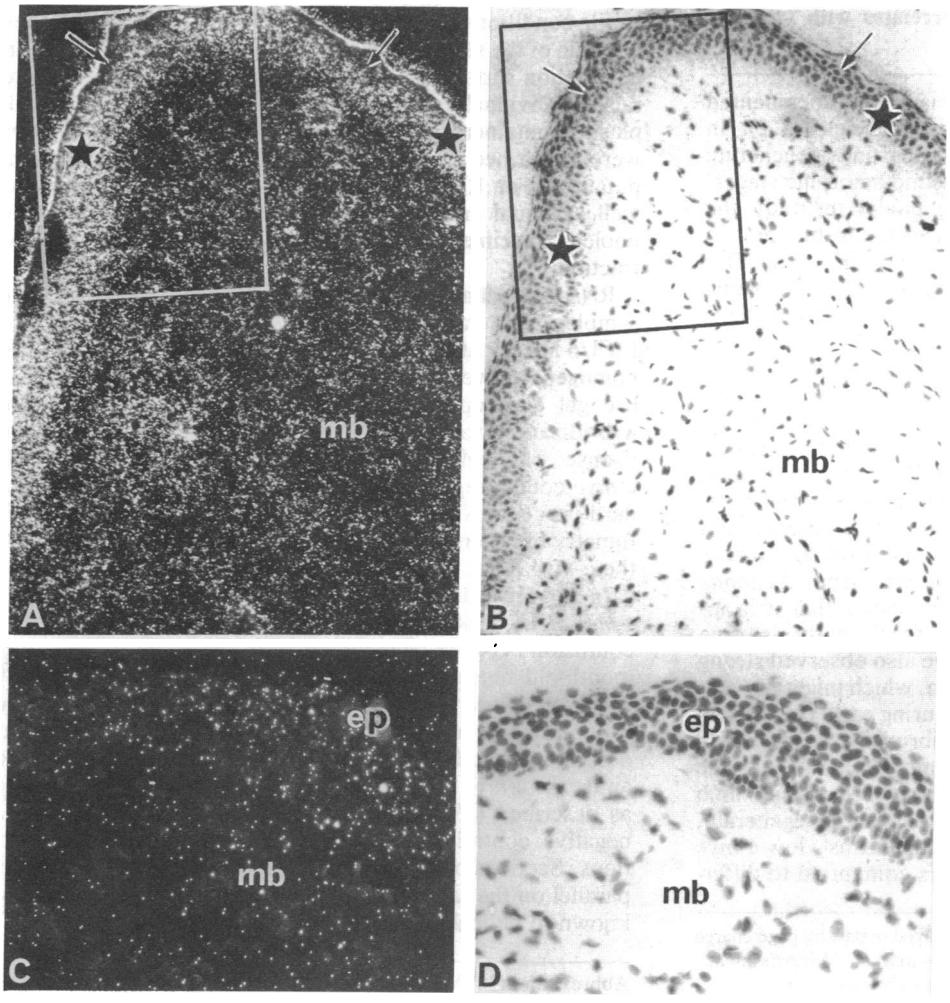

FIG. 2. Localization of c-*myc* RNA in the limb regenerate. *In situ* hybridizations were carried out as described in the text. (*A* and *B*) Sections were hybridized with a ^{35}S-labeled antisense c-*myc* RNA probe. (*A*) Regenerate under dark-field illumination. A high level of labeling was detected in the germinative layer and intermediate layers of the epithelium (star). The signal was much lower in the outermost layers (arrows). A moderate labeling was also detected in the mesenchymal cells (mb). (×160.) (*B*) The same regenerate under bright-field illumination. The rim of autoradiographic grains lining the tissue sections is an artifact created during the procedure of autoradiography (drying of the emulsion). (×160.) (*C* and *D*) Control sections of the regenerate hybridized with a ^{35}S-labeled sense c-*myc* RNA probe. (*C*) Dark-field illumination. Labeling is not above background in the epithelium (ep) and in the mesenchyme of the outgrowth (mb). (×200.) (*D*) Bright-field illumination. (×200.)

oocytes (Fig. 1, lane 6). The oocytes also express a 1.8-kb c-*myc* transcript as described (10).

Densitometric quantification was done by scanning different autoradiographs of the gel (ref. 10; see *Materials and Methods*). From these results the level of c-*myc* RNA from one stage II oocyte is equivalent to the level of c-*myc* RNA found in 10^5 *Xenopus* somatic cells in culture, in agreement with our previous observations. The same quantification procedure was applied to the other samples, and the signal obtained was normalized to the amount of RNA loaded in each lane. A 7- to 8-fold increase in the expression of c-*myc* was observed in the whole regenerate, relative to control limbs (Fig. 1, lanes 1 and 2). In contrast, a very weak expression was detected in the stump tissues.

It is rather difficult to compare the level of c-*myc* expression during regeneration and cell proliferation in culture. However, the signal obtained from eight regenerates processed in the gel (around 400,000 cells) was equivalent to the signal with 300,000–400,000 *Xenopus* somatic cells in their growing phase in culture. As all of the cells of the regenerate are not expressing c-*myc* (see below), we conclude that the level of c-*myc* RNA in the regenerate is at least equivalent to the c-*myc* RNA level in growing cells in culture.

Cellular Localization of c-*myc* RNA in the Limb Regenerate. *In situ* hybridization analysis provided specific tissue localization of the c-*myc* RNA detected by Northern blot hybridization. c-*myc* RNA was not evenly expressed in the regenerate and only specific cells exhibited a high level of c-*myc* RNA (Figs. 2 and 3). c-*myc* RNA is primarily localized to the wound epithelium as compared with the mesenchymal cells of the regenerate. A relatively high autoradiographic grain density was observed in the innermost layers (two or three cell layers) of the wound epithelium. About 15–20 silver grains were counted above these cells. The outermost layers were not labeled. A reduced level of c-*myc* RNA is also observed in the mesenchymal cells of the blastema (apical cell accumulation). Grain density is evenly distributed in these cells, as no gradient was detected. Control slides hybridized with a c-*myc* sense RNA probe showed a low background (Fig. 2 *C* and *D*).

Cellular Localization of c-*myc* RNA in the Limb Stump. c-*myc* RNA was detected in low relative amounts in total RNA from the limb stumps (Fig. 1*B*). *In situ* hybridization analysis indicated that soft tissues of the stump located between the skeleton and the epidermis (i.e., muscles and dermis) exhibit a grain density equivalent to background. However, a significant signal is apparent in the basal layers of the stratified epidermis, whereas the outermost layers are approximately equal to background (Fig. 3 *C* and *D*). Therefore, the pattern of c-*myc* RNA localization is qualitatively similar to that of the regenerate but differs quantitatively.

Ultrastructure of the Regenerate. TEM showed that the ultrastructure of the epithelial cells is different in the outermost layers as compared with the region below (Fig. 4). The cytoplasm of the superficial cells is filled with cytokeratine tonofilaments, which are the most abundant cell components identified. The chromatin of the elongated nucleus is very electron-dense and therefore not indicative of a high level of transcription. In cells of the basal (germinative) layer and intermediate layers, a large density of free ribosomes was

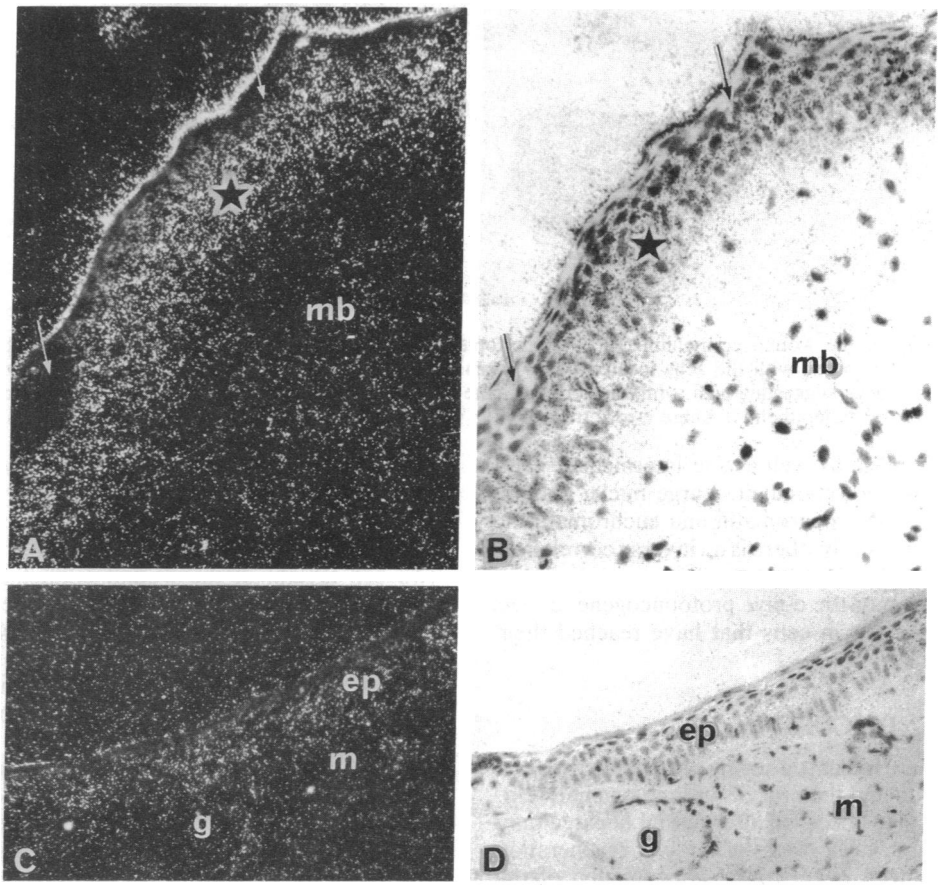

FIG. 3. Localization of c-*myc* RNA in the limb regenerate and stump. (*A* and *B*) Enlarged regions of the regenerate shown in Fig. 2. (×320.) (*C* and *D*) Localization of c-*myc* RNA in the limb stump after hybridization of sections with a ^{35}S-labeled antisense c-*myc* probe. (*C*) Stump under dark-field illumination. Epidermis labeling (ep) is much weaker than in the regenerate epithelium. Labeling is not above background in the mesenchymal tissue (m). Glands of epidermal origin located in the dermis are not labeled. (×160.) (*D*) Bright-field illumination of the same section. (×160.)

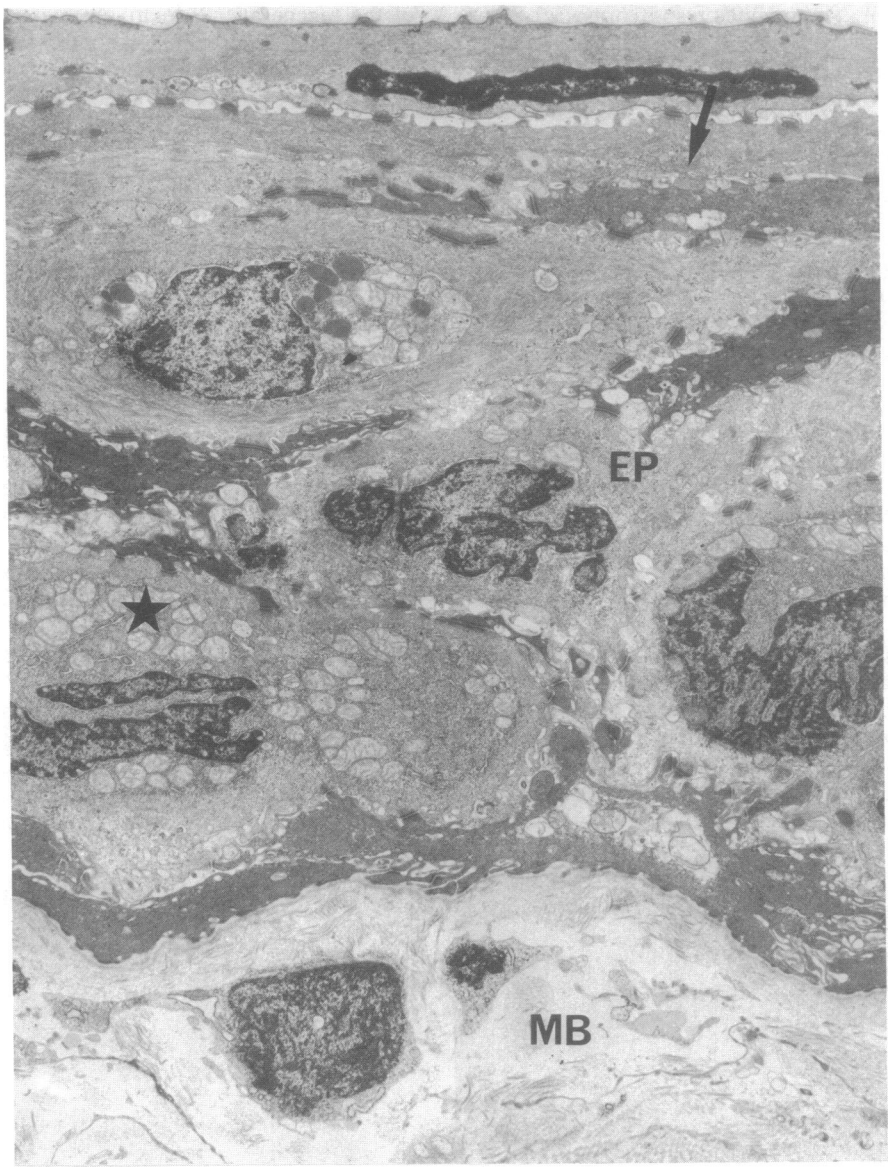

FIG. 4. Ultrastructure of the wound epithelium (EP). The cytoplasmic content is different in the outermost cells rich in cytokeratin tonofilaments (arrow) as compared with the cells located below (star). There, in addition to intermediate filaments, mitochondria and free ribosomes are quite prominent, suggesting high synthetic activity. Note the changes in size and electron density of the nucleus from the basal region toward the surface of the epithelium where c-*myc* transcription is no longer observed (MB, mesenchymal cells). (× 7360).

detected and mitochondria as well as the intermediate filaments described above are abundant. Large nuclei exhibit patches of perinuclear heterochromatin and euchromatin in large quantities. Consequently, there is an inverse correlation between the level of cell differentiation in the wound epithelium and the expression of the c-*myc* protooncogene. c-*myc* RNA is no longer present in cells that have reached their differentiated state.

DISCUSSION

The data presented here indicate a strong correlation between c-*myc* gene expression and the process of limb regeneration. A similar result was also observed after partial hepatectomy in the rat (18), although early stages of rat liver regeneration produce a compensatory hypertrophy phenomenon of the remaining lobules rather than a true epimorphic regeneration. The *in situ* hybridization results revealed a predominance of c-*myc* RNA in the inner layers of the wound epithelium, with a greater intensity in the regenerate than in the stump. These are two regions where cell morphology is distinctly different. There are large intercellular spaces in the wound epithelium (19) but this feature in cell topography is apparently independent of the expression of the c-*myc* protooncogene.

A low signal is observed in the superficial layers (one or two cell layers) of the wound epithelium. These cells are already differentiated into keratinocytes (Fig. 4) and are probably not very transcriptionally active, as judged by nuclear morphology and chromatin appearance. Moreover, the cytoplasm apparently no longer contains organelles involved in protein synthesis such as ribosomes. c-*myc* RNA is expressed in the inner layers of the wound epithelium, although only cells in the deepest (i.e., basal or germinative) layer are actively dividing. As the expression of c-*myc* correlates with cell proliferation, or with commitment to divide (9), these observations indicate that the inner layers of the wound epithelium are in a state competent to undergo cell proliferation. This interpretation is in agreement with the large variety of organelles observed to be present in the cytoplasm and with the nuclear ultrastructure.

In the mesenchymal cells of the outgrowth, c-*myc* RNA is present in a relatively low level. The lack of a gradient of

c-*myc* RNA favors the hypothesis that all of the cells are still in the cell cycle, although it is not yet possible to determine in which phase (20, 21). The decreased level of c-*myc* RNA expressed in these cells might reflect the fact that these cells are entering the differentiation program.

In the newt *Notophthalmus viridescens*, all of the cell layers of the wound epithelium express a high level of c-*myc* transcription (22). This may indicate that in the newt limb regenerate, all of the cells, including the outermost ones, are competent to undergo cell proliferation, in agreement with the high regenerative potential of this animal. In accordance with this observation, it is known that in normal human intestinal epithelium, an actively dividing tissue, c-*myc* is detected uniformly throughout the entire thickness of the colon epithelium. Its expression is not restricted to the regions located at the base of the crypt where proliferation occurs for cellular renewal (23).

Although the froglet is in a growing stage at the time of limb amputation, the relative abundance of c-*myc* RNA is increased in the regenerating limb compared to the nonregenerating limb. The biochemical and cytological data provide measures of only the steady-state levels of c-*myc* RNA. Thus we do not know if the regulation of c-*myc* expression is transcriptional or posttranscriptional; both levels of control can be involved in alteration of c-*myc* expression (6–9, 24–26). At the time of amputation c-*myc* is already being transcribed in the normal growing limb. However, it is clear that during regeneration a new regulation of c-*myc* expression is superimposed on that gene. In this sense, this system may be analogous to that of 3T3L1 preadipocytes transfected with the c-*myc* gene under control of Rous sarcoma virus. Enhanced expression of c-*myc* prevents the differentiation of these cells by compelling them to cycle (8). A similar result is reported during the formation of the eye during *Xenopus* development. A high level of c-*myc* RNA during organ ontogenesis may prevent cells from entering the terminal differentiation phase, a situation not compatible with the overall growth of the embryo (13). Differential expression of the c-*myc* protooncogene in specific cell layers may, therefore, be involved in the delicate balance that must be maintained between cellular growth and differentiation during the process of regeneration.

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