

Relationship Between Localized Cellular Growth and Patterning in Salamander Limbs

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2000-2001

This Senior Thesis is presented to the Biology Department as partial fulfillment of the requirements for graduation with a biology degree from Hartwick College.

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ABSTRACT

Considerable research has been done examining the cellular and molecular mechanisms of pattern formation in the tetrapod limb. Pattern formation in vertebrate limbs is epimorphic, and so dependent on cell division. The pattern of the dividing cells should therefore reflect the effects of patterning genes. Sonic Hedgehog, for example, has an expression pattern that is restricted to the zone of polarizing activity in the limb bud. Fibroblast growth factors (FGFs) are expressed in various regions of the limb bud mesenchyme and epidermis. Homeobox (Hox) genes have inter-nesting expression patterns in the tip of the limb bud. However, the actual patterns of dividing cells in developing or regenerating limbs have not been examined. Salamanders provide ideal material for such a study because of their large cell sizes and the fact that they regenerate their limbs. I used bromodeoxyuridine (BrdU) immunocytochemistry to analyze the relationship of dividing cells to pattern formation in the regenerating limbs of salamanders.

INTRODUCTION

In recent years, considerable research has been done looking at the process of limb outgrowth and development. Attention has been paid to the genetic cause of pattern formation in the limbs. Sonic Hedgehog (SHH) has been shown to be expressed in the posterior region of the limb bud. This region is often called the zone of polarizing activity, and it has been shown to function in establishing the antero-posterior patterns of the limb, possibly via the induction of Bone Morphogen Proteins (BMPs) (Riddle et al., 1993; Imokawa and Yoshizato, 1997; Drossopoulou et al., 2000).

Fibroblast Growth Factors (FGFs) have been shown to be necessary for initiation of limb outgrowth, via a feedback loop between FGF10 in the mesenchyme and FGF8 in the epidermis. FGF10 expression induces FGF8, which induces further FGF10, and will eventually lead to limb outgrowth (Ohuchi et al., 1997). FGF4 in the posterior apical ectodermal ridge (AER) has been shown to operate in a similar feedback loop with SHH expressed in the zone of polarizing activity (ZPA) (Zunige et al., 1999).

Homeobox (Hox) genes consist of four families of similar genes. These genes are expressed in the limb in complex nesting patterns (Schwabe et al., 1997). It has been shown that Hox gene mutants had limb bones with reduced growth, especially those bones that were made last, suggesting that the mutations resulted in a reduction of the prechondrogenic precursor cells (Capecchi, 1997). It has also been shown that the expression of these genes may be redundant, as individual mutations in either the *Hoxd-11* or the *Hoxa-11* genes will result in minor defects in the radius and ulna. Both genes need to be knocked out for major problems to occur (Johnson and Tabin, 1997). Hox genes are also known to be involved in muscle formation, especially the Hox A gene cluster, which is found in both forelimb muscle and hind-limb muscle, and the Hox C gene cluster, which is, interestingly, found only in the hind-limb muscles (Houghton and Rosenthal, 1999).

These three gene groups are not the only ones that are important for limb development. For instance, BMPs play important roles in pattern formation. The FGF4/ SHH feedback loop that signals between the AER and the ZPA is negatively controlled by BMP signaling (Zunige et al., 1999). BMPs are also necessary for chondrogenesis (Pizette and Niswander, 2000). T-box genes have been shown to be important in the determination of limb identity. That is, they may help to determine whether the limb is a forelimb or hind limb. It has been shown that TBX4 and TBX5 are both important limb outgrowth regulators, and that TBX4 is exclusively found in the hind-limb, while TBX5 is exclusively found in the forelimb, indicating that they may play a role in limb identity (Rodriguez-Esteban et al., 1999). These genes, and many others all play important roles in the genetic process of limb patterning.

While many people have looked at the genetic causes of pattern formation, very few have looked to see if any of the developing patterns will show up in the patterns of the dividing cells in the developing limb. Sessions (unpublished data, personal communication) has shown a correlation between bone formation and the number of dividing cells in a limb blastema. Limb buds were removed from developing axolotls, and ipsilateral and contralateral grafts were performed. The Limbs with the contralateral grafts produced mirror image duplications, and therefore were building more bone structures. This was reflected in copious amounts of dividing cells present, especially in comparison to the number of dividing cells present in the ipsilateral grafts, which produced relatively normal limbs. The dividing cells also seemed to

have their highest concentration where the new digits were forming (fig. 1). Assuming the dividing cell patterns would reflect the developing limb's patterns, I operated on 11 red backed salamanders and allowed them to regenerate. At certain stages of limb development- mid bud, palette, early digit, or late digit- the salamanders were injected with bromodeoxyuridine (BrdU), a thymine analogue that incorporates into replicating DNA (DNA replication usually occurs just before mitosis). The limbs were then fixed, serially sectioned, and put on slides. The BrdU labeling was then revealed using immunocytochemistry methods. For the mid bud stage limbs, the pattern of cellular growth strongly reflected the early pattern forming gradient genetically established. In all stages, a correlation between the presence of blood vessels and a higher density of cellular division was noticed. Finally, we unexpectedly saw dividing cells in mature, non-regenerating tissue, especially noteworthy in the mature muscle tissue.

MATERIALS AND METHODS

I amputated the right hind limb at the mid-thigh level of 11 salamanders, *Plethodon cinereus*. The salamanders were allowed to regenerate in moist dishes and were fed fruit flies twice a week. When the salamanders reached certain stages of development (mid bud, palette, early digits, and late digits- fig. 2) they were injected with 1% BrdU, and allowed to rest at an appropriate temperature for six hours. They were then euthanized with 2-phenoxy ethanol. The regenerating limb was dissected out, fixed in 3:1 ethanol/ acetic acid fixative, and kept at freezer temperatures. For serial sectioning, the limbs were dehydrated using 3 one-hour washes in 100% ethanol, and 2 one-hour washes in xylene. The tissues were then soaked in hot paraplast over night at 60°. The tissues were then imbedded in wax, sectioned into 10 µm sections and affixed on slides.

Immunostaining- The sections were rehydrated by washing twice in xylene for 2 minutes each, twice in 100% ethanol for 2 minutes each, and washed once for one minute each in 95% ethanol and 70% ethanol. They were then washed in PBS for no more than one hour. The sections were hydrolysed in 4N HCl for 30 minutes, neutralized in 0.1 M Na₂B₄O₇ for 2 minutes, and washed in PBST three times for 3 minutes each. The slides were then incubated for one hour at 4°C with anti-BrdU diluted in blocking solution at a 1:30 dilution, with at least 30 µl of solution per section. The slides were incubated in a moist chamber and supported on glass pipettes. Large coverslips were used to keep the solution evenly spread. The slides were then washed three times at 3 minutes each with PBS. They were then incubated with solution 1 from the Vectastain ABC kit for 30 minutes in a moist chamber supported on glass pipettes, at room temperature, and with no coverslips. The slides were then washed again with PBS three times at 3 minutes each. They were then incubated with solution 2 from the Vectastain ABC kit, also in the moist chamber supported by pipettes, at room temperature and with no coverslips, for 30 minutes. This was followed by 3 PBS washes at five minutes each. The slides were then incubated with the AEC substrate for 30 minutes in a moist chamber supported by glass pipettes, at room temperature, and with no coverslips. The slides were finally rinsed with distilled water. Coverslips were affixed with aquamount, and the slides were analyzed under light microscopy.

RESULTS

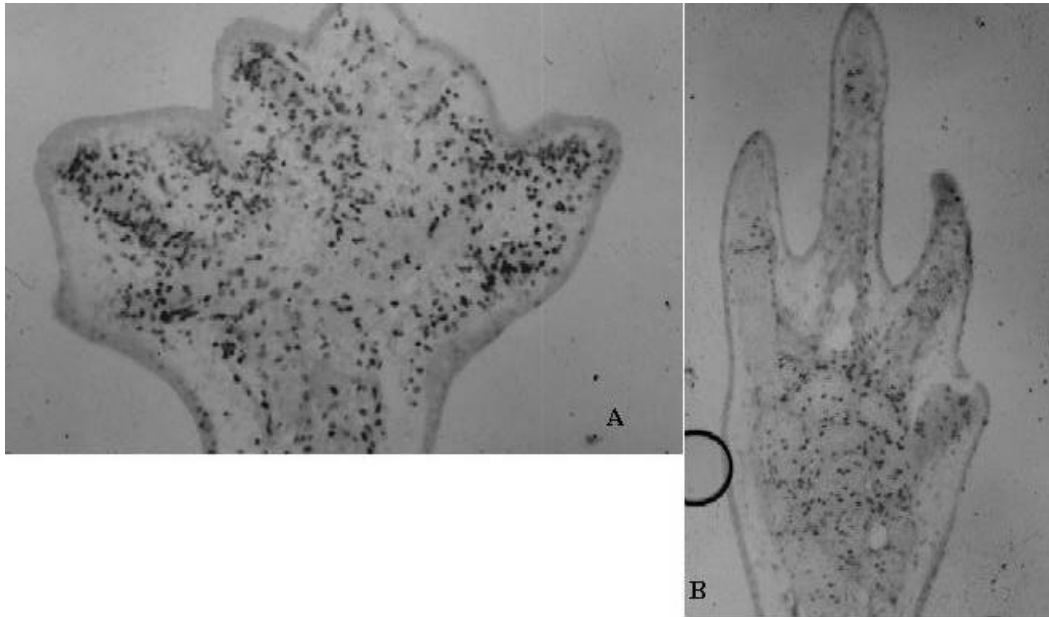


Figure 1- Images from Sessions ipsilateral/contralateral grafting experiment. A shows the contralateral graft at a late development stage. Note the large number of BrdU labeled cells, and that they show up in a general pattern that the new digits will take. B shows the ipsilateral graft at a late development stage. Note the smaller number of labeled cells, and that they too show the general pattern of the developing digits (Images courtesy of S.K. Sessions).

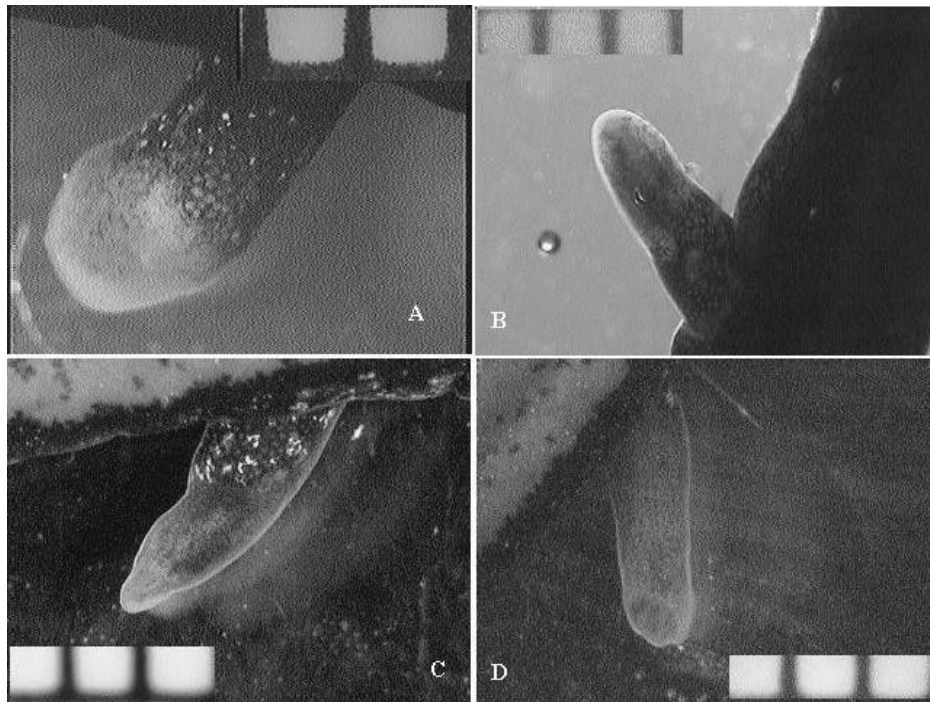


Figure 2- The different stages of limb regeneration. A is mid bud stage. B is palette stage. C is early digit stage. D is later digit stage. The black lines on the separate scales are 1 millimeter apart.

In the mid bud stage salamanders, there was a density of dividing cells along the posterior side of the limb bud blastema. Analysis showed that there were clearly more dividing cells in the posterior portion of the limb bud than in the anterior part of the limb bud (fig. 3).

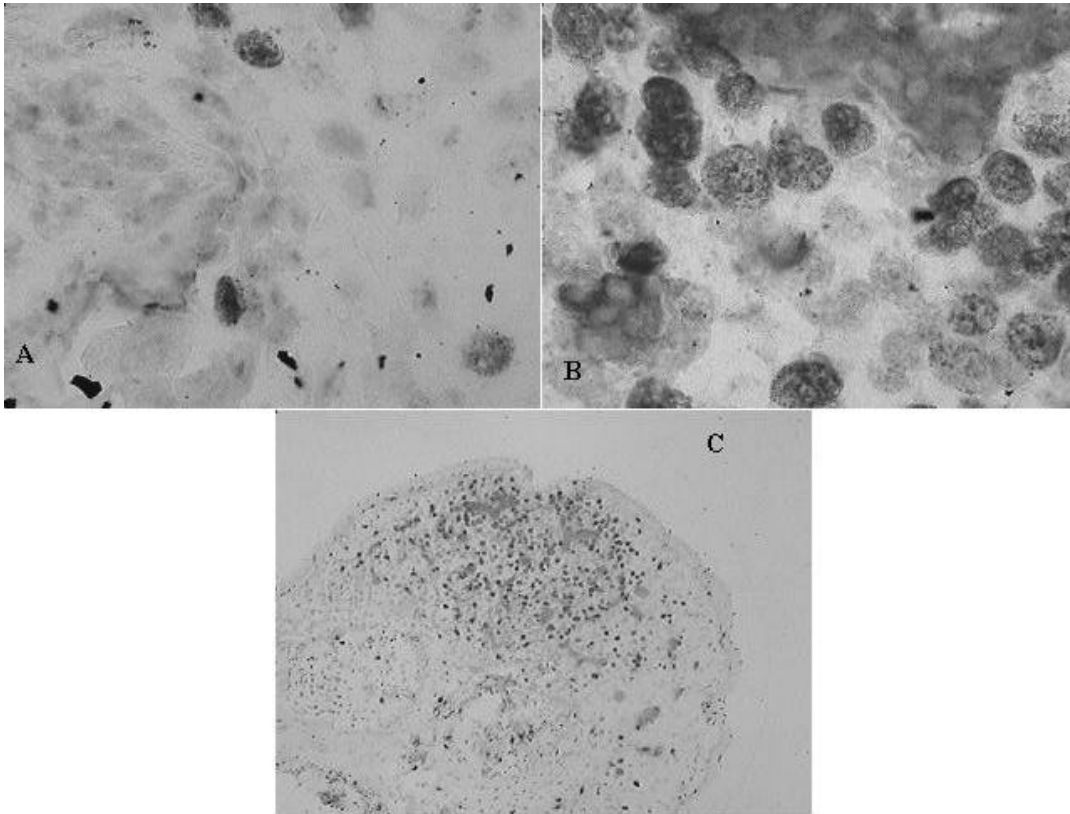


Figure 3- From a mid bud stage limb. C shows the overall view of the limb bud, showing that the dividing cells are concentrated in the posterior region of the limb bud. A shows a close up of the anterior, showing a lower level of the dividing cells. B shows a close up of the posterior, showing the higher levels of dividing cells. It also shows a stained blood vessel at the top of the image. A and B taken at 40x magnification. C taken at 4x magnification.

Later stage limbs showed no overt patterns that were readily discernable from microscopic evaluation of the slides. There was usually a homogenous mixture of BrdU labeled cells with no real pattern visible. The only exception was in the early and late digit stages where the differentiating cartilage of the developing bones could be seen, but the actual pattern of labeled cells in the cartilage and in the surrounding tissue had the same unremarkable pattern. Interestingly, approximately 50% of all cells appeared to be labeled (fig 4).

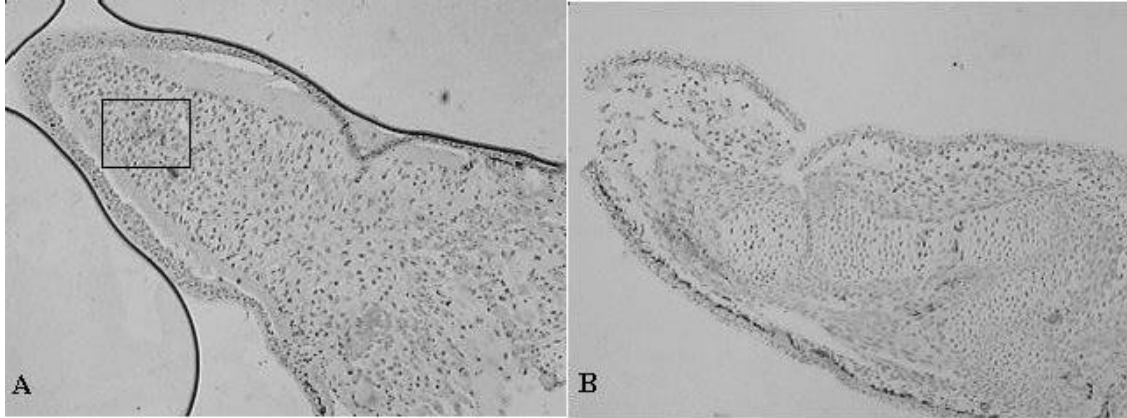


Figure 4- Shows 4x magnification images of palette (A) and late digit (B) stage limbs. Note the lack of patterns visible in the dividing cells. A blood vessel hot spot area is visible in A in the box outline. Also of note is the differentiated cartilage visible in B.

All stages where blood vessels were noticed exhibited an increased number of dividing cells located near or around the blood vessels. The blood vessels stained lightly because of endogenous peroxidase activity that reacted with the stain. This was one of the only other patterns that were noticeable (figs. 3 and 4).

In addition to the observation that there were a large number of stained cells in the regenerating tissues, such as the regenerating skin and cartilage, there were also a large number of labeled cells seen in mature tissue. Labeled cells were observed at high numbers in mature skin and bone tissue (fig. 5). In addition to those tissue types, labeled cells were also observed in mature muscle tissue (fig. 6).

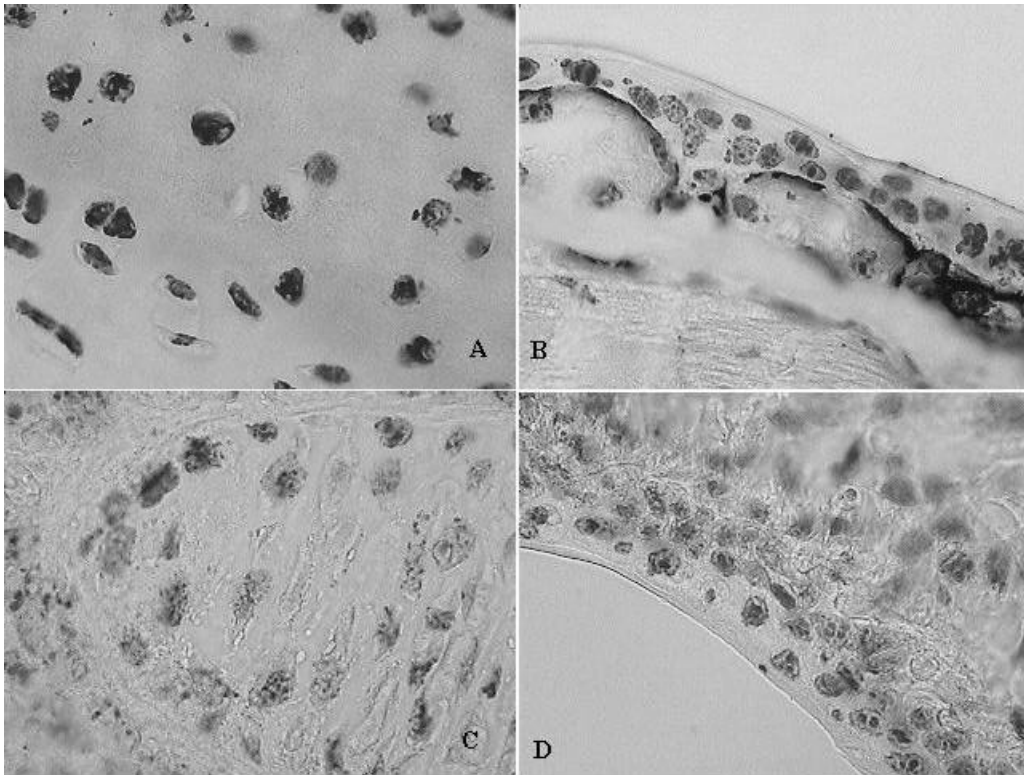


Figure 5- Shows 40x close-ups of dividing cell regions. A and B are both mature tissues, while C and D are both from regenerating tissue. A and C both show cartilage/bone tissue with high amounts of labeled cells. B and D both show skin cells with high amounts of labeled cells. Notice that both the mature and regenerating cells have similar amounts of dividing cells.

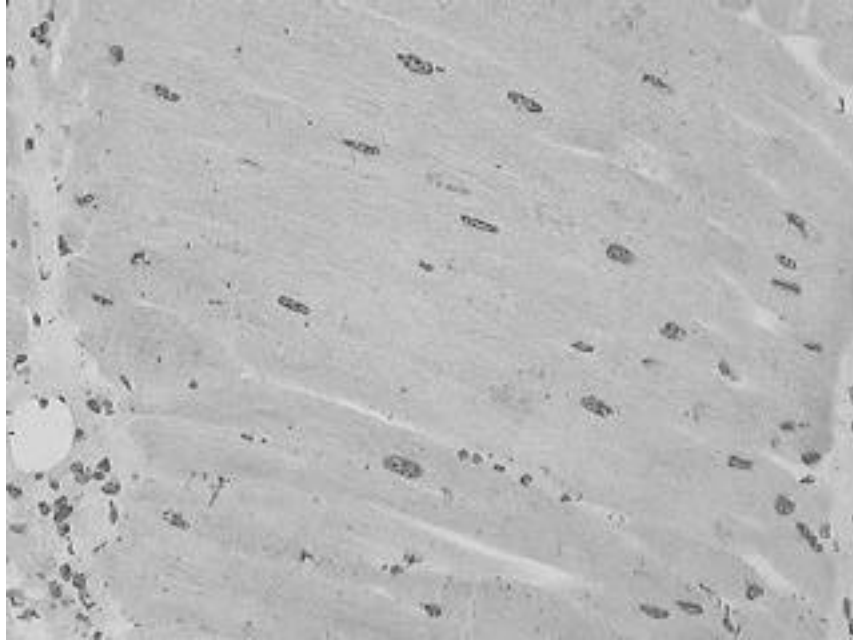


Figure 6- From a mid bud stage salamander. This shows the mature muscle of the salamander, which clearly shows labeled muscle nuclei, indicating that they were dividing, despite the fact that as mature muscle cells, they should not have been dividing.

DISCUSSION

The results from the mid-bud sections are very encouraging, and support the hypothesis that BrdU labeling experiments can be used to analyze patterns of dividing cells. The experiment also confirms that the BrdU staining process works in plethodontid salamanders. The posterior region of cellular growth seen in figure 2 most likely corresponds with the region of SHH expression that marks the zone of polarizing activity in the limb bud. Despite the lack of any other meaningful patterns in this particular experiment, these results show that the process can work.

The increase in the number of labeled cells near blood vessels is very interesting. The blood vessels themselves appeared “labeled” because of endogenous peroxidase in the blood, which reacted with the staining reagent. There could be a number of reasons why there was an increase in the number of labeled cells near the blood vessels. It could be that the cells nearest to the blood vessels are receiving the most nutrients from the incoming blood, and therefore are dividing more often. It could also be that the peroxidase from the blood got into those cells, giving them a background stain like the blood vessel itself. Further experiments would be needed to determine the exact cause of this phenomenon.

The sheer number of labeled cells also causes a problem. The excess labeling probably obscured any actual patterns that were present, such as the pattern seen in the mid bud stage salamanders. The species of salamander studied here, *Plethodon cinereus*, has a genome size of 22.3 picograms (Sessions and Larson, 1987). This is a very large genome size, especially when compared to the 1.9 picograms humans have (Weaver and Hedrick, 1997). This large genome size leads to these salamanders needing a long period of time for DNA replication. It is possible that this long period of DNA replication leads to the problems with over labeling of the cells. If we were to decrease the time of incubation with the BrdU to four hours, it would probably cut down on the excess labeling, as you would get only the most recently dividing cells labeled. Due to the short period of time we had for this experiment we were not able to test this hypothesis, so further trials are probably needed to help determine the best time of BrdU incubation for salamanders.

A final problem involved the number of adult cells that were seen dividing. In the mature skin cells, the number of dividing cells would be expected, as the outer most skin layer is constantly being replaced. The number of dividing cells seen in the mature bone, however, cannot be explained in this way. It is possible that the number of dividing bone cells could be explained by problems we had with the length of the BrdU incubation. This solution does not, however, explain the labeled cells seen in the mature muscle, which certainly should not have any reason for replicating its DNA, and absolutely should not be

dividing. According to current theory, the muscle cells do not divide after they fuse together to form the myotubes (Gilbert, 1997). It is possible that the labeled cells seen in the muscle, and possibly also the cells seen in mature bone and skin, are part of some unknown process that is part of the limb regeneration process. Further studies are needed to determine the exact reason for the labeled muscle cells, possibly looking to see if there is some kind of migration of divided muscle cells to the regenerating blastema. It may also be that the muscle cells are just replicating their DNA to increase the production of some gene product that is secreted and necessary for the limb regeneration process. Whatever the case may be, further study of this interesting phenomenon is needed.

ACKNOWLEDGEMENTS

I would like to thank Dr. Stanley K. Sessions. Without his help and guidance this project would have never happened. I would like to thank the rest of the Hartwick College Biology Department for their support and any helpful comments and suggestions they made along the way. I would like to thank Rob Hunt, who helped make some of the technical details of this project work out. Finally, I would like to thank the rest of the Hartwick College Biology Class of 2001. Thank you all for being there to talk about various problems along the way, and for helping to make the whole process seem a little bit easier than it really was.

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