Spatial Pattern of Nerve Differentiation in *Hydra* Is Due to a Pattern of Nerve Commitment

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The pattern of nerve differentiation along the body column of *Hydra* was investigated. Nerve precursors in late S phase were labeled with [³H]thymidine and their distribution compared with that of newly differentiated nerves. The two distributions were found to be the same. Based on independent evidence that nerve commitment occurs in mid- to late S phase (G. Venugopal and C. David, 1981, *Develop. Biol.* 83, 361–365) it was concluded that the pattern of nerve differentiation along the body column of *Hydra* is due to differences in nerve commitment in different body regions. Furthermore, the level of nerve commitment in head and foot tissue is sufficiently high to deplete stem cells in these regions as is observed.

INTRODUCTION

Interstitial stem cells in *Hydra* continuously give rise to differentiated nerves and nematocytes (see Bode and David (1978) for review). Averaged over the whole animal, 30% of stem cell daughters enter the nematocyte pathway, 10% enter the nerve pathway, and 60% remain stem cells in each stem cell generation (David and Gierer, 1974). Stem cells are distributed uniformly throughout the gastric region but are notably depleted in the head and foot regions and immediately adjacent tissue (David and Plotnick, 1980). Nematocyte differentiation parallels the stem cell distribution and thus is localized in the gastric region (David and Challoner, 1974). By comparison, nerve differentiation is distributed rather uniformly along the body column (David and Gierer, 1974). In particular, extensive nerve differentiation occurs in both the head and foot regions despite the dramatically lower concentration of stem cells in these regions.

There appear to be two simple explanations for the high level of nerve differentiation in head and foot tissue: (1) the fraction of stem cells committed to nerve differentiation is higher in head and foot tissue than in gastric tissue thus compensating for the lower concentration of stem cells in these regions; (2) committed nerve precursors migrate from the body column into head and foot regions thereby increasing the rate of nerve differentiation in these regions over that expected from the low concentration of stem cells. Indeed, there are several reports in the literature of interstitial cell migration into heads and regenerating tips (Tardent and Morgenthaler, 1966; Herlands and Bode, 1974; Yaross and Bode, 1978).

These two possibilities can be distinguished by comparing the distribution of nerve precursors with the distribution of newly differentiated nerve cells. If migration occurs we expect these two distributions to differ; if enhanced nerve commitment is responsible for the high levels of nerve differentiation in head and foot, we expect nerve precursors and newly differentiated nerves to be distributed similarly.

Nerve precursors can be labeled with [³H]thymidine 18–30 hr prior to terminal differentiation (David and Gierer, 1974). We have, therefore, compared the distribution of labeled nerve precursors with the distribution of newly differentiated nerves. The results indicate no detectable redistribution of nerve precursors between late S phase and terminal differentiation. Since independent evidence (Berking, 1979; Venugopal and David, 1981b) indicates that nerve commitment occurs in mid- to late S phase, we conclude that the pattern of nerve differentiation in *Hydra* is due to an increase in the fraction of stem cells committed to nerve differentiation in head and foot tissue compared to gastric tissue.

MATERIAL AND METHODS

Hydra were cultured at 18–19°C in 10^{-3} M CaCl₂, 10^{-3} M NaHCO₃, and 10^{-5} M EDTA. Cultures were fed daily with *Artemia* nauplii and washed 6 hr later. Experimental animals were maintained in closed petri dishes at 20–21°C.

Budless *Hydra* selected from the culture 24 hr after the last feeding were used in all experiments. The animals contained about 8000 epithelial cells and about 40,000 total cells. Sizes of individual sections (expressed
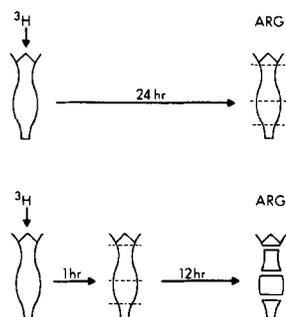
FIG. 1. Schematic representation of experimental protocol. See text for details. Top, control *Hydra*; bottom, sectioned *Hydra*.

in terms of epithelial cells) are given in the figure legends.

Counting nerves, stem cells, and epithelial cells in tissue sections. Tissue maceration (David, 1973) was used to identify and count individual cell types. Maceration dissociates *Hydra* tissue into single cells and small clusters of interstitial cells. Cells are fixed and retain their *in vivo* morphology in macerations; thus, they are easily counted by phase contrast microscopy.

The number of epithelial cells is a convenient measure of epithelial surface area or piece size. Thus we normalize all cell numbers to the number of epithelial cells in a sample in order to make comparison between experiments.

The detailed technique for preparing macerations and counting cells is described elsewhere (Sproull and David, 1979a; Venugopal and David, 1981a).

Labeling with [³H]thymidine. Hydra were labeled with [³H]thymidine (100 μCi/ml; 30Ci/mmole) by injecting 0.1 μl of isotope solution into the gastric cavity (David and Campbell, 1972). Under these conditions, all the isotope is taken up within about 45 min.

To score labeled nerve cells, tissue samples were macerated, dried on slides, and autoradiographed using Kodak AR10 stripping film.

RESULTS AND DISCUSSION

Distribution of Nerve Precursors and Newly Differentiated Nerves in Hydra

The experimental protocol is outlined schematically in Fig. 1. To determine the distribution of newly differentiated nerves, control *Hydra* were pulse-labeled with [³H]thymidine and incubated for 24 hr to permit differentiation of labeled nerves. The *Hydra* were then cut into sections as shown, macerated, and scored for labeled nerves by autoradiography. To determine the distribution of nerve precursors, *Hydra* were pulse-labeled as above and then cut into sections 1 hr later to prevent redistribution of the labeled precursors. The sections were then incubated for 12 hr to permit dif-

ferentiation of labeled nerves, macerated and scored, by autoradiography.

Nerve differentiation continues normally in isolated pieces of tissue (Venugopal and David, 1981a). However, the time lag from the end of S phase to nerve differentiation is shortened from about 18 hr in control *Hydra* to about 9 hr in isolated pieces (Venugopal and David, 1981b). Thus, in the protocol outlined in Fig. 1, nerve precursors labeled during the last 6 hr of S phase are scored in control *Hydra*; precursors labeled during the last 4 hr of S phase are scored in sectioned *Hydra*. Isolated pieces can not be incubated longer since, after 12 hr, the differentiation of newly committed nerves induced by head and foot regeneration of the isolate begins (Venugopal and David, 1981a).

Results of two independent experiments are shown in Figs. 2A and 3A. Animals were cut into four or five sections as shown, processed according to the protocol in Fig. 1, and scored for labeled nerves cells (Ne*) and epithelial cells (Epi). The ratio of Ne*/Epi is plotted along the body column. We have plotted the results from control *Hydra* and sectioned *Hydra* on different ordinates in order to compensate for the larger number of labeled nerves in control *Hydra* (due to longer incubation time). The results indicate no difference in the distribution of Ne*/Epi between control *Hydra* and sec-
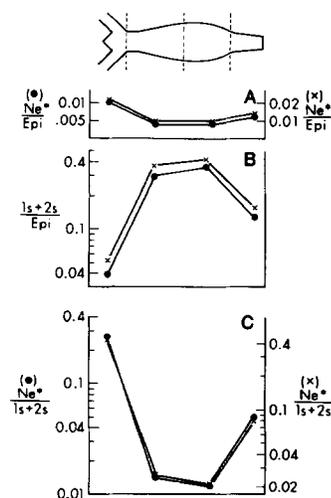

FIG. 2. Distribution of labeled nerves and stem cells along the body column. Control *Hydra* (x) or sectioned *Hydra* (●) were pulse-labeled with [³H]thymidine, incubated, and sampled as shown in Fig. 1. The numbers of labeled nerves (Ne*) and 1s+2s were normalized to the number of epithelial cells (Epi) in each section. Abscissa represents position along the body column in units of epithelial cells. It corresponds roughly to the *Hydra* outlined at the top. Average size of each piece starting from the head is: 1900, 2600, 2600, and 1000 epithelial cells. Each experimental value is positioned in the center of the piece on the abscissa. (A) Ne*/Epi, results of control and sectioned *Hydra* are plotted on different ordinates. (B) 1s+2s/Epi. (C) Ne*/1s+2s, results of control and sectioned *Hydra* are plotted on different ordinates.
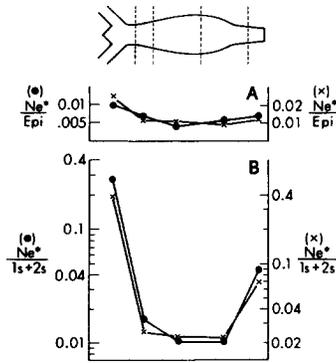
FIG. 3. Distribution of labeled nerves (Ne*) and the ratio of Ne*/1s+2s along the body column. Control *Hydra* (×) and sectioned *Hydra* (●) were labeled and sampled as in Fig. 2. Average size of each piece starting from the head is: 1800, 900, 2000, 2000, and 900 epithelial cells. (A) Ne*/Epi. (B) Ne*/1s+2s; note different ordinates for control and sectioned *Hydra*.

tioned *Hydra*. Thus, late S phase nerve precursors are distributed along the body column in a pattern which is identical to that of newly differentiated nerve cells. We conclude, therefore, that no redistribution of nerve precursors occurs after late S phase. If any redistribution occurs, it must be complete by mid-S phase of the precursor cell cycle.

It is important to note the limitations of our data in view of earlier reports of cell migration in *Hydra* (Tardent and Morgenthaler, 1966; Herlands and Bode, 1974; Yaross and Bode, 1978). Our experiment is not sensitive enough to detect low levels of migration. For example, if 10–20% of the Ne* scored in head and foot tissue were due to migration of nerve precursors from the body column, it would probably go undetected in our experiment against the large background of Ne* already present in head and foot tissue. Furthermore, our experiment only scores migration over a short time interval (12 hr) compared to the longer time intervals used in earlier studies. Thus, our results are not in disagreement with the interstitial cell migration that has been reported by others. Rather they suggest that most of that migration is probably due to stem cells rather than committed precursors (see below). Our results do, however, indicate that migration of committed precursors does not contribute significantly to the observed pattern of nerve differentiation in *Hydra*.

The Pattern of Nerve Commitment

In order to use the above results to determine whether the pattern of nerve differentiation along the body column of *Hydra* is due to a pattern of nerve commitment or to precursor migration, we need to know the timing of the commitment event in the precursor cell cycle. If nerve commitment occurs before mid-S

phase, then redistribution of committed cells could contribute to the observed pattern of Ne*/1s+2s. However, if commitment occurs in mid-S phase or later, then the observed pattern of Ne*/1s+2s can only be due to differences in the level of nerve commitment in different regions of the body column.

Three independent lines of evidence indicate that nerve commitment occurs in mid-S phase or later. (1) Regeneration induces extensive nerve differentiation starting 12 hr after cutting (Bode *et al.*, 1973; Venugopal and David, 1981a). Since this differentiation is induced by regeneration, nerve commitment cannot precede nerve differentiation by more than 12 hr. Hence nerve commitment can occur no earlier than late S phase of the precursor. (2) Berking (1979) has shown that nerve differentiation is affected by an inhibitory morphogen before but not after mid-S phase of the precursor cell cycle. After mid-S phase cells appear "committed" to nerve differentiation. (3) Finally, Venugopal and David (1981b) have used an assay for self differentiation to show that differentiation of nerve precursors becomes independent of exogenous signals, i.e., committed, at mid-S phase. Taken together these results localize nerve commitment to a point in mid- to late S phase of the precursor cell cycle. Thus, the nerve precursors whose distribution we measured in Figs. 2A and 3A were newly committed cells. The fact that these cells have the same distribution as newly differentiated nerves rules out any major contribution of cell migration to the pattern of nerve differentiation. The pattern must, therefore, be due to strong differences in the level of nerve commitment in different regions of the body column.

The fraction of stem cells committed to nerve differentiation can be estimated from the ratio of labeled nerves to stem cells (estimated as 1s+2s) (Sproull and David, 1979b). Figure 2B shows the distribution of 1s+2s along the body column. The results are similar to previous observations (Bode *et al.*, 1973; David and Plotnick, 1980). Figure 2C shows the ratio of Ne*/1s+2s calculated from the results in Figs. 2A and B. The ratio is strongly patterned: in head and foot tissue the ratio is 10- to 20-fold higher than in gastric tissue. Because the gastric region was not further subdivided in the experiment shown in Fig. 2, it is possible that the drop in Ne*/1s+2s is not as steep as the results in Fig. 2C indicate. To test this we examined, in a second experiment, a small piece of tissue at the distal end of the gastric region next to the head. The results in Fig. 3B indicate that, also in this case, the drop in Ne*/1s+2s is almost 15-fold. Thus the transition from a low to high rate of nerve commitment appears to occur over a very short distance.

From the Ne*/1s+2s ratio in Figs. 2C and 3B it is

possible to estimate that the fraction of stem cells undergoing nerve commitment in head and foot regions is >0.7 compared to <0.05 in the gastric region (Sproull and David, 1979b). Under these conditions the stem cell population in head and foot tissue declines with every cell generation. Thus, nerve commitment localized in head and foot tissue may be responsible for the observed depletion of stem cells in these regions (David and Plotnick, 1980). The small stem cell population which continues to exist in the head and foot is probably carried into these regions by slow distal and proximal movement of tissue from the gastric region (Campbell, 1967).

The pattern of nerve commitment is qualitatively similar to the pattern of morphogenetic potential in *Hydra*. Nerve commitment is high in head and foot regions which are morphogenetically dominant and low in gastric tissue which is morphogenetically neutral. This parallel also extends to changes in morphogenetic potential during head regeneration (Venugopal and David, 1981a). Thus it appears likely that nerve commitment is controlled by signals which are closely related to the signals which control morphogenesis in *Hydra*.

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