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BRIEF NOTES
Nerve Commitment in *Hydra*
II. Localization of Commitment in S Phase

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The kinetics of nerve differentiation were investigated during head regeneration in *Hydra*. In particular the cell cycle parameters of stem cells undergoing nerve commitment were determined. Head regeneration induces extensive nerve commitment localized at the regenerating tip (G. Venugopal and C. David, 1981, *Develop. Biol.* 83, 353-360). The appearance of committed nerve precursors is followed 12 hr later by the appearance of newly differentiated nerves. Under these conditions the time from the end of S phase to nerve differentiation is about 9 hr and the time from the beginning of S phase to nerve differentiation is about 18 hr. Thus nerve commitment occurs in mid- to late S phase of the stem cell precursor.

INTRODUCTION

Nerve cells in *Hydra* differentiate from a population of uncommitted interstitial stem cells. Commitment of individual stem cells to the nerve pathway appears to be controlled by the same signals which control tissue morphogenesis and regeneration. These signals change dramatically at the site of regeneration (MacWilliams, 1981a,b) and lead to dramatic increases in the fraction of stem cells differentiating nerves (Bode *et al.*, 1973; Yaross and Bode, 1978; Venugopal and David, 1981). In the present report we have used the large change in nerve commitment induced by regeneration to investigate the timing of commitment in the precursor cell cycle. Our results indicate that nerve commitment occurs in mid- to late S phase of the stem cell precursor.

MATERIALS AND METHODS

All materials and methods are described in the accompanying report (Venugopal and David, 1981).

RESULTS

1. Time Lag between Nerve Commitment and Nerve Differentiation

Nerve differentiation increases markedly at the site of head regeneration beginning 12 hr after cutting (Bode *et al.*, 1973). This effect is shown in Fig. 1. Budless *Hydra* were decapitated close to the tentacle ring and allowed to regenerate. At various times, regenerating tips were macerated and the ratio of nerves to epithelial cells (Ne/Epi) was determined. Beginning at 12 hr of regeneration there is an increase in the Ne/Epi ratio; between 12 and 24 hr the ratio doubles from 0.06 to 0.12. Since regenerating tips contain about 1600 epithelial cells, this increase represents the differentiation of about 90 new nerves at the site of regeneration.

The increase in nerve differentiation is preceded by an increase in committed nerve precursors at the site of regeneration. The kinetics of appearance of these precursors is described in the accompanying report (Venugopal and David, 1981) and is included schematically in Fig. 1 for comparison. The appearance of committed nerve precursors parallels the appearance of differentiated nerves. From the time lag between the two curves we conclude that nerve commitment precedes terminal differentiation by about 12 hr.

2. Localization of S/G2 Boundary

The kinetics of nerve differentiation in normal *Hydra* have been described (Campbell and David, 1974; David and Gierer, 1974). Under nonregenerating conditions the nerve precursor has a cell cycle of about 24 hr consisting primarily of S phase (12 hr) and G2 (12 hr). Following mitosis, both daughter cells differentiate as nerves in about 6 hr. Under these conditions the time lag between [^{3}H]thymidine labeling of the precursor and the appearance of newly differentiated labeled nerves is about 18 hr.

If the kinetics of nerve differentiation were similar in regenerating tips and normal animals, then the observed 12-hr lag between nerve commitment and differentiation would place the commitment event itself in the G2 phase of the precursor. Such a conclusion, however, does not agree with the observation that treatment with hydroxyurea blocks nerve commitment during regeneration (Venugopal and David, 1981). This latter result suggests that commitment occurs in S phase

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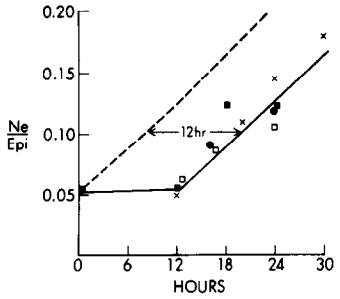

FIG. 1. Appearance of newly differentiated nerve cells in regenerating tips. Budless *Hydra* were decapitated and allowed to regenerate. At the times indicated, the regenerating tips were excised, macerated, and scored for nerves and epithelial cells. The tip fragments contain about 1600 epithelial cells. Different symbols indicate independent experiments. The dotted line indicates the appearance of committed nerve precursors in regenerating tips (from Venugopal and David (1981), Fig. 2).

of precursor. To resolve this discrepancy we have investigated the kinetics of nerve differentiation in regenerating tissue.

Budless *Hydra* were pulse-labeled with [^{3}H]thymidine. One hour after labeling, heads were removed from half the animals to initiate regeneration. Regenerates and whole animal controls were incubated and samples were macerated at 12, 18, 24, and 36 hr for determination of labeled nerve cells. The results are shown in Fig. 2. Labeled nerve cells began to appear in control animals at 18 hr and increased to 7% by 24 hr with kinetics very similar to previous reports (David and Gierer, 1974; Berking, 1979). By comparison, labeled nerves began to appear at about 9 hr in regenerating pieces and increased more rapidly than in the controls to a value of 25% by 24 hr. The results indicate that the time from the end of the S phase to terminal differentiation is shortened from 18 hr in control animals to about 9 hr in regenerating tissue.

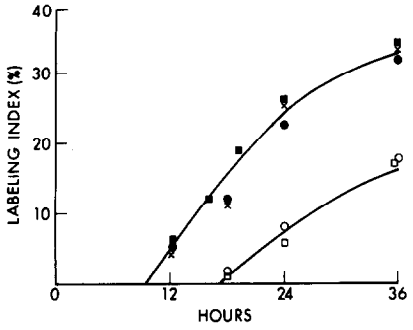

FIG. 2. Labeling index of nerve cells in regenerating and nonregenerating control *Hydra*. Animals were pulse-labeled at time 0 with [^{3}H]thymidine. Regenerating tips and equivalent pieces from nonregenerating control *Hydra* were macerated at the times indicated and scored for labeled nerve cells by autoradiography. (×) tip from regenerating animal; (●, ■) distal one-fifth of gastric region regenerating as isolated pieces; (○, □) distal quarter of gastric region from nonregenerating control *Hydra*.

By combining the results in Figs. 1 and 2 it is possible to locate the S/G2 boundary of the nerve precursor population during regeneration. This boundary must parallel the appearance of new nerves in Fig. 1 but occurs 9 hr earlier. Since the lag between nerve commitment and nerve differentiation is 12 hr (Fig. 1), it is clear that commitment occurs before the S/G2 boundary of the precursor. Thus nerve commitment induced by regeneration is expected to be sensitive to hydroxyurea treatment as was observed (Venugopal and David, 1981).

Figure 2 also shows the kinetics of nerve differentiation in small isolated pieces of tissue regenerating both head and foot. A small region of the body column was isolated from *Hydra* pulse labeled with [^{3}H]thydmidine. The isolates were incubated and samples were macerated at various times to determine labeled nerve cells. The results in Fig. 2 indicate that the kinetics of nerve differentiation in isolated pieces is identical to the kinetics in regenerating tips; the time lag between the end of S phase and nerve differentiation is 9 hr in both cases.

3. Localization of the G1/S Boundary

In order to localize the G1/S boundary of the precursor population which is committed to nerve differentiation as a result of regeneration we have used the [^{3}H]thymidine-labeling technique outlined schematically in Fig. 3. Regenerating tissue was exposed to [^{3}H]thymidine for increasing lengths of time beginning 1 hr before and extending up to 11 hr after the start of regeneration. Although the [^{3}H]thymidine was given

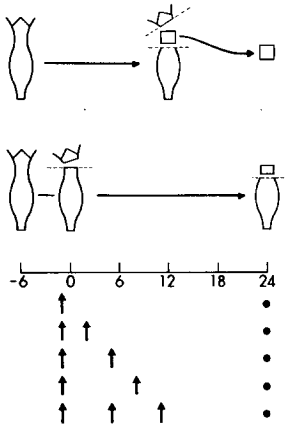

FIG. 3. Procedure for determining the G1/S boundary of nerve precursor population during regeneration (see text). Regenerating and nonregenerating control *Hydra* were labeled with [^{3}H]thymidine (↑) at times indicated. Regenerating tips or equivalent pieces from control *Hydra* were macerated at 24 hr (●) and scored for labeled nerve cells by autoradiography.

in pulses, the procedure amounts to continuous labeling over the interval between the pulses since all precursors which enter S phase during that period will be labeled. After 24 hr of regeneration the regenerating tips were excised, macerated, and scored for labeled nerves by autoradiography.

In the procedure outlined in Fig. 3 nerve precursors which are in S phase at the time of the first pulse or which enter S phase during the labeling interval are labeled; precursors which enter S phase after the labeling interval are not labeled. As the labeling interval is extended to progressively later times successive groups of nerve precursors enter S phase and become labeled. Thus the increase in labeled nerves at 24 hr is a measure of the time at which successive groups of nerve precursors enter S phase, i.e., the G1/S boundary of the precursor population.

Figure 4A shows the results of the experiment outlined in Fig. 3. The number of labeled nerve cells is plotted at the time the labeling period ended. A single [^{3}H]thymidine pulse given at −1 hr labeled only a portion of the newly differentiated nerves at 24 hr; as the labeling period was lengthened, the number of labeled nerves increased from 0.02 to 0.07 Ne*/Epi. Since regenerating tips contain about 0.12 Ne/Epi at 24 hr, of which about 0.07 are newly differentiated (Fig. 1), the results indicate that continuous labeling from −1 to +11 hr of regeneration labels all the precursors to newly differentiated nerves.

A striking feature of the results is the significant number of nerves labeled by a single pulse given before regeneration started (−1 hr). This suggests that precursors which are already in S phase can differentiate nerves in response to regeneration. However, to be certain of this point we need to exclude the possibility that

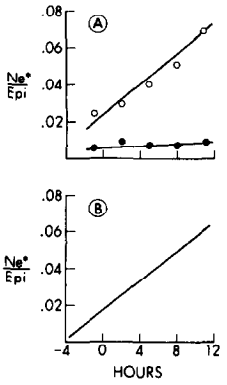

FIG. 4. Appearance of labeled nerve cells in regenerating (○) and nonregenerating (●) *Hydra* labeled with [^{3}H]thymidine according to protocol outlined in Fig. 3. All samples were macerated at 24 hr and scored for labeled nerves. Results are plotted at the time the animals were last labeled. Regeneration started at 0 hr. (A) Ne*/Epi, experimental data. (B) Difference between Ne*/Epi curves.

the −1-hr labeling is due to nerve differentiation occurring as a result of normal tissue expansion. We therefore examined nerve differentiation in nonregenerating control tissue. We labeled control *Hydra* in parallel with regenerating animals (Fig. 3). Pieces of tissue from the gastric region equivalent to the regenerating tip were excised from control animals at 12 hr, incubated as isolated pieces and macerated at 24 hr in parallel with samples from regenerating *Hydra*. The 12-hr period of isolation serves to make the differentiation kinetics of nerve cells the same in both controls and regenerating tips (Fig. 2).

The results in Fig. 4 indicate a slow increase in labeled nerves in nonregenerating tissue compared to the rapid increase in regenerating tips. At all the points from −1 to +11 hr, the number of labeled nerves in the controls was less than in regenerating tips. Subtracting the control curve from the regenerating curve (Fig. 4B) yields the G1/S boundary for successive groups of nerve precursors which differentiate specifically as a result of the regeneration stimulus. Even after subtracting nerve differentiation in the controls, it is clear that the G1/S boundary of some nerve precursors occurs before the beginning of regeneration. This clearly shows that precursors which entered S phase before regeneration started can still undergo nerve commitment. Thus nerve commitment must occur after the beginning of S phase. Since the G1/S boundary extrapolates back to −4 hr, the results suggest that nerve commitment occurs, at the earliest, after 4 hr of S phase.

DISCUSSION

Following removal of a *Hydra's* head, tissue at the cut surface regenerates a new head. Although complete regeneration requires 2–3 days, changes occur in the cell composition of the tissue much earlier (Bode *et al.*, 1973). One of the earliest and most dramatic cellular changes is the appearance of newly differentiated nerves in the regenerating tip starting about 12 hr after cutting (Fig. 1). This increase is preceded by an increase in committed nerve precursors which occurs about 12 hr earlier (Venugopal and David, 1981). Since the increase in nerve commitment is blocked by hydroxyurea (Venugopal and David, 1981) it appears likely that nerve commitment occurs in S phase of the nerve precursor.

In the present experiments we have localized the time of nerve commitment more precisely within the cell cycle of the precursor. We have performed two experiments: one to localize the G1/S boundary of the nerve precursor and one to determine the S/G2 boundary. The results of both experiments are summarized graphically in Fig. 5. The G1/S boundary for cells undergoing nerve commitment during 0–12 hr of regeneration lies about

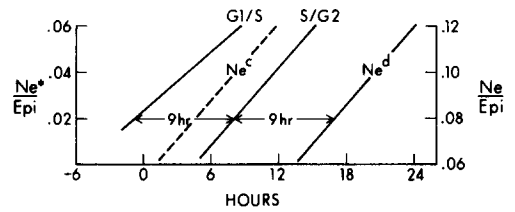

FIG. 5. Schematic representation of cell cycle of nerve precursor during regeneration. Data were derived from this report and the accompanying report (Venugopal and David, 1981). Ne^d, Appearance of new nerves during head regeneration (Fig. 1); Ne^c, appearance of committed nerve precursors during head regeneration (Venugopal and David, 1981); G1/S boundary (Fig. 4); S/G2 boundary derived by subtracting 9-hr nerve differentiation time (Fig. 2) from Ne^d curve (Fig. 1). Ordinates are displaced relative to each other to account for the background of unlabeled nerves (0.06 Ne/Epi) present in tissue before the experiment. Abscissa: hours of regeneration.

18 hr before terminal differentiation. The S/G2 boundary lies about 9 hr before terminal differentiation. Nerve commitment assayed by the isolation technique (Venugopal and David, 1981) increases between the boundaries of the S phase and indicates that commitment occurs approximately in mid-S phase.

Localization of commitment in mid-S agrees well with other results on *Hydra* stem cells. Berking (1979) investigated the differentiation of nerves induced by a feeding stimulus. He showed that a small well-defined cohort of stem cells differentiated nerves in response to a feeding stimulus and that this cohort of cells was in mid-S phase at the time of feeding. Since he was able to argue that the differentiation signal was probably of short duration and confined to a period close to feeding itself, Berking's results support the conclusion that an event signaling nerve differentiation occurs in mid-S phase. In addition Berking was able to show that treatment of *Hydra* with low concentrations of an endogenous inhibitor of *Hydra* morphogenesis (Berking, 1977) would inhibit nerve differentiation induced by feeding when added at the time of feeding or up to 4 hr later. Thereafter the differentiation of nerve precursors became insensitive to inhibition. On the basis of these observations Berking concluded that stem cells could respond to signals for nerve differentiation up to the middle of S phase but that thereafter they were insensitive to exogenous signals and therefore "committed" to nerve differentiation. It is interesting that our localization of commitment based on the isolation technique agrees with Berking's localization based on loss of inhibitor sensitivity.

In addition, it is important to note that M. Yaross, B. Baca, and H. Bode, (personal communication) have also localized commitment to S phase using an approach formally similar to ours but differing in experimental techniques. The similarity of the results from all three experiments provides a strong basis for the conclusion that nerve commitment from multipotent stem cells in *Hydra* occurs in the S phase.

Shortening of the Cell Cycle

The present experiments confirm earlier observations (Schaller, 1976a,b) that the regeneration environment dramatically alters the cell-cycle kinetics of nerve precursors. In well-fed nonregenerating *Hydra*, the lag between pulse labeling with [^{3}H]thymidine and appearance of labeled nerve cells is about 18 hr (David and Gierer, 1974). During regeneration, this lag is shortened from 18 to about 9 hr (Fig. 2). The lag consists of G2 and a period of postmitotic differentiation. Since the G2 phase of the interstitial cell cycle has been shown to vary in length (Campbell and David, 1974), it appears likely that shortening of the lag is due to shortening in G2. This possibility is supported by observations that a factor released during head regeneration stimulates interstitial cell mitosis and thereby effectively shortens G2 of the interstitial cell population (Schaller, 1976a). Further experiments showed that treatment with the factor shortened the lag between [^{3}H]thymidine labeling and the appearance of labeled nerve cells (Schaller, 1976b).

In addition to a shortened G2, the present results suggest that S phase may also be shortened during regeneration. The length of S phase based on results in Fig. 5 is about 9 hr compared to estimates of 12 hr in well-fed nonregenerating *Hydra* (David and Campbell, 1972; Campbell and David, 1974). However, because the shortening is small and the errors in S-phase determinations are large, it is not possible at present to assess the significance of the difference.

Comparison with Other Systems

Some information is available about commitment events in other systems. In murine erythroleukemia cells (Harrison, 1976; Gusella *et al.*, 1976; Levenson *et al.*, 1980) and myoblasts (Nadel-Ginard, 1978), commitment involves a choice between differentiation versus further proliferation. Evidence now indicates that this choice is made in G1 and leads either to a further round of cell division or to differentiation. In both cases commitment involves only expression of an already programmed type of differentiation. Such cells do not appear capable of choosing alternate pathways of differentiation and thus the "commitment" event may be quite different from that undertaken by a multipotent stem cell such as that in *Hydra*. Thus, it may not be surprising that commitment of *Hydra* stem cells occurs in S phase while commitment of erythroleukemia cells or myoblasts occurs in G1.

At present it is not possible to assess the significance

of commitment in S phase. One may speculate, however, that commitment events involving a choice between differentiation pathways require significant rearrangement of chromatin structure or perhaps even transposition of genetic elements. The extended state of chromatin during the replication process might be more conducive to such modification than other phases of the cycle.

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