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Regulation of the self-renewal probability in *Hydra* stem cell clones

(differentiation/negative feedback model/computer simulation/interstitial cell)

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ABSTRACT *Hydra* interstitial stem cells continuously give rise to daughter stem cells as well as precursors for nerve and nematocyte differentiation. Growth of the stem cell population is controlled by the self-renewal probability (P_s): P_s is the fraction of stem cell daughters that remain stem cells in each generation. We have determined P_s for *Hydra* interstitial stem cells by using a novel technique based on the cell composition of clones. Stem cell clones were grown in aggregates of nitrogen mustard-inactivated *Hydra* tissue. They contain several hundred cells after 14 days of growth, including stem cells, differentiating nematocytes, and differentiating nerve cells. Clone size, size variability, and the ratio of differentiating cells to stem cells are sensitive measures of P_s. We have prepared standard curves relating these parameters to P_s, using computer simulations of clone growth. Comparison of the experimentally observed parameters of clones to these curves indicates that P_s decreases from 0.8 in 5- to 6-day clones to 0.6 in 10- to 12-day clones. The decrease in P_s coincides with the increase in clone size and suggests that P_s may be regulated by the density of stem cells in clones. Such a mechanism could be responsible for the observed homeostasis of stem cell populations *in vivo*.

Stem cells are cells that can proliferate in an undifferentiated state, as well as give rise to differentiated products. Experimental evidence suggests that the density of some stem cell populations is homeostatically regulated. Thus, if the hematopoietic stem cells of a mouse are destroyed by X-irradiation a few transplanted cells will proliferate to reestablish the stem cell population at its original density (1, 2). A similar population recovery is observed in *Hydra* after interstitial stem cells are depleted by treatment with hydroxyurea (3).

In *Hydra* it has been shown that the length of the cell cycle is essentially the same during the recovery phase as it is when the stem cell population is stable (3). This suggests that population recovery proceeds *via* a mechanism involving changes in the frequency with which stem cells give rise to daughter stem cells (the self-renewal probability, P_s). Specifically, it is attractive to suppose that the self-renewal probability is high at low stem cell density and lower in the "stationary state."

Both hematopoietic stem cells and *Hydra* interstitial stem cells form clones in tissue from which stem cells have been removed. Developing clones would seem an ideal system for studying density-dependent regulation of the self-renewal probability, because the number of stem cells present undergoes drastic changes during clone growth. No evidence has been found thus far, however, for changes in the self-renewal probability in hematopoietic clones in the spleen. Here we report evidence for density-dependent regulation of the self-renewal probability in *Hydra* interstitial cell clones. Our work also suggests that the self-renewal probability in hematopoietic clones is variable, contrary to prevailing views.

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METHODS

Cloning. Interstitial stem cells were cloned in aggregates of nitrogen mustard-treated *Hydra* tissue, using an input of 30–40 live cells per aggregate as previously described (4). Under these conditions less than 20% of the aggregates contain one clone and aggregates containing two or more clones are rare.

Analysis of Clones. The cell composition of clones was determined by the maceration technique (5). After 13–14 days of growth, aggregates containing clones were dissociated individually in 0.2 ml of maceration solution and fixed with formaldehyde, and the entire volume was dried on a 1-cm^2 patch on a microscope slide. After addition of a coverslip, the entire patch was scanned under phase contrast for interstitial cells.

Cell Generation Times. The generation time of interstitial cells was estimated from the [^{3}H]thymidine labeling kinetics of populations "continuously" exposed to [^{3}H]thymidine as previously described (6).

RESULTS

The *Hydra* Stem Cell System. Asexually growing *Hydra* each contain 3500 interstitial stem cells; this is about 4% of total cells (4, 7). These cells have a 27-hr cell cycle (6) and a population doubling time of 3.5 days, which coincides with the growth rate of *Hydra* tissue (8). Under these conditions of exponential growth, 60% of stem cell daughters remain stem cells ($P_s = 0.6$) and 40% differentiate.

The cell flow pattern of *Hydra* stem cells is shown in Fig. 1. Stem cells occur as single or paired interstitial cells. Following determination for nerve differentiation, stem cells complete the cell cycle, undergo mitosis, and differentiate nerve processes within about 6 hr (7). Nematocyte determination initiates a series of synchronous cell divisions that create nests of 4, 8, and 16 interstitial cells. Cells in such nests are interconnected by cytoplasmic bridges (9) and differentiate in synchrony. Accompanying nematocyte determination is a shortening of the stem cell cycle from 27 hr to 18 hr in nests of 4 and 8 cells and probably also in nests of 2 (6).

The maceration technique (5) for dissociating *Hydra* tissue permits morphological classification and quantitation of interstitial cells. Interstitial cells occur in macerations as single cells, in pairs, and in clusters of 4, 8, and 16 cells; we refer to these classes as 1s, 2s, 4s, etc. The class of large single and paired interstitial cells (Fig. 1) is a mixture of stem cells and precursors for nerve and nematocyte differentiation. We use the total number of cells in this class (1s + 2s) to estimate the stem cell pool. Cells in nests of 4 (4s) are exclusively nematocyte precursors (Fig. 1) and thus the ratio of 4s/(1s + 2s) can be used as an index of nematocyte differentiation.

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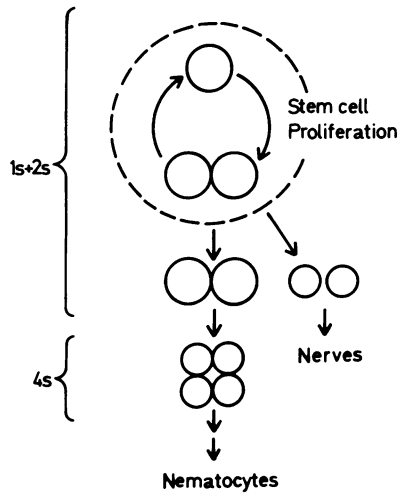

FIG. 1. Cell flow diagram for interstitial stem cells in *Hydra*, showing stem cell self-renewal and the two principal differentiation pathways to nerves and nematocytes. Brackets on left indicate cells that occur in macerations as 1s + 2s and 4s. See text for detailed description [modified from David and Gierer (7)].

Interstitial stem cells can be cloned using a modified feeder-layer technique in aggregates of nitrogen mustard-treated *Hydra* tissue (4). Clones increase in size rapidly and may contain several hundred cells after 12 to 14 days, including stem cells, differentiating nematoblasts, and differentiating nerve cells. A notable property of *Hydra* stem cell clones is their relative similarity in size. We have suggested elsewhere (4) that this may be due to feedback regulation of the self-renewal probability of stem cells. In the present experiments we have used a simple procedure based on measurements of the cell composition of clones to investigate changes in the value of P_s during the growth of clones.

Cell Composition of Stem Cell Clones. The value of the self-renewal probability strongly influences the cell composition of clones. In particular, the clone size, the size variability, and the proportion of differentiating cells in clones vary with changes in P_s. For example, if the P_s were 1.0, then clones would contain 2^n cells after n generations; all clones would be of identical size (i.e., no size variability) and no differentiated cells would be present. At lower values of P_s, clones would contain fewer than 2^n cells; statistical fluctuations would ensure that individual clones differed in size; and differentiated cells would be present. Thus, the value of P_s in clones can be determined by comparing the observed characteristics ofinterstitial cell clones with the results of computer simulations.

Single clones were grown in nitrogen mustard-treated host aggregates, macerated after 14 days, and scored quantitatively for their content of interstitial cells (1s, 2s, and 4s). The cell compositions of clones from two independent experiments are recorded in Table 1. The average number of 1s + 2s in 10 clones in experiment I was 142; in 7 clones in experiment II, 221. The variation in clone size in each experiment was relatively small, yielding coefficients of variation of about 0.5. In both experiments there was extensive nematocyte differentiation, the ratio of 4s/(1s + 2s) varying from 0.2 to 0.8.

Cell Cycle of Interstitial Cells in Clones. In order to use the data in Table 1 for determining P_s, the generation times of 1s, 2s, and 4s must be known. We have determined these parameters using a simple "continuous" labeling technique in which the duration of the cell cycle is estimated from the kinetics of labeling of a population "continuously" exposed to [^{3}H]thymidine. The procedure has been shown to label all interstitial cells in whole *Hydra* and to yield an estimate of the cell cycle similar to estimates obtained by summing the individual phases (6).

Table 1. Cell composition of 13- and 14-day interstitial cell clones

Exp.	Clone	Total cells occurring as 1s + 2s	Total cells occurring as 4s	4s/(1s + 2s)
Exp. I (13-day clones)	1	253	44	0.17
	2	205	80	0.39
	3	176	36	0.20
	4	30	8	0.27
	5	45	16	0.36
	6	90	40	0.44
	7	166	128	0.77
	8	126	44	0.35
	9	159	62	0.39
	10	191	84	0.44
Average		142	53	0.38
Exp. II (14-day clones)	1	141	56	0.40
	2	302	188	0.62
	3	202	112	0.55
	4	122	100	0.82
	5	164	60	0.37
	6	172	92	0.53
	7	447	248	0.55
Average		221	122	0.55

Clones were prepared and analyzed for interstitial cells as described in *Methods*. The number of interstitial cells in clusters of 1 and 2 (1s + 2s) and in clusters of 4 (4s) was recorded. The values in the table refer to the number of cells in each category. In Exp. I, 10 clones were found in a total of 52 aggregates; in Exp. II, 7 clones in 46 aggregates.

Fig. 2 shows the results of "continuous" labeling of clones

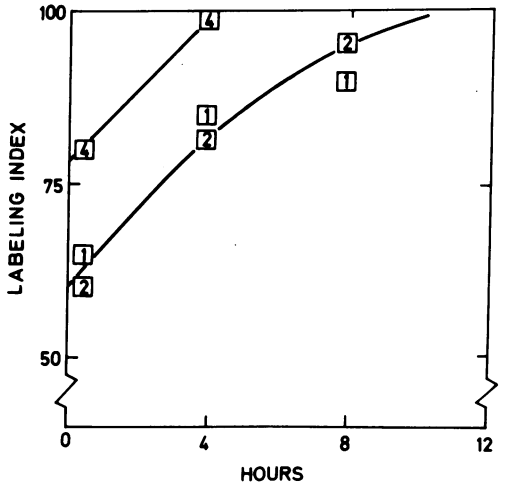

FIG. 2. "Continuous" [^{3}H]thymidine labeling of interstitial cells in clones. Nitrogen mustard aggregates containing an average of 5 clones each were labeled by injecting [^{3}H]thymidine (0.01 μCi in 0.1 μl; 6 Ci/mmol) into the central cavity at t_0 and at 1 hr prior to sampling. Aggregates incubated for long periods (see Table 2) were reinjected every 12 hr. This labeling schedule catches all cells once in their cell cycle, because the S phase of interstitial cells is about 12 hr (6). Five aggregates were macerated for each time point and the labeling index of the 1s, 2s, and 4s was determined following autoradiography. The results shown are for day 13 of clone growth.

Table 2. Estimated generation time of interstitial cells in clones

Clone growth, days	Generation time, hr	
	1s + 2s	4s
0–4	24*	—
7	24†	19†
9	24†	17†
13	22†	16†
Standard *Hydra* (6)	27‡	17

* Cell cycle of 1s + 2s determined by direct count of increase in cell number in very early clones (data derived from figure 4 in ref. 4).
† Generation time was estimated from continuous labeling experiments (see Fig. 2) in which the time to reach 100% labeling is equal to the generation time minus S phase. S was assumed to be 12 hr (6).
‡ Cell cycle of 1s + 2s, which are probably stem cells.

starting on day 13 of clone growth. Fours are ~80% labeled at time t_0 and completely labeled by 4 hr, commensurate with their short cell cycle, of which a major part is S phase. Fifty to 60% of 1s + 2s are labeled at t_0 and >90% by 8 hr. Similar experiments performed at 7 and 9 days of clone growth yielded results essentially identical to Fig. 2. The cell generation times have been estimated from these labeling results as the time to reach 100% labeling plus 12 hr for the S phase (6). These results are summarized in Table 2. An estimate of the generation time during the first 4 days of clone growth has been obtained directly from the growth rate of the population of 1s + 2s in clones (see figure 4 in ref. 4). The results in Table 2 demonstrate that the cell generation time of interstitial cells is very similar in clones and in normal *Hydra*. In addition, the results demonstrate that, at least up to 13 days, there is no significant change in cell generation time during clone growth.

Procedures for Determining P_s in Clones. We have developed computer programs to simulate clone development, based on the cell flow diagram in Fig. 1 and the cell cycle results in Table 2. The computer begins each simulated clone with one stem cell. To create the next generation, the computer generates a random number between 0 and 1. If the number falls between 0 and P_s, the stem cell "divides" to give two stem cells. If the number is between P_s and 1.0, the stem cell "differentiates." A second random number call determines whether the cell enters the nematocyte or nerve pathway. The computer repeats this process for each stem cell to create the succeeding generation. After each generation the number of stem cells and the number of each type of differentiated cell are recorded. Corrections are applied to this data for differences in cell cycle time. The computer reiterates this process for 10^3–10^4 clones, and finally calculates the average number of 1s + 2s per clone, the coefficient of variation of this number, and the average ratio of 4s/(1s + 2s). By repeating the simulations using different values for P_s between 0.5 and 1.0, we gave generated standard curves relating P_s to these three parameters (Fig. 3). Computer simulations using models in which stem cells can also give rise to one stem cell and one differentiated cell (7) give essentially the same results.

The reliability of these procedures for estimating P_s can be assessed by comparing them to normal animals for which the value of P_s has been independently determined. The ratio of 4s/(1s + 2s) in standard *Hydra* is 0.4, yielding an estimate of P_s of 0.6, in good agreement with previous estimates (7). Similarly, the observed doubling time of the population of 1s + 2s in normal *Hydra* is 3.5 days, yielding an estimate of P_s of 0.6 in Fig. 3, again in good agreement with previous estimates. Thus, the computer simulation procedures utilized here for

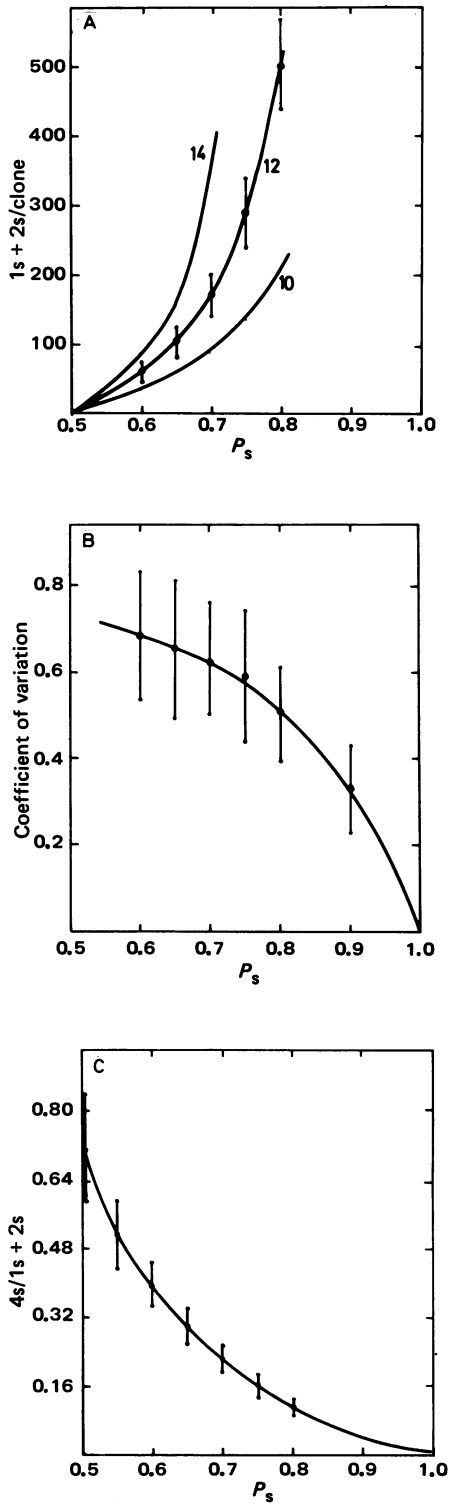

FIG. 3. Standard curves relating P_s to the total number of 1s + 2s per clone (*A*), the coefficient of variation of 1s + 2s per clone (*B*), and the ratio of 4s/(1s + 2s) (*C*). A computer was programmed to simulate clone growth, using the cell flow model in Fig. 1 (see text). Stem cells were assumed to have a 27-hr cell cycle; differentiating 2s and 4s were assumed to have an 18-hr cell cycle. At least 10^3 clones were simulated at each value of P_s. Clones were "grown" for 12 generations except in *A*, where results for 10, 12, and 14 generations are shown. To estimate the range of results expected experimentally, "10-clone" experiments were simulated: clones were generated in groups of 10 and then the three parameters were calculated. The error bars indicate the standard error for 100 such "10-clone" experiments at each value of P_s.

Table 3. Calculation of P_s in interstitial cell clones

Exp.	Total 1s + 2s/clone	4s/(1s + 2s)	Coefficient of variation of 1s + 2s/clone
Exp. I (10 clones)	142 $P_s = 0.70$	0.38 $P_s = 0.61$	0.49 $P_s = 0.81$
Exp. II (7 clones)	221 $P_s = 0.71$	0.55 $P_s = 0.54$	0.54 $P_s = 0.78$
David and Murphy (4) (9 clones)	ND	0.44 $P_s = 0.58$	0.45 $P_s = 0.84$

Three parameters of stem cell clones are summarized from the results in Table 1. Results from an additional set of clones scored by David and Murphy (4) are included for comparison. The total number of 1s + 2s is not included for the latter data (ND) because a washing step involved in preparation of these clones for autoradiography causes uncontrolled cell loss from slides. Values of P_s were estimated for each experimental result by using the standard curves in Fig. 3. A stem cell generation time of 27 hr (6) has been used in the calculations.

clones yield correct estimates when applied to normal animals.

Only 7–10 clones were examined in each of our actual experiments. Due to this limited sample size, the average values of the clone size, the coefficient ofvariation, and the 4s/(1s + 2s) that we measured experimentally are expected to contain statistical errors. To determine the magnitude of these errors, we have simulated "10-clone experiments" on the computer directly. The variations we encountered in these simulations are plotted as the standard deviations in Fig. 3. These simulated errors indicate graphically the range of values within which the results from single experiments may fall and suggest the accuracy of the estimates of P_s.

If the value of P_s is constant during the growth of a clone, then estimates of P_s based on the three parameters displayed in Fig. 3 will be identical. If, on the other hand, P_s varies during clone development, then the three estimates may not be identical, because they measure P_s at different times during clone growth. The size variation among clones is primarily generated when clones are small and subject to statistical fluctuations in the number of stem cells. For example, we have found, in further simulations, that shifting P_s from 0.8 to 0.6 after the sixth generation does not increase the variation among clones over that observed when P_s remains constant at 0.8, whereas shifting P_s at earlier generations does significantly increase this variation. Thus, the coefficient of variation measures P_s in early clones. By comparison, the ratio of 4s/(1s + 2s) estimates P_s at the time when the precursors to the 4s were determined to leave the stem cell pool. Because there are 2 generations between a determined stem cell and a nest of 4 nematocyte precursors (Fig. 1), this ratio estimates P_s 2 days prior to the moment at which the 4s are scored, or after 10 generations in our experiments. The total clone size is affected by the value of P_s throughout the clone growth and the clone size thus yields an estimate of P_s that is an "average" of all the values that P_s has assumed.

Calculation of P_s in Clones. Table 3 summarizes the parameters of the two sets of clones listed in Table 1 and another set scored independently by David and Murphy (4). Below each experimental value in Table 3 is an estimate of P_s obtained by using the standard curves in Fig. 3. The experimental results for the three sets of clones are in good agreement, indicating the reproducibility of the cloning procedure. However, the values of p_s differ, depending on which parameter of the clones is used to make the estimate. This strongly suggests that the value of P_s is not constant during clone growth. In particular, the coefficient of variation suggests that P_s is 0.8 in young clones, the clone size procedure estimates a value of 0.7 for the "average" of P_s during clone growth, and the ratio of 4s/(1s + 2s) suggests that P_s is 0.6 in old clones.

Our data permit us to reject the hypothesis that P_s is constant during clone growth with either of two independent statistical tests: (*i*) All our measurements show a consistent trend of decreasing P_s during clone growth. The probability of obtaining this result by chance, given constant P_s, is less than 0.015. (*ii*) A search was performed to find an "optimum" value of P_s that gives the best fit to all experimental measurements. Goodness of fit was measured by the sum of squared deviations between the optimum value and the experimental measurements; the deviations were measured in units equal to the errors given in Fig. 3. If P_s were constant, this goodness of fit for the optimum value would be expected to have a χ^2 distribution with 7 degrees of freedom. The actual value obtained was 57.3, which when compared to the χ^2 distribution gives $P < 0.001$.

The validity of these tests depends on the assumption that our measurements are not strongly influenced by systematic errors; we feel that this assumption is justified. Our standard curves (Fig. 3) are steep precisely in the range where they are being used to estimate P_s; this minimizes the effect of experimental errors. Our standard curves for clone size and for 4s/(1s + 2s), furthermore, give values for normal *Hydra* that are in good agreement with previous, independent estimates, as mentioned above.

Our coefficient of variation measure could, in principle, be affected by poor technique, because any departure from uniformity in the handling of clones would tend to increase its value. Furthermore, variations in the stem cell cycle time would also increase the coefficient of variation by causing differences in the number of generations in clones at the time of sampling. If, however, our measured values of the coefficient of variation are too high, this only strengthens our contention that P_s changes during the development of stem clones in *Hydra*.

Estimates of P_s based on clone size (Fig. 3A) are affected by errors in the determination of the stem cell generation time. Nevertheless, such errors, which are unlikely to be greater than ±3 hr, cannot remove the discrepancy among the three estimates of P_s. For example, lengthening the stem cell generation from 27 to 34 hr reduces the age of clones from 12 to 10 generations and thus increases the estimate of P_s from 0.7 to 0.8 (Fig. 3A). However, the estimate of P_s based on 4s/1s + 2s becomes even lower when the stem generation is lengthened and thus the difference in P_s between early and late clones remains. A similar argument applies to shortening the stem cell generation time.

A final possible source of error involves the assumption, in the computer simulations, that differentiating stem cells are partitioned 3:1 to the nematocyte and nerve pathways. We justify this assumption on the basis of the results of David and Murphy (4), who observed in a similar cloning experiment (see table 3 in ref. 4) nerve differentiation at a rate comparable to normal tissue. Furthermore, even assumptions of zero nerve differentiation in the present experiments would only raise the estimates of P_s from 0.6 to 0.66, which is still significantly lower than 0.8 observed early in clone development.

DISCUSSION

Self-Renewal Probability during Clone Growth. The present experiments demonstrate that the self-renewal proba-

bility decreases during the growth of interstitial stem cell clones from a value of 0.8 during the first 6 generations to a value of 0.6 by 10 generations. This result could be due to the increase in interstitial cell density during clone growth or to a change in the nitrogen mustard-treated host tissue due to starvation or aging during the experiment. The latter possibility can be tested experimentally. The apparent cloning efficiency of stem cells is directly related to P_s: decreasing P_s increases the probability that a stem cell will "extinguish" by differentiating before founding a clone. Therefore, if the observed decrease in P_s were due to some property of host tissue, then the apparent cloning efficiency of stem cells should be lower in old than in young nitrogen mustard-treated hosts. The magnitude of this change would be about 2-fold (10). No such change in cloning efficiency has been observed in host tissue up to 7 days after nitrogen mustard treatment (4). Thus, it appears likely that the observed decrease in P_s is due to the increase in interstitial cell density during growth of the clone.

A Model for the Control of P_s. The cloning results suggest a model in which the value of P_s is regulated by negative feedback from the neighboring cells in the clone. The feedback signal appears likely to emanate from stem cells, because decreasing the number of stem cells increases the self-renewal probability. Such a signal would provide a mechanism to account for the observed homeostasis in stem cell concentration in *Hydra* tissue (3), as well as accounting for observed changes in P_s during clone growth. The idea that differentiating product cells are the source of the feedback signal in clones is unattractive, because a feedback system in which products depressed the self-renewal probability would not give rise to homeostasis. Hence, we favor the idea that stem cells are the source of the feedback signal.

The nature of the feedback signal cannot be directly deduced from the present results. Feedback could be due to a diffusible factor, secreted by stem cells, to which stem cells are also sensitive. In this case, the value of P_s in any given stem cell would be set by the ambient concentration of the factor. In the case of isolated stem cells with few or no stem cell neighbors, the factor concentration would be low and P_s would be high. Conversely, for a cell surrounded by numerous neighboring stem cells, the factor concentration would be high and P_s correspondingly lower. Alternatively, the feedback might be mediated by direct contact between neighboring stem cells similar to contact inhibition of cells in culture. The equilibrium concentration of stem cells in *Hydra* ectoderm is such that there are about 3500 stem cells in an epithelium containing 10,000 ectodermal cells (7). Because stem cells are smaller than ectodermal cells, it appears unlikely that stem cells are continuously in direct contact under conditions of normal growth. However, the epithelium undergoes frequent movement and contractions such that the stem cells, which are freely floating in spaces between ectodermal cells, may come in repeated contact with each other. A model in which the strength of feedback signal is mediated by the frequency of stem–stem contacts would, thus, be consistent with all the observed results.

Comparison of *Hydra* and Bone Marrow Stem Cells. The model proposed for *Hydra* stem cells, in which P_s can vary in clones, differs from the stochastic model proposed by Till *et al.* (11) for bone marrow stem cells in which the value of P_s was assumed to be constant, at least in the "quasi steady-state" of exponential clone growth. Because the *Hydra* and bone marrow systems have yielded different conclusions based on similar experimental procedures, we were prompted to reexamine the bone marrow experiments.

Till *et al.* (11) proposed the stochastic model in order to explain the large variation in stem cell content of bone marrow clones: the experimentally determined coefficients of variation for clones ranged from 1.3 to 2.2 (12). Our own calculation of clone variation (Fig. 3*B*) yielded maximum values for the coefficient of variation of 0.8 for the stochastic model, which are much lower than values of 2.0 obtained in computer simulations by Till *et al.* (11) and analytically by Vogel *et al.* (10). It appears to us that the latter authors arrived at a value of 2.0 by including extinguished clones (clones in which all stem cells differentiate) in the computed clone size variation; when we include extinguished clones in our calculations we also arrive at a value of 2. Because the extinguished clones cannot be observed experimentally, however, and therefore are not included in the experimentally determined clone size variation, they should be deleted from the simulations used to determine P_s. When this is done it is clear that the variation in bone marrow clones is much larger than any variation that stochastic models could generate. Such variation could be generated if the spleen were heterogeneous and contained microenvironments characterized by different values of P_s (13). Whether there is, in addition, any form of feedback control of P_s for bone marrow stem cells cannot be determined from the present data, but would seem to merit investigation in light of the *Hydra* results.

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