Cell Cycle Length, Cell Size, and Proliferation Rate in Hydra Stem Cells

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We have analyzed the cell cycle parameters of interstitial cells in *Hydra oligactis*. Three subpopulations of cells with short, medium, and long cell cycles were identified. Short-cycle cells are stem cells; medium-cycle cells are precursors to nematocyte differentiation; long-cycle cells are precursors to gamete differentiation. We have also determined the effect of different cell densities on the population doubling time, cell cycle length, and cell size of interstitial cells. Our results indicate that decreasing the interstitial cell density from 0.35 to 0.1 interstitial cells/epithelial cell (1) shortens the population doubling time from 4 to 1.8 days, (2) increases the [3H]thymidine labeling index from 0.5 to 0.75 and shifts the nuclear DNA distribution from G2 to S phase cells, and (3) decreases the length of G2 in stem cells from 6 to 3 hr. The shortened cell cycle is correlated with a significant decrease in the size of interstitial stem cells. Coincident with the shortened cell cycle and increased growth rate there is an increase in stem cell self-renewal and a decrease in stem cell differentiation. © 1990 Academic Press, Inc.

INTRODUCTION

Interstitial stem cells in hydra are multipotent cells which continuously differentiate nerve cells, nematocytes, and gland cells in asexual animals (see Bode and David, 1978; David *et al.*, 1987 for reviews) and egg and sperm in sexual animals (Bosch and David, 1987). Stem cells are distributed uniformly along the body column in the ectoderm; differentiation of nerve cells, nematocytes, and gland cells, however, is strongly localized in specific regions of the animal.

The interstitial cell population grows at the same rate as the epithelial tissue in hydra so that, over many generations of asexual budding, daughter animals contain an essentially constant ratio of stem cells to epithelial cells. Experimental perturbation of this ratio leads to changes in the rate of stem cell proliferation and hence recovery of the typical level of stem cells in tissue (Bode *et al.*, 1977; Sproull and David, 1979). In *Hydra vulgaris* (formerly *Hydra attenuata*) the altered growth rate of the stem cell population is mediated by a change in the fraction of daughter cells per generation which remain stem cells (self-renewal probability, P₃) (Bode *et al.*, 1977; Sproull and David, 1979). To date there has been no evidence for a change in cell cycle parameters associated with altered growth rates.

In the course of experiments done with another species (*Hydra oligactis*), we have observed for the first time changes in the cell cycle parameters of interstitial cells associated with altered population growth rates. Our results indicate that the cycle is shortened under conditions of faster than normal stem cell proliferation. The shortening affects only stem cells and not differentiating products of the stem cell pool.

Shortening of the stem cell cycle is accompanied by a marked decrease in the average size of stem cells. As a result stem cells in hydra have different sizes depending on growth conditions and hence cell size is not a reliable indicator of stem cell function.

MATERIAL AND METHODS

Animal culture. All experiments were carried out using a male strain of *H. oligactis* originally collected from Lake Zürich in Switzerland in 1979 by Dr. Lynne Littlefield and kindly provided by Dr. Pierre Tardent 1983. The animals were cultured in M-solution, adjusted to pH 7.8 (Sugiyama and Fujisawa, 1977). Budless hydra were used in all experiments. Animals were selected from the culture 24 hr after the last feeding.

Identification of cell types by maceration. The cell composition and cell number in pieces of hydra tissue were determined in macerated preparations (David, 1973). Interstitial cells occur in macerations as single cells and in clusters of 2, 4, 8, and 16 cells. Cells in clusters of 4, 8, and 16 cells (referred to as 4s, 8s, 16s) are proliferating precursors in the nematocyte differentiation pathway ("nematoblasts").

For the experiments reported here 100-1000 cells or cell clusters were counted in a given sample. The concentration or "density" of interstitial cells in hydra tissue is expressed as the ratio of interstitial cells (1s + 2s) to epithelial cells (epi).
Production of monoclonal antibodies. Monoclonal antibodies were raised using a modification of the procedure by Oi and Herzenberg (1980) as described by Schmidt and David (1986). Monoclonal antibody IX8c was produced against H. oligactis. Supernatants from hybridoma cultures were used directly for antibody staining or diluted appropriately in PBS (phosphate-buffered saline).

Visualization of antibody binding. Using indirect immunofluorescence, binding of monoclonal antibodies to gamete precursors was visualized on macerated cells by staining with FITC-labeled goat anti mouse IgG/IgM as described elsewhere by Hobmayer et al. (1990).

Determination of cell size. The size of cells and nuclei was determined as described by Bosch and David (1984). The area of individual cells or nuclei was traced on a digitizing tablet and the areas were measured with a Leitz Dialux microscope equipped with a variable diaphragm which was adjusted to the size of large interstitial cell nuclei. The photometer was connected to a Kontron PSI 80 microcomputer which calculated areas and stored the data. The error in measurement was less than 0.1%.

Determination of nuclear DNA content. Macerated preparations of hydra cells were washed three times for 10 min in PBS buffer and stained overnight with the DNA-specific fluorochrome DAPI (0.5 μg/ml, Serva Heidelberg) (Leeman and Ruch, 1982) in McIlvaine’s citrate buffer (pH 7.0). The slides were washed briefly in PBS, covered with a drop of PBS-glycerin (1:3), and kept in the dark until measured. The fluorescence of nuclei was measured with a Leitz MPV microscope photometer fitted with a camera lucida and 40X phase objective. The digitizing tablet was connected to a Kontron PSI 80 microcomputer which calculated areas and stored the data.

RESULTS

Rapid Growth of Interstitial Cells at Lowered Cell Density in Hydra oligactis

To analyze the growth of interstitial cells at lowered cell densities we grafted small pieces of tissue from the gastric region of normal animals into the body column of nitrogen mustard (NM)-treated, interstitial cell-free animals. The animals were fed every day and growth was analyzed by counting the numbers of epithelial cells and interstitial cells (1s + 2s) daily. Pieces of grafted tissue contained about 1500 1s + 2s which corresponds to roughly one-fourth of the total number of 1s + 2s in a standard hydra.

In normal animals with an interstitial cell density of 0.35 1s + 2s/epi interstitial cells have a population doubling time of 4 days, which coincides with the growth rate of the surrounding epithelial tissue (Fig. 1A). Grafting of normal tissue into nitrogen mustard-treated animals lowers the interstitial cell density three- to fourfold and leads to an immediate increase in the interstitial cell growth rate (Fig. 1B). During the first 4 days after grafting, the interstitial cell population increased with a doubling time of 1.8 days compared to 4 days in control animals (Fig. 1A). As a result of this rapid growth, the interstitial cell population reached an essentially normal density of 0.30 1s + 2s/epi on Day 4 (Fig. 2A). From this point on, the growth rate of the interstitial cell population decreased significantly and paralleled the growth rate of the host tissue (Fig. 1B). The host tissue in these transplants has a doubling time of about 6 days which is significantly longer than normal control animals (Fig. 1A). This is due to the fact that the host tissue is a mixture of NM-treated epithelial cells, which are slowly dying, and untreated epithelial cells, which were introduced with the transplant and which are proliferating normally.

Changes in Nematocyte Differentiation during Rapid Growth

Previous experiments in Hydra vulgaris have shown that rapid growth of interstitial cells is accompanied by a decreased proportion of stem cells entering the nematocyte differentiation pathway (David and MacWil-
Fig. 1. Increase of epithelial cell number and interstitial cell (1s + 2s) number in normal animals (A) and in NM host animals (B). The animals were fed daily, macerated at the times indicated and scored for epithelial cells (▲) and interstitial cells (●). Each value is the average of 10 animals.

Williams, 1978; Sproull and David, 1979). To investigate nematocyte differentiation in our NM grafts we determined the number of nests of 4s (4s), the first cell type exclusively restricted to the nematocyte pathway (David and Gierer, 1974). Figure 2B shows that the ratio of 4s/1s + 2s decreased dramatically from 0.13 on Day 0 to 0.07 on Day 3. When the interstitial cell population recovered to normal density on Days 4 to 5 (Fig. 2A), the ratio of 4s/1s + 2s also began to increase and reached control values on Day 9. Thus, rapid growth of interstitial cells under conditions of reduced cell density in Hydra oligactis is correlated with a decrease in the proportion of interstitial cells entering the nematocyte pathway.

Proportion of Cells in S and G2 in Rapidly Proliferating Interstitial Cell Populations

To analyze the cell cycle parameters of rapidly proliferating interstitial cells we first determined the proportion of cells in S phase by pulse labeling with [3H]-thymidine. Figure 3 shows that the labeling index of interstitial cells in NM grafts increased from 0.50 at the

Fig. 2. Increase in the density of interstitial cells (1s + 2s/epi) (A) and changes in the rate of nematocyte differentiation (4s/1s + 2s) (B) under conditions of rapid growth in NM host animals. At the times indicated animals were macerated and scored for epithelial cells (epi), interstitial cells (1s + 2s), and nests of 4s (4s).

Fig. 3. [3H]thymidine pulse labeling index of interstitial cells (1s + 2s) growing in NM host animals. The data are from the same experiments shown in Fig. 2.
time of grafting to 0.75 day after grafting. The high labeling index persisted on Day 2 but then slowly decreased as the interstitial cell density increased (Fig. 2A) and the growth rate decreased (Fig. 1B). Thus, under conditions of rapid growth, 1.5-fold more interstitial cells are in S phase than in normal control animals.

Since proliferating hydra cells have no measurable G1 phase (David and Campbell, 1972, Campbell and David, 1974) the increased labeling index in Fig. 3 suggests that a smaller fraction of interstitial cells are in G2. To confirm this we determined the proportion of cells in S phase and G2 by measuring the nuclear DNA contents of interstitial cells under conditions of normal and rapid growth. Figure 4 shows the results of such an experiment. All values were normalized to the DNA content of nerve cells (Fig. 4A), which are postmitotic daughter cells with G1 DNA content (David and Gierer, 1974). Under conditions of normal growth, roughly half the interstitial cells had S phase and half had G2 DNA levels; very few cells had G1 DNA levels (Fig. 4B). Such a distribution is typical for interstitial cells in hydra and reflects the fact that these cells have a cell cycle consisting primarily of S and G2 (Campbell and David, 1974). By comparison, under conditions of rapid growth the proportion of cells with G2 DNA levels was decreased and the proportion with S phase DNA levels increased (Fig. 4C). Assuming that the duration of S phase does not change, these results suggest that the duration of G2 in rapidly proliferating interstitial cells is decreased.

Length of G2 in Rapidly Proliferating Interstitial Cells

Because hydra cells lack a G1 phase it is possible to determine the length of G2 by a simple continuous labeling experiment with [3H]thymidine (David and Campbell, 1972; Campbell and David, 1974). In such an experiment the time to 100% labeling is a measure of G2. Figure 5A shows the results of continuous labeling experiments for both normally and rapidly proliferating interstitial cells in H. oligactis. In both cases the continuous labeling curve is complex suggesting the existence of several populations with different G2 lengths.

In normally proliferating cells, three major populations can be identified which we refer to as short, medium, and long G2 cells. The length of the G2 for these populations was estimated from the times at which the labeling curve changed slope. In control animals short G2 cells had a G2 of 6 hr, medium G2 cells a G2 of 12 hr, and long G2 cells a G2 of >24 hr. As discussed below, the long G2 cells are sex-cell precursors. These cells are recognized by the monoclonal antibody IX8c and constitute about 10% of the total interstitial cell population in H. oligactis. The remaining 90% of interstitial cells are IX8c- (dotted line in Fig. 5A); these cells are 100% labeled by 12 hr.

The labeling curve of rapidly proliferating cells is markedly altered compared to normal cells (Fig. 5A). The fraction of cells in S phase is higher at t₀ and the labeling curve changes slope at 3 hr rather than 6 hr. Both results indicate that the G2 phase of the short G2 population is decreased compared to normal animals. By comparison, the medium G2 population appears similar to that in normal animals; it is fully labeled by 12 hr. The labeling kinetics of the long G2 population was not investigated in these experiments.

The identification of three populations with different labeling kinetics in Fig. 5A raises the question of whether these populations are associated with particular functions. To investigate this question we carried out two experiments to determine the function of subpopulations of interstitial cells.

Identification of Long G2 Cells as Sex-Cell Precursors

Previous work of Littlefield et al. (1985) has demonstrated the presence of significant numbers of sex-cell
precursors in the interstitial cell population of *H. oligactis* cultured at 18–22°C. To detect these cells we used the monoclonal antibody IX8c (see Materials and Methods). In control animals the population of 1s + 2s contained an average of 9.7% IX8c+ cells (Table 1). As shown in Fig. 5B, the pulse-labeling index of IX8c+ 1s + 2s was markedly lower than that of total 1s + 2s indicating a significantly longer G2. Consistent with this interpretation, the labeling index increased slowly during continuous labeling, such that after 24 hr only 45% IX8c+ cells were labeled. Extrapolating this labeling curve to 100% indicates that IX8c+ cells have a G2 length of 2–3 days. Thus they correspond to the long G2 population identified in Fig. 5A. It is interesting to note that their cell cycle parameters are similar to epithelial cells.

**Identification of Short G2 Cells as Stem Cells and Medium G2 Cells as Nematocyte Precursors**

To determine the function of short and medium G2 cells we followed the fate of these cells in an [3H]thymi-

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**TABLE 1**

<table>
<thead>
<tr>
<th>Cell Composition of <em>Hydra oligactis</em></th>
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<tr>
<td><strong>Number/Hydra</strong></td>
</tr>
<tr>
<td>epithelial cells</td>
</tr>
<tr>
<td>interstitial cells</td>
</tr>
<tr>
<td>total 1s + 2s</td>
</tr>
<tr>
<td>nests of 2s</td>
</tr>
<tr>
<td>sex cell precursors (1s + 2s)</td>
</tr>
<tr>
<td>nests of nematocyte precursors (4s)</td>
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*Note.* The number of cells and nests is determined in macerates. Sex cell precursors were identified by labeling with the monoclonal antibody IX8c (see Materials and Methods).
dine pulse-chase experiment (Figs. 6A, 6B). To distinguish the two populations hydra were labeled for 6 hr at which time all short G2 cells were labeled while some medium G2 cells (and long G2 cells) were still unlabeled. Since nematocyte precursors (4s) have a short G2 (Campbell and David, 1974), these cells were also nearly 100% labeled at 6 hr. After removal of the label at 6 hr the labeling index of cells in the stem cell pool (1s + 2s) continued to increase from 78 to 90% by 12 hr after which it remained roughly constant (Fig. 6B). At the same time the labeling index of nematocyte precursors (4s) decreased from 100% at 6 hr to 80% at 12 hr (Fig. 6A).

The increase in the labeling index of 1s + 2s between 6 and 12 hr indicates that unlabeled cells exited from this population. The coincident decrease in labeling index of 4s indicates that unlabeled cells entered this pool. These changes are consistent with the interpretation that at least some unlabeled 2s divided to become 4s in the nematocyte differentiation pathway. The decrease in the labeling index of 4s (Fig. 6A) can be used to estimate the length of the G2 of 2s that become 4s. Since unlabeled 2s continued to enter the pool of 4s from 6 to 12 hr, the G2 phase of these nematocyte precursors must be roughly 12 hr. Thus nematocyte precursors constitute part of the medium G2 population. After 12 hr the labeling index of 4s remained constant indicating that labeled 2s entered the pool of 4s at the same rate as labeled 4s completed the cycle and left the pool.

The increase in the labeling index of 1s + 2s between 6 and 12 hr (Fig. 6B) supports the interpretation that short G2 cells are stem cells. Were the unlabeled cells at 6 hr (i.e., medium and long G2 cells) stem cells, then they would have remained in the stem cell pool (1s + 2s), and the labeling index of this pool would have decreased between 6 and 12 hr as a result of mitoses in unlabeled cells. This clearly did not occur; rather, the labeling increased from 78 to 90%. Hence we conclude that short G2 cells, which are 100% labeled at 6 hr, are stem cells. The increase in labeling index in Fig. 6B did not continue to 100% due to the presence of unlabeled sex precursors which have a very long cell cycle (Fig. 5B).

**Rapidly Growing Interstitial Cells Are Smaller**

When interstitial cells under conditions of rapid proliferation were counted by phase microscopy, they appeared smaller in size than interstitial cells in normal animals. To quantitate this visual impression we measured the size distribution of the interstitial cell population under conditions of rapid growth in NM grafts. The outlines of individual cells were traced on a digitizing tablet and the area was computed. Representative size distributions of the interstitial cell population on Day 0 and Day 4 in NM grafts are shown in Fig. 7. The distributions demonstrate a significant decrease in mean cell size from 190 \( \mu \text{m}^2 \) on Day 0 (Fig. 7A) to 135 \( \mu \text{m}^2 \) on Day 4 (Fig. 7B). Figure 8A shows that the decrease did not occur in one step but continuously over the course of several days of rapid cell growth. Under the same conditions the nuclei of interstitial cells did not change markedly in size (Fig. 8B).

**DISCUSSION**

**Length of the Interstitial Cell Cycle in Hydra oligactis**

The results of cell cycle measurements in the present report are in good agreement with previous observations in *H. vulgaris* (Campbell and David, 1974). \(^{3}H\)thymidine pulse labeling and nuclear DNA determinations (Figs. 3 and 4) indicate that in normal animals about half the interstitial cells are in S and half in G2. There is no G1 phase. The complex increase in the labeling index during continuous \(^{3}H\)thymidine labeling (Fig. 5A) indicates that the length of G2 is not uniform in all interstitial cells. Rather there appear to be three major subpopulations: one with a G2 of about 6 hr, one with a G2 of about 12 hr and one with a G2 of >24 hr. Campbell and David (1974) reported the presence of two subpopulations in *H. vulgaris* with G2 of 6- and 12-20 hr
on the basis of the results of a labeled mitosis experiment. These results applied specifically to 2s, but since 2s comprise about 80% of the population of 1s + 2s (Table 1; see also David and Challoner, 1974), the results are roughly consistent with the observations reported here for the short and medium G2 populations. Long G2 cells are sex cell precursors and appear not to be present in H. vulgaris.

In view of the close agreement between cell cycle parameters of interstitial cells in H. vulgaris and H. oligactis, it seems likely that the S phase duration is also similar in both species. Assuming an S phase of 12 hr (Campbell and David, 1974) and the G2 parameters given above, then in normal H. oligactis the cell cycle of short-cycle cells is about 18 hr, that of medium-cycle cells about 24 hr, and that of long-cycle cells about 3 days.

Short-Cycle Cells Are Stem Cells; Medium- and Long-Cycle Cells Are Differentiation Products

The interstitial cell population in hydra consists of stem cells and committed precursors to several differentiation pathways (David and Gierer, 1974; Bosch and David, 1987; Littlefield and Bode, 1986). In terms of cell numbers, most interstitial cells (1s + 2s) are either stem cells or nests of two precursors (2s) to nematocyte differentiation (David and Gierer, 1974). In H. oligactis there are in addition significant numbers of 1s + 2s which are precursors to gamete differentiation (Littlefield et al., 1985). Whether these major subpopulations of interstitial cells—stems cells, nematocyte precursors, and sex-cell precursors—have different cell cycle parameters has not been known. However, the data presented here (Fig. 5) together with the results of Campbell and David (1974) provide good evidence for the presence of subpopulations of interstitial cells with different cell cycle parameters. We refer to these subpopulations as short-, medium- and long-cycle cells.

Using a sex cell-specific monoclonal antibody it was possible to identify long-cycle cells as sex-cell precursors (Fig. 5B). Nematocyte precursors were shown to be medium-cycle cells on the basis of their G2 length (Fig. 6A). Finally, it was possible to identify short-cycle cells as stem cells on the basis of the fact that the stem cell pool remained fully labeled following removal of [3H]-thymidine (Fig. 6B).

The Stem Cell Cycle Is Shortened under Conditions of Rapid Growth

The results here demonstrate for the first time a change in cell cycle parameters under conditions of rapid interstitial cell proliferation. The G2 of short-cycle cells decreases from about 6 hr in normal animals to about 3 hr under these conditions (Fig. 5A). By comparison, G2 of the medium- and long-cycle populations appears not to change.

Previous measurements of cell cycling under different growth conditions (Bode et al., 1977; David and MacWilliams, 1978; Sproull and David, 1979) failed to observe changes in the cell cycle using continuous labeling methods. This appears to be due to insufficiently precise measurements and to the fact that the change in cycling only occurs in one population, namely the short-cycle cells. Reexamination of the earlier data in light of the present results indicates that they agree quite well with these results.

It is possible to estimate quantitatively the transfer of medium-cycle cells to the nematocyte pathway (4s) from the changes in the labeling index between 6 and 12 hr (Fig. 6A, 6B, Table 1). The results indicate that about 50% more 2s exited the 1s + 2s than entered the pool of 4s. Thus some 2s with a medium cell cycle must have other functions, possibly as precursors to nerve cells or gland cells.
Stem Cell Cycle and Stem Cell Size

Our results demonstrate that proliferating interstitial cell populations differ in mean size depending on the growth conditions. Rapidly proliferating cells have a mean size of 135 μm² while slowly proliferating cells have a mean size of 190 μm² (Fig. 7). Thus cell size does not appear to be rigidly controlled in hydra. The two populations are interconvertible with changes in the size distribution occurring over the course of several cell generations (Fig. 8). The size which cells reach after several days of rapid proliferation may reflect a minimal size at which stem cells can proliferate. This idea is supported by independent experiments which show that stem cells in peduncle tissue have a similar size and retain this size under conditions of rapid proliferation (Holstein and David, 1990).

The observed size differences between interstitial cell populations are accompanied by changes in stem cell cycle length. Since only stem cells reduce their cell cycle length under conditions of rapid growth (Fig. 5A), the size shift in the population of interstitial cells (Fig. 7) must be due to a size shift of stem cells. If growth is constant during the cell cycle, then such size changes would be a natural consequence of the shortened cycle: short-cycle cells grow less per cycle and hence become smaller over the course of several generations (Fig. 8A). As a result, the size of interstitial cells is not a reliable parameter with which to identify stem cells: stem cells fluctuate in size depending on cell cycle and growth rate.

A correlation of cell growth with cell cycle length has been previously demonstrated for budding yeast (Johnston et al., 1977) and fission yeast (James et al., 1975; Nurse and Thuriaux, 1977): cells that are abnormally small at birth have a longer cell cycle and hence grow back to normal size. This relationship has also been observed in Amoeba, Tetrahymena, and Physarum (Prescott, 1976). Genetic analysis of the yeast cell cycle (cdc mutants) has also demonstrated that cell size is regulated by the length of the growth phase in G1 (Fantes and Nurse, 1977, 1978).

Density Dependent Control of the Stem Cell Growth Rate

Interstitial cells in hydra form a constant proportion of total cells; in asexual animals this concentration is about 0.3–0.4 ls/epi (David and Bode, 1978; David et al., 1987). Maintenance of this constant stem cell density requires that the rate of stem cell growth be closely matched to the growth of epithelial tissue.

A feedback mechanism in which stem cell proliferation is controlled by stem cell density has been demonstrated previously in H. vulgaris (Bode et al., 1977; David and MacWilliams, 1978; Sproull and David, 1979). In these experiments a reduction in stem cell density was found to cause an increase in stem cell proliferation; the increase in growth rate was inversely related to the density of stem cells in tissue. The results presented in Figs. 1 and 2 confirm this observation for stem cells introduced into NM-treated tissue of H. oligactis. Reduction of the stem cell density caused faster stem cell proliferation while restoration of normal levels of stem cells caused slower proliferation.

Stem Cell Growth in Hydra oligactis Is Regulated by Cell Cycle Length and Pₕ

In principle there are two mechanisms by which stem cell growth can be regulated: regulation of the stem cell cycle and regulation of the fraction of daughter cells that remain stem cells (the probability of self-renewal, Pₕ). While doubling the rate of stem cell proliferation requires a twofold shortening of the cell cycle, the same result can be achieved by increasing Pₕ from 60 to 70% (Sproull and David, 1979).

Although the results presented here have demonstrated a shortening of the stem cell cycle, the change is too small to account for the twofold increase in the growth rate of the stem cell population. Thus, as in earlier experiments with H. vulgaris, it is necessary to postulate changes in Pₕ to explain the increase in growth rate of the stem cell population. Consistent with this conclusion, the ratio of differentiating nematocyte precursors to interstitial cells (4s/ls + 2s) decreased in rapidly proliferating populations and increased with recovery to normal stem cell levels and lower stem cell growth rates. The magnitude of the decrease is consistent with similar observations in H. vulgaris (Sproull and David, 1979).

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