Three types of interstitial stem cell subpopulation were isolated from Hydra magnipapillata, and their roles in sex determination were examined. A subpopulation of interstitial stem cells restricted to the sperm differentiation pathway was isolated previously from strain nem-1 (male). Another subpopulation restricted to the egg differentiation pathway was also isolated from the same strain. Hydroxyurea treatment was used for isolation in both cases. “Pseudoepithelial hydra” containing only sperm- or egg-restricted stem cells but no other interstitial stem cell types were maintained by force-feeding for 2 years. Sex reversal from egg- to sperm-restricted stem cells occurred three times during this period. Both of these two stem cell types are numerous in the central gastric region of the pseudoepithelial hydra, but absent in the foot region below the budding zone. Foot tissue was cut out from normal nem-1 polyps (male) and allowed to regenerate. The regenerates produced eggs but no sperm upon sex induction. These and other results suggest that the foot tissue contains multipotent stem cells capable of differentiating into eggs during sexual differentiation. These observations suggest that strain nem-1 (male) contains three types of interstitial stem cell subpopulations: (1) sperm-restricted stem cells, (2) egg-restricted stem cells, and (3) multipotent stem cells capable of differentiating into nerve cells, nematocytes, and eggs. Upon sex induction, however, differentiation of eggs by the latter two types is suppressed, and only sperm are produced by the sperm-restricted stem cells. Evidence is presented which suggests that similar “phenotypic males,” which normally only produce sperm but contain the stem cell types capable of differentiating into eggs, occur widely in Hydra magnipapillata. A possible relationship between phenotypic male hydra and hermaphroditic hydra is discussed.

INTRODUCTION

Hydra reproduce asexually, by budding, and sexually. Interstitial stem cells give rise to three types of somatic cells, nerve cells, nematocytes, and gland cells, during asexual growth and to germ cells, sperm, and eggs in sexual reproduction.

In order to examine differentiation capability of individual interstitial stem cells, cloning experiments were done. The results obtained have shown that the interstitial stem cell population contains at least two types of subpopulations. One type is sperm- or egg-restricted stem cells capable of differentiating into sperm or eggs, respectively, but not into any other types of cells. Polyps which contain only sperm- or egg-restricted stem cells in the tissue and are free from any other type(s) of interstitial stem cells have been obtained by two different procedures. One is by treatment of normal Hydra with hydroxyurea (HU), which selectively kills rapidly proliferating interstitial stem cells without severely affecting more slowly proliferating epithelial cells (Littlefield, 1985, 1991; Nishimiya-Fujisawa and Sugiyama, 1993). The other is by restrictive temperature treatment of a strain containing temperature-sensitive mutant interstitial stem cells (Nishimiya-Fujisawa and Sugiyama, 1993). In both procedures, excessive treatment eliminates the entire interstitial stem cell population, turning the treated animals into “epithelial hydra” (Campbell, 1976). Epithelial hydra lacking the interstitial stem cells and their differentiation products, nerve cells, and nematocytes cannot move or feed. However, they can be maintained by force-feeding (Marcum and Campbell, 1978).
The present study is an extension of this initial observation. In this paper we first report the isolation and characterization of egg-restricted stem cells from strain nem-1 (male). We then report another unexpected observation made with this strain. In addition to the egg-restricted stem cells, this strain apparently also contains multipotent stem cells which can differentiate into eggs but not into sperm. Thus, although male in phenotype, strain nem-1 contains two types of stem cells capable of differentiating into eggs. However, their differentiation into eggs is normally suppressed. We propose to call this type of hydra, which is male in phenotype but contains multipotent stem cells capable of differentiating into eggs, phenotypic males.

**FIG. 1.** Relative numbers of three polyp types produced by treatment of nem-1 polyps in 2.5 mM HU. The abscissa represents the length of the treatment (days). The ordinate represents the relative numbers (%) of incomplete removal (solid bars), pseudoepithelial hydra (cross-hatched bars) and epithelial hydra (open bars) produced as the result of the treatment. Polyp types were examined by an indirect FITC staining procedure using mab C41 (see main text). The sample size was 15.

In theory, it is possible to control the treatment to produce individual polyps from which a majority of interstitial stem cells are eliminated, except for only one (or a very few) cell(s). Polyps containing only sperm- or egg-restricted stem cells are produced in this way. Nonfeeding polyps produced by the treatment are maintained by force-feeding for several weeks to allow the remaining stem cells to proliferate. Any polyps recovering self-feeding capacity during this period are discarded. Nonfeeding polyps are then selected for sperm or egg production upon sex induction.

The second type of interstitial stem cell subpopulation is multipotent stem cells which can give rise to somatic cells (nerve cells and nematocytes) during asexual growth and also to sperm or eggs during sexual differentiation. This cell type was cloned by the procedure involving cell aggregates. Hydra can regenerate from the aggregates of dissociated cells (Noda, 1971; Gierer et al., 1972). Bosch and David (1986, 1987) produced aggregates of epithelial cells, to which a low number of interstitial stem cells was added. The stem cells cloned by this procedure all gave rise to both somatic cells (nerve cells and nematocytes) and gametic cells (sperm or eggs). No sperm- or egg-restricted stem cells were isolated by this procedure, presumably either because these cells are much less numerous than the multipotent stem cells or because they cannot survive the aggregate cloning procedure.

In our previous study of sperm-restricted stem cells, we made an unexpected observation. HU treatment of strain nem-1 (male) produced mainly sperm-restricted stem cells. However, it also yielded egg-restricted stem cells. This observation was described in a preliminary note in Nishimiya-Fujisawa and Sugiyama (1993).

**FIG. 2.** Photographs of sexually differentiated nem-1 polyps. (A) A normal male polyp carrying two nipple-shaped testes (indicated by arrows); (B) a pseudoepithelial polyp carrying two testes; (C) a pseudoepithelial polyp carrying two round eggs (indicated by asterisks); and (D) a pseudoepithelial polyp bearing a testis and an egg. A cleavage furrow is visible in the egg in D. Live animals were gently pressed under a cover glass and photographed under a standard microscope (4X objective). When examined using phase-contrast optics (20–40X objective), highly motile sperm were observable in the testes (Sugiyama and Sugimoto, 1985). Scale, 1 mm.
TABLE 1

Induction of Sexual Differentiation of Nonfeeding (Pseudoepithelial) Hydra

<table>
<thead>
<tr>
<th>HU treatment (days)</th>
<th>No. examined</th>
<th>No. forming testes (%)</th>
<th>No. forming eggs (%)</th>
<th>No. forming both (%)</th>
<th>No. forming neither (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>18</td>
<td>16 (89)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>2 (11)</td>
</tr>
<tr>
<td>2.5</td>
<td>23</td>
<td>6 (26)</td>
<td>2 (9)</td>
<td>2 (9)</td>
<td>13 (56)</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>3 (20)</td>
<td>2 (13)</td>
<td>0 (0)</td>
<td>10 (67)</td>
</tr>
</tbody>
</table>

Evidence will also be presented which suggests that phenotypic males are not rare but probably common in Hydra magnipapillata. A possible relationship between phenotypic males and hermaphroditic hydra is discussed.

MATERIALS AND METHODS

The materials and methods described in a previous paper by Nishimiya-Fujisawa and Sugiyama (1993) were used, except for those listed below;

(1) Strains

Ten strains belonging to H. magnipapillata (vulgaris group; Campbell, 1987) were used. The main experiments were done using strain nem-1. This strain was originally female (Sugiyama and Sugimoto, 1985), but since then had changed its sex spontaneously to male. This male nem-1 strain was used in our previous study (Nishimiya-Fujisawa and Sugiyama, 1993) and also in this study. During this study, females arose from this strain twice, one spontaneously, nem-1 f1 (female), and the other by HU treatment, nem-1 f2 (female). SSB (female), SSC (female), SSE (male), and ms-1 (male) were described in Sugiyama and Sugimoto (1985). SSC m (male) arose by spontaneous sex reversal from SSC (female). A new strain nB-2 (male) was obtained by a sexual cross of nem-1 (male) and SSC (female). All of the strains were selected for their relative stability of sexual phenotypes and ready response to sex induction by a combination of gentle aeration of the culture solution and reduced feeding (Sugiyama and Fujisawa, 1977; Sugiyama and Sugimoto, 1985; Nishimiya-Fujisawa and Sugiyama, 1993).

Prior to the study, each strain was subcloned starting from a single polyp, and the sex of each subcloned population was confirmed. All experiments were done using these subcloned populations.

(2) BrdU Labeling and Staining of BrdU-Labeled Cells

Several polyps were placed in 7 ml of culture solution containing 5 mM BrdU (Aldrich Chem.) and kept in the dark for 3 days. Polyps were fed once a day, and the culture solution containing BrdU was replaced with fresh solution a few hours later. At the end of the treatment, the gastric cavity of the polyps was washed thoroughly by flushing the fresh culture solution using a finely drawn plastic tube. This was done three times at half-hour intervals, just prior to using the polyps in a grafting experiment.

BrdU-labeled cells in whole mounts of hydra were examined by an immunohistochemical enzyme staining procedure using an anti-BrdU antibody and alkaline phosphatase, according to the procedure of Holstein et al. (1991).

RESULTS

Pseudoepithelial hydra are defined as hydra containing proliferating interstitial cells but no nerve cells, nematoblasts, or nematocytes in their tissue (Nishimiya-Fujisawa and Sugiyama, 1993). They resemble epithelial hydra (Campbell, 1976) in morphology and the inability to move or eat. Unlike epithelial hydra, however, they can produce sperm or eggs during sexual differentiation.

(1) HU Treatment

HU treatment was used to produce pseudoepithelial hydra. A population of mature nem-1 polyps was maintained, and new young polyps produced by this population were used for the treatment. Soon after collecting all of the young polyps for the treatment, about 1/3 (23) of the parental population was subjected to induction of sexual differentiation. All of them produced testes, reconfirming that the sex of the parental population was male.

Groups of 30–40 young polyps were treated in 2.5 mM HU for 2–3 days. The treated animals were then maintained in the normal manner by feeding them once a day. The ability to capture and ingest food gradually decreased in the treated polyps, reflecting decreases in the numbers of nerve cells and nematocytes. A few animals which lost all feeding ability were force-fed by the procedure of Marcum and Campbell (1978). New buds which developed and detached from the initial treated polyps were discarded, and only the latter were kept.

At the 10th day after the end of the HU treatment, 15 polyps were randomly selected from each group and subjected to examination of interstitial cells in their tissue. Indirect FITC staining was employed to visualize the inter-
strial cells using a monoclonal antibody C41 which specifically recognizes these cells (David et al., 1991; Nishimiya-Fujisawa and Sugiyama, 1993).

The HU-treated polyps were classified into three groups as described previously (see Fig. 1 in Nishimiya-Fujisawa and Sugiyama, 1993). Group 1: incomplete removal. Both large and small interstitial cells are present in the tissue of this group. The latter cell type represents proliferating nematocyte precursors and indicates the presence of multipotent stem cells in the tissue. Group 2: pseudoepithelial hydra. The tissue of these animals contains only large interstitial cells but no small interstitial cells, indicating the absence of the multipotent stem cells. Group 3: epithelial hydra. The tissue of this type is completely free from both large and small interstitial cells.

The relative numbers of the three polyp types produced by the HU-treatment is shown in Fig. 1. With an increase in the length of treatment, the number in the incomplete removal group decreased, whereas the number of epithelial hydra increased. The number of pseudoepithelial hydra did not change appreciably with length of treatment, remaining at about 60%.

(2) Sexual Differentiation Test

The remaining polyps were maintained for an additional 20 days by feeding each polyp once a day by force-feeding (Marcum and Campbell, 1978). The stem cells remaining in the treated polyps increased in number during this period. Some polyps recovered self-feeding capacity during this period. These animals were discarded after recording their numbers.

At about 30 days after the treatment, all nonfeeding animals were subjected to sex induction by starvation (Nishimiya-Fujisawa and Sugiyama, 1993). Some polyps differentiated sexually in the first test. Others, however, failed to respond to the induction and became smaller and thinner by starvation. The nonresponding polyps were returned to the daily force-feeding culture, allowed to recover to normal size, and then resubjected to sexual induction. The same process was repeated for nonresponding animals three or four times during a period of about 80 days (30-110 days after HU treatment).

Examples of sexually differentiated pseudoepithelial hydra are shown in Fig. 2. A typical male pseudoepithelial hydra carrying two large nipple-shaped testes is shown in Fig. 2B. A typical female pseudoepithelial hydra bearing two large round eggs is shown in Fig. 2C. Fig. 2D shows a pseudoepithelial polyp carrying both a testis and an egg showing a cleavage furrow. All of these pseudoepithelial hydra have a larger (bloated) hypostome and thinner tentacles (Figs. 2B-2D) than normal hydra (Fig. 2A).

The numbers of pseudoepithelial hydra which produced testes or eggs are summarized in Table 1. With an increase in the length of HU treatment, the relative number of testis-forming pseudoepithelial hydra decreased, whereas the relative number of the egg-producing pseudoepithelial hydra increased.

(3) Long-Term Maintenance of Pseudoepithelial Hydra

In the sex induction experiment described above, one male pseudoepithelial polyp producing testes and two female pseudoepithelial polyps producing eggs were selected, and each was used as a founder to start a clonal line. The founder was force-fed once daily until it produced several buds. One large and vigorous bud was selected and used as

![FIG. 3. Long-term maintenance of clonal lines of pseudoepithelial hydra. Each line was started from a single nonfeeder determined to contain germ-restricted cells in the sex induction test after HU treatment: clone S1 from a sperm-producer and clones E1 and E2 from egg-producers. Each generation in each line consists of a single polyp, which was force-fed daily until it grew and produced several buds, one of which was selected and used as the polyp for the next generation. This process was repeated for 2 years. Each symbol represents one generation. Squares and circles represent polyps which produced testes and eggs, respectively. Triangles represent polyps which were not subjected, or did not respond, to sex induction. Testis-producing polyps which appeared in the female line, clone E2, are shown by filled squares. Asterisks represent polyps subjected to stem cell examination by monoclonal antibody staining.](image-url)
the polyp of the second generation. This second generation polyp was force-fed to allow it to produce buds, from which the one for the third generation was selected. The same process was repeated from one generation to the next in the three independent lines for 2 years as shown in Fig. 3.

(4) Histology

During the passage, parental polyps, after producing buds for the next generation, or siblings of the buds selected for the next generation were not needed for line maintenance. Some of them were examined for stem cells using the indirect FITC staining procedure described above. Polyps subjected to this examination are marked by asterisks in Fig. 3. Large numbers of C41 cells were found in all of the specimens examined, without exception.

Figure 4 shows photomicrographs of C41 cells present in the strain nem-1 hydra. The following features should be noted. (1) In normal hydra, the C41 cells are numerous and distributed evenly throughout the entire tissue, except for two small regions at the extreme tips of the hypostome or foot (Fig. 4A). These regions free from the interstitial cells were named "clear zones" by David and Plotnick (1980). The inset shows the foot tissue at a higher magnification. The clear zone without any C41 cells above the basal disk is clearly shown. (2) Figure 4B shows an epithelial hydra in which the C41 cells are totally absent. (3) Figure 4C shows a male pseudoepithelial hydra. The C41 cells are present but less numerous than in normal hydra (Fig. 4A). They are also unevenly distributed. In the parental tissue, they are localized mainly in the central part of the body column, but are absent in the upper gastric tissue near the hypostome and in the foot tissue below the budding zone. Their complete absence in the foot tissue is clearly shown in the inset in Fig. 4C. The bud tissue appears to contain fewer C41 cells than the parental tissue, although every bud examined contained some of these cells. (4) Figure 4D shows a female pseudoepithelial hydra. The C41 cells are distributed in a manner similar to that in the male pseudoepithelial hydra (Fig. 4C). They are present in the central part of the body column, but not in the upper part near the hypostome or the lower part below the budding zone.

Some polyps were also examined after toluidine blue staining (Diehl and Burnett, 1964) as in the previous study (Nishimiya-Fujisawa and Sugiyama, 1993) (not shown). No differences were noted in the distribution pattern between the toluidine blue-positive and C41-positive interstitial cells in the pseudoepithelial hydra. By either staining procedure, no pseudoepithelial hydra was found which contained interstitial cells in the foot tissue below the budding zone.

(5) Sexual Differentiation

Some polyps not needed for line maintenance were also utilized to examine their ability to differentiate sexually. Testis formation by S1 males occurred readily by 4-10 days of starvation in most cases. Egg formation in E1 and E2 females required a longer time of starvation (10-15 days). A little over half of the polyps tested produced eggs in the first test. Those that failed to sexually differentiate in the first test were discarded without reexamination.

Individual polyps which differentiated sexually are indicated in Fig. 3. Two important features should be noted. (1) Gamete-restricted stem cells maintained their restricted differentiation capacity for at least 2 years. (2) Sex was stable in one male (clone S1) and in one female (clone E1) line. However, sex reversal took place in the other female line (clone E2). Testes were formed instead of eggs at least three times in this line. In one case, offspring from the sex-reversed female was maintained for five generations. All offspring produced testes.

The results described in sections 3 to 5 show that gamete-restricted stem cells can proliferate vigorously for a long period of time and that they are stably transmitted from parental polyp to bud in each generation. They also maintain their capacity to differentiate into gametes for a long period of time. However, sex reversal can occur, at least from female to male, in these cells.

(6) Masculinization

Masculinization is a phenomenon of sex reversal from female to male which occurs when male tissue is temporarily grafted to female tissue (Goetsch, 1922) (see Fig. 5). The interstitial stem cells migrating from male to female tissue during grafting are thought to be responsible for the masculinization process (Sugiyama and Sugimoto, 1985).

A masculinization experiment was carried out using male

![Fig. 4](image-url) Interstitial cells present in nem-1 polyps indirectly stained using mab C41. Insets show the foot tissue below the budding zone at a higher magnification. (A) A normal male polyp bearing two buds. Large numbers of C41 cells are present throughout the entire body column tissue. In the foot tissue (inset), the C41 cells are numerous in the upper part, become progressively less numerous in the lower part, and are absent in the clear zone (David and Plotnick, 1980) adjacent to the basal disk. (B) An epithelial polyp carrying six buds without any C41 cells in the entire tissue. (C) A male pseudoepithelial polyp carrying five buds. (D) A female pseudoepithelial polyp (clone E1) carrying four buds. In C and D, C41 cells occur in the body column of the parental polyp only above the budding zone. Total absence of these cells below the budding zone can clearly be seen in insets. Scales, 1 mm in the main part; 0.2 mm in the inset.
pseudoepithelial hydra (clone S1) as the interstitial stem cell donor, as shown schematically in Fig. 5. The results, summarized in Table 2, show that male pseudoepithelial hydra (clone S1) are capable of masculinizing both normal (nem-1·f2 and SSB) and pseudoepithelial female strains (clone E1). This observation suggests that sperm-restricted stem cells present in pseudoepithelial hydra can masculinize females. A similar observation was previously reported for H. oligactis (Littlefield, 1986).

(7) Migration of Gamete-Restricted Stem Cells

The histological examination described in section 4 has shown that the gamete-restricted stem cells are absent from the foot tissue below the budding zone in pseudoepithelial hydra (Fig. 4). The absence of these cells in the foot tissue may be produced by the inability of these cells to migrate into and/or to proliferate in the foot tissue. To examine their migration ability, BrdU-labeled gamete-restricted stem cells were introduced into normal hydra by grafting (Fig. 6), and their migration in the host tissue was followed by staining them using an anti-BrdU antibody. The results obtained are presented in Fig. 7. The following features should be noted in the distribution patterns exhibited by the migrating cells in the host polyps. (1) The sperm-restricted stem cells produced a distribution pattern in the new hosts closely resembling their original distribution pattern in pseudoepithelial hydra (Figs. 7A and 7B). These cells are localized mainly in

TABLE 2
Sex Reversal of Females into Males by Temporary Grafting to Male Tissue (Masculinization)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Type</th>
<th>Strain</th>
<th>Type</th>
<th>No. examined</th>
<th>No. forming testes</th>
<th>No. forming eggs</th>
<th>No. forming both</th>
<th>Dead or lost</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1 (♀)</td>
<td>P</td>
<td>S1 (♂)</td>
<td>P</td>
<td>11</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>nem-1·f2 (♀)</td>
<td>N</td>
<td>S1 (♂)</td>
<td>P</td>
<td>10</td>
<td>8</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>SSB (♀)</td>
<td>N</td>
<td>S1 (♂)</td>
<td>P</td>
<td>12</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E1 (♀)</td>
<td>P</td>
<td>nem-1 (♂)</td>
<td>N</td>
<td>12</td>
<td>9</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>E1 (♀)</td>
<td>P</td>
<td>SSB (♂)</td>
<td>N</td>
<td>13</td>
<td>0</td>
<td>12</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

*P and N stand for pseudoepithelial and normal hydra, respectively.
FIG. 6. Axial grafting procedure for the interstitial cell migration experiment. Donor tissue containing BrdU-labeled interstitial cells was axially grafted into the host to allow these cells to migrate into the host tissue.

the central part of the host body column, but not in the upper gastric part near the hypostome or the lower part near the basal disk. This was true when the host was epithelial hydra (Fig. 7A) or normal female (Fig. 7B). It was also true when the host was normal male, although the migrating cells were less numerous (not shown). (2) The egg-restricted stem cells also produced a similar distribution pattern when the host was an epithelial hydra (Fig. 7C). (3) However, the egg-restricted stem cells produced a drastically different pattern when the host was a normal male (Fig. 7D) or a normal female (not shown); the number of the migrating cells was very low and their range of migration was limited to short distances from the graft border. (4) The BrdU-labeled cells from normal male hydra migrated extensively, occupying almost the entire host (Figs. 7E and 7F). Only the basal disk and small adjacent region (clear zone) were not occupied by them (not clearly visible in the figures). The BrdU-labeled migrating cells from the normal tissue presumably include not only interstitial stem cells, but also nerve cell precursors and nematocytes and their precursors. All BrdU-labeled cells migrating into the tentacles are presumably nematocytes.

Similar examinations were done on many other specimens of various donor-host combinations and various periods of migration times (3-12 days). No migration of the BrdU-labeled gamete-restricted stem cells into the foot tissue was observed.

(8) Foot Tissue Regeneration

Since the gamete-restricted stem cells are absent from the foot tissue of pseudoepithelial hydra (Fig. 4), and since they do not migrate into the foot tissue of normal hydra (Fig. 7), they may be also absent from the foot tissue of normal hydra.

To examine this possibility, we cut out foot tissue from normal nem-1 polyp and allowed it to regenerate. A number of different outcomes could be anticipated depending on the stem cell type(s) present in the foot tissue. (1) If no stem cells are present, the regenerates should turn into epithelial hydra. (2) If only gamete-restricted stem cells are present, the regenerates should turn into pseudoepithelial hydra. (3) If only multipotent stem cells are present, the regenerates should turn into normal hydra. (4) If only somatic cell-restricted stem cells are present, the regenerates should turn into hydra that have nerve cells and nematocytes, can move and feed, but cannot differentiate sexually.

To insure the absence of gamete-restricted stem cells in the regenerates, the foot tissue was obtained by an amputation made at approximately 3/4 of the length from the basal disk to the budding zone, as shown in Fig. 8. The foot tissue pieces obtained in this way, if left alone, failed to regenerate. However, we succeeded in making them regenerate by force-feeding (Marcum and Campbell, 1978). A pair of fine forceps was used to gently and slowly make a small opening in the apical healing end of the excised foot tissue, and a piece of finely chopped brine shrimp tissue was gently pushed through the opening into the gastric cavity. When force-fed in this way once a day for about a week, regeneration occurred in some of the foot tissue. The regenerates were then fed normally. When they grew larger and produced several buds, they were induced to differentiate sexually by starvation.

The result obtained is presented in the top row in Table 3. The majority of the regenerates from the nem-1 foot tissue produced eggs. This was rather surprising since the regenerating tissue came from male polyps.

Similar foot tissue regeneration experiments were carried out using several other male and female strains (Table 3). The results obtained were also surprising. The regenerates of two male strains (SSC·m and n8·2) produced only eggs and no sperm. In another strain (SSE), the majority of the
FIG. 7. Migration of interstitial cells in the normal and epithelial hydra tissue of strain nem-1. BrdU-labeled interstitial cells migrating from the donor tissue (marked by bold arrowheads) into the host tissue were examined by visualizing these cells by an indirect enzyme staining (Materials and Methods). Front boundaries of the migrating cells are indicated by fine arrows. The graft combination (donor/host) and the length of grafting are: (A) male pseudoepithelial hydra/epithelial hydra, 8 days; (B) male pseudoepithelial hydra/female normal hydra, 4 days; (C) female pseudoepithelial hydra/epithelial hydra, 10 days; (D) female pseudoepithelial hydra/male normal hydra, 8 days; (E) male normal hydra/epithelial hydra, 6 days; and (F) male normal hydra/female normal hydra, 8 days. Scale, 0.5 mm.

regenerates produced testes, but one produced eggs. The regenerates of female strains produced only eggs (nem-1·f1, SSC and SSB).

These observations demonstrate that stem cells capable of differentiating into eggs are present in some male strains of H. magnipapillata. A similar observation was previously made in the aggregate cloning experiment by Bosch and David (1986, 1987).

DISCUSSION

(1) Gamete-Restricted Stem Cells

Sperm- and egg-restricted stem cells were isolated from strain nem-1 (male) by HU treatment (Table 1). The same two stem cell types were previously isolated from male and female strains of H. oligactis, respectively (Littlefield, 1985, 1991). However, this is the first report for the isolation of both types from a single male strain.

In theory, the ideal condition for the HU treatment is that which eliminates the entire interstitial stem cell population from the majority of the treated polyps, except for one or a very few interstitial stem cells remaining in a minority of the polyps. Polyps treated under this condition have a high probability of containing a clone arising from a single stem cell. In the present study, treatment with 2.5 mM HU for 3 days was close to this ideal condition (Fig. 1). This treatment yielded only egg-restricted stem cells. Sperm-restricted stem cells were isolated mostly by a slightly shorter duration of treatment (Table 1).
This observation can be explained by the difference in HU sensitivity or in the numbers of stem cell types. If the sensitivity increases in the order of egg-restricted stem cells, sperm-restricted stem cells, and other stem cell type(s) or if the cell number decreases in the same order, the egg-restricted stem cells have the highest probability of being isolated under the ideal treatment condition. Sperm-restricted stem cells will be isolated under slightly gentler conditions.

(2) Long-Term Maintenance and Sex Reversal

Sperm-restricted and egg-restricted stem cells continued to proliferate over 2 years in force-fed pseudoepithelial hydra (Fig. 3). Polyps randomly selected and examined during this period all contained large numbers of interstitial cells in their tissue. These observations suggest that germ stem cells have a strong and long-lasting proliferating capacity and that they are stably transmitted from parental to bud tissue from one generation to another. Similar observations were also reported for the germ stem cells in *H. oligactis* (Littlefield, 1985, 1991).

Of the two clones of egg-restricted stem cells, sex was stable in clone E1. In clone E2, however, sex reversal to sperm-restricted stem cells was observed three times (Fig. 3). Assuming the cycle time of 2 days for the stem cells (Holstein and David, 1990) and the presence of 100 stem cells per polyp, the three reversals in the 2 egg-restricted clones in 2 years give a rate of reversal of $4 \times 10^{-5}$ per cell per generation.

The mechanism of this sex reversal is not clear. It may be produced by somatic mutation in the gene determining sex or alteration in the expression of the gene (transdifferentiation).

No sex reversal from sperm- to egg-restricted stem cells was observed in clone S1. However, reversal occurring in this stem cell type, even if present, is hard to detect due to suppression of their differentiation by masculinization (see below).

Spontaneous sex reversal was observed in normal nem-1 hydra in routine cultures; female polyps appeared in a small population of males twice in 5 years, and males appeared in a small population of females once in 2 years. These rates seem to be comparable to, or a little lower than, the rate observed in pseudoepithelial hydra mentioned above.

Sex reversal in gonochoristic species of hydra (species having separate male and female polyps) have been reported by many investigators (for example, see Hyman, 1931; Ewer, 1948; Tardent, 1966). The present result shows that the reversal can occur at the level of the gamete-restricted stem cells.

(3) Masculinization

Masculinization is a phenomenon of sex reversal from female to male which occurs when male tissue is temporarily grafted to female tissue (Goetsch, 1922). During grafting, the interstitial stem cells originally present in the male tissue migrate into the female tissue. In sexual differentia-

---

**TABLE 3**

Sexual Differentiation of Foot Tissue Regenerates

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sex</th>
<th>No. examined</th>
<th>Testes</th>
<th>Eggs</th>
<th>Both</th>
<th>Neither</th>
</tr>
</thead>
<tbody>
<tr>
<td>nem-1</td>
<td>♂️</td>
<td>31</td>
<td>1</td>
<td>29</td>
<td>1*</td>
<td>0</td>
</tr>
<tr>
<td>SCC</td>
<td>♂️</td>
<td>28</td>
<td>0</td>
<td>21</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ms-1</td>
<td>♂️</td>
<td>8</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SSE</td>
<td>♂️</td>
<td>24</td>
<td>23</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

---

*a Induction of sexual differentiation was done when each regenerate had produced several mature offspring.

*b One offspring produced eggs while four others produced testes.

*c This clone of regenerates all turned into epithelial hydra unable to move, feed, or differentiate sexually.
tion, they differentiate into sperm, suppressing egg differentiation from the interstitial cells present in the female tissue (Sugiyama and Sugimoto, 1985). In the present study, the temporary tissue grafting experiment carried out using pseudoepithelial hydra (Fig. 5) has shown that sperm-restricted stem cells present in these hydra can "masculinize" females and convert them into males (Table 2). Similar observations were previously reported for H. oligactis (Littlefield, 1986).

(4) Female Multipotent Stem Cells

In addition to sperm- and egg-restricted stem cells, strain nem-1 appears to contain another stem cell type. It is female multipotent stem cells which give rise by differentiation to somatic cells (nerve cells and nematocytes) during asexual growth, and also to eggs (via egg-restricted stem cells) in sexual differentiation.

Existence of this stem cell type in strain nem-1 was previously shown by aggregate cloning by Bosch and David (Table 4 in Bosch and David, 1987). The result of the foot regeneration experiment (Table 3) supports the presence of this stem cell type in the nem-1 foot tissue. Sperm- and egg-restricted stem cells were shown to be absent in the foot tissue of pseudoepithelial hydra by histological examination (Fig. 4). These cells were unable to move into the foot when introduced into normal hydra by grafting (Fig. 7). These observations suggest that the foot tissue of normal nem-1 hydra may also be free from these cells.

To test this possibility, foot tissue was cut out from normal nem-1 polyps (male), allowed to regenerate, and then induced to differentiate sexually. All of the regenerates could move and feed, indicating that the foot tissue contains (multipotent) stem cells differentiating into nerve cells and nematocytes. The majority of the regenerates produced eggs but no sperm (Table 3), suggesting that sperm-restricted stem cells or male multipotent stem cells are absent in the foot tissue. This is because, if they were present, egg differentiation should have been suppressed by masculinization.

The result also showed that the sex of the nem-1 polyps changed from male to female in the foot tissue regeneration. The simplest interpretation of this observation is to assume that the nem-1 foot tissue contains female multipotent stem cells previously isolated by aggregate cloning by Bosch and David (1987). In normal nem-1 polyps, differentiation of these cells into eggs is suppressed by the presence of the sperm-restricted stem cells. However, this suppression is removed when the foot tissue is cut off from the rest of the polyp. Thus, these cells give rise to nerve cells and nematocytes in the regenerates and also to eggs (via egg-restricted stem cells) upon induction of sexual differentiation.

Taken together, the results of the foot tissue regeneration (Table 3) and aggregate cloning experiments (Bosch and David, 1987) suggest strongly that strain nem-1 contains female multipotent stem cells capable of differentiating into eggs (via egg-restricted stem cells) but not into sperm.

(5) Relationships of the Three Stem Cell Types

The discussion given above supports the view that three stem cell types are present in strain nem-1: sperm- and egg-restricted stem cells and female multipotent stem cells. Assuming that this view is correct, we suggest that the three stem cell types are related to each other as schematically shown in Fig. 9.

The multipotent stem cells give rise by differentiation to the egg-restricted stem cells. During asexual growth, both types proliferate stably, and the former differentiate into somatic cells (nerve cells, nematocytes, and gland cells). The latter may, or may not, give rise (by transdifferentiation or somatic mutation) to the sperm-restricted stem cells.

Sexual differentiation differs significantly depending on whether it occurs before or after the appearance of the sperm-restricted stem cells. (a) Before their appearance, the egg-restricted stem cells differentiate into eggs. (b) After their appearance, this process is suppressed, and sperm are produced instead of eggs.

The original nem-1 polyps used for HU treatment correspond to (b), whereas the nem-1 foot tissue regenerates (Table 3) and female nem-1 polyps (nem-1-f1) correspond to (a). A switch from the egg- to sperm-restricted stem cells was observed in clone E2 during long-term maintenance of pseudoepithelial hydra (Fig. 3).

(6) Phenotypic Male (Spontaneous Masculinization)

In the scheme shown in Fig. 9, sexual phenotype is initially female. However, it changes to male by spontaneous masculinization by the appearance of the sperm-restricted stem cells. We propose to call this type of male the phenotypic male.

Surprisingly, phenotypic males may not be rare in H. magnipapillata. Five male strains were used in the foot tissue regeneration experiment (Table 3). Two of them (nem-1 and SSC-r-m) were known to have a history of sex reversal. Of the five strains used, nem-1 and two other strains (SSC-m and nB-2) produced eggs after foot tissue regeneration, suggesting that these two strains, like nem-1, are also phenotypic males containing female multipotent stem cells.

The remaining two male strains (SSE and ms-1) produced only or mainly testes after regeneration. Two simple explanations are possible for this observation. First, the two strains may not be phenotypic males. Instead, they may be normal males containing male multipotent stem cells. Alternatively, the two strains may be phenotypic males like the other three strains. However, tissue localization of the sperm-restricted cells may be different in the two groups. Some sperm-restricted stem cells may be present in the foot tissue in the two strains. In either case, testes will be produced before or after foot tissue regeneration.
FIG. 9. Possible relationships of the three interstitial stem cell types present in strain nem-1 (phenotypic male). During asexual growth, the multipotent stem cells give rise by differentiation to the three somatic cell types and the egg-restricted stem cells. The sperm-restricted stem cells may or may not arise from the egg-restricted stem cells by transdifferentiation (or somatic mutation). In sexual differentiation, the egg-restricted stem cells differentiate into eggs in the absence of the sperm-restricted stem cells. This differentiation, however, is suppressed in the presence of the sperm-restricted stem cells and is replaced by sperm differentiation.

In theory, the two cases can be distinguished by aggregate cloning of the multipotent cells and HU cloning of the germline-restricted cells from the two strains. These experiments, however, have not been done.

In any event, at least three (nem-1, SSC·m, and nB-2) of the five male laboratory strains examined are phenotypic males (Table 3). It may be interesting to ask how widely, or rarely, phenotypic males occur in natural populations of H. magnipapillata or other gonochoristic species of hydra (hydra having separate male and female polyps). No experiment, however, has been done to answer this question.

ACKNOWLEDGMENTS

We are indebted to Drs. L. Littlefield, T. Bosch, and C. David for critically reading the manuscript and giving valuable comments. We also thank the latter two for a generous gift of the monoclonal antibody C41. This work was supported by a grant from the Japanese Ministry of Education, Science and Culture (No. 06454689) and by a grant from the Sumitomo Foundation.

REFERENCES


Gierer, A., Berkling, S., Bode, H., David, C. N., Flick, K., Hansmann,

Received for publication March 6, 1995
Accepted June 7, 1995