

Spatial Distribution and Differentiation Potential of Stem Cells in Hatchlings and Adults in the Marine Platyhelminth *Macrostomum* sp.: A Bromodeoxyuridine Analysis

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Stem cells (neoblasts) in Platyhelminthes are pluripotent, and likely totipotent, undifferentiated cells which retain throughout adult life the capacity to proliferate and from which all somatic cells as well as the germ cells derive. However, basic data on the pool and heterogeneity of neoblasts, their rates of differentiation into sets and subsets of differentiated cells, and their migration to different body regions are still lacking. To fill this gap, S-phase cells in the macrostomid *Macrostomum* sp. were labeled with the thymidine analog 5-bromo-2'-deoxyuridine (BrdU). S-phase cells were found to be neoblasts and to be distributed in two bands along the lateral sides of the body leaving unlabeled the median axis of the body and the region anterior to the eyes. This distribution is parallel to that of mitotic cells demonstrated using an antibody to phosphorylated histone H3. At different chase times, clusters of BrdU-labeled cells appear, labeled cells migrate to formerly unlabeled areas, and they differentiate into several somatic cell types and into germ cells. Finally, continuous exposure to BrdU shows an extensive renewal of the epithelial cells. Altogether, these results strengthen the idea of platyhelminth neoblasts as an unparalleled stem-cell system within the Animal Kingdom calling for further investigation. © 2000 Academic Press

Key Words: stem cells; neoblasts; platyhelminthes; *Macrostomum*; bromodeoxyuridine; cell cycle; cell labeling; cell renewal; cell migration

INTRODUCTION

Stem cells are, by definition, undifferentiated cells which have the capacity to self-renew and to generate differentiated progeny (reviewed in Morrison *et al.*, 1997). They are found in many metazoan taxa, from sponges to vertebrates (reviewed in Potten, 1997). In the adult organism, the mechanisms by which stem cells proliferate and differentiate into other cell types have been extensively studied in certain model systems such as the mammalian hematopoietic system (Morrison *et al.*, 1995), gut epithelium (Potten and Loeffler, 1990), and epidermis (Jones, 1997; Jensen *et al.*, 1999), as well as in lower Metazoa such as the coelenterate hydra (Bode, 1996). Recent, renewed interest in stem cells, namely in mammals, has been fostered by their finding in unexpected tissues (e.g., the central nervous system; McKay, 1997) and by their potential to produce transgenic organisms (Mansour *et al.*, 1988; Grasreld and Kollias, 1992)

and to be used as vectors for gene therapy (Keller and Snodgrass, 1999).

Platyhelminthes possess a stem-cell system which may be considered unique in the Animal Kingdom (Baguña, 1981). This is because their stem cells, or neoblasts (see Brønsted, 1969, for general references) are the only cells in the adult that divide and differentiate into all cell types, including germ cells (Sauzin-Monnot, 1973; Hori, 1997). In free-living Platyhelminthes (the so-called "Turbellaria"), neoblasts are small, ovoid, undifferentiated basophilic cells found within the parenchyma, the tissue filling the space between the epithelium and the gut (Pedersen, 1959; Rieger *et al.*, 1991a, 1999; Ehlers, 1995; Hori, 1997). After whole organisms are dissociated into single cells by enzymatic treatment or maceration (Baguña and Romero, 1981) neoblasts are easily identified by their small size and very high nuclear to cytoplasmic ratio. Ultrastructurally, neoblasts bear a big nucleus with a prominent nucleolus, and a scanty

rim of cytoplasm filled with free ribosomes, a few mitochondria, and some or no endoplasmic reticulum (Pedersen, 1972; Palmberg, 1990; Morita, 1995; Hori, 1997). Cells similar to neoblasts, the so-called "germinative cells," have also been reported in several parasitic Platyhelminthes, and their paths of differentiation to some somatic cell types and to germ cells were described (Gustafsson, 1976).

Neoblasts have been particularly well studied in flatworms of the order Tricladida, namely freshwater planarians. In quantitative terms they comprise from 20 to 30% of the total number of cells (Bagaña and Romero, 1981). More than 50% of their neoblasts are postmitotic, 40% form a transit amplifying population, and a mere 3–5% represent the likely true stem cell. This renewal system gives rise to all differentiated cell types, maintains its own population by cell proliferation, and, therefore, plays a key role in the unending processes of growth and degrowth. In addition, freshwater planarians and some microturbellarians show high powers of regeneration (reviewed in Bagaña, 1981, 1998; Gremigni, 1988; Bagaña *et al.*, 1994). In this process, neoblasts seem to be the main, if not the sole, source from which blastema cells originate (Bagaña *et al.*, 1989). With the exception of the totipotent embryonic stem cells from mammals (Heath *et al.*, 1990; Snodgrass *et al.*, 1992), no other renewal system in the Animal Kingdom matches the potentialities of the neoblast system. Indeed, even in the thoroughly studied Hydra (Bode, 1996), interstitial cells, a sort of stem cells similar to neoblasts, give rise to germ cells but only to a limited repertoire of somatic cells because epidermal and gastrodermal cells are differentiated cells which retain the capacity to proliferate.

Detailed analysis of the pool and heterogeneity of neoblasts, their rate of proliferation and differentiation into sets and subsets of differentiated cells, and their eventual migration to different body regions needs efficient methods of labeling cells. Tritiated thymidine and, more recently, bromodeoxyuridine (BrdU), both labeling cells at the S period of the cell cycle, seem the most suitable markers. In Platyhelminthes, though, their application has been scanty. Thymidine labeling and electron microscopy were used by Palmberg (1990) in the microturbellarian *Microstomum lineare* to trace, up to 45 h of regeneration, differentiation of neoblasts to epithelial, muscle, neuron, rhabdite, and flame cells. In the polyclad *Notoplana humilis*, replacement of the epidermis was followed after thymidine incorporation (Drobysheva, 1988). And in the cestode *Diphyllobothrium dendriticum*, cell cycle analysis, DNA synthesis, and cytodifferentiation of germinative cells to different somatic cell types were studied by thymidine labeling (Gustafsson, 1976; Wikgren and Gustafson, 1967). Attempts to identify cycling cells with thymidine or BrdU in the well-studied freshwater planarians have been unsuccessful (Best *et al.*, 1965; Coward, 1979; Bagaña *et al.*, 1994). Recently, the successful incorporation of BrdU into neoblasts in different freshwater planarians has been reported (Newmark and Sanchez Alvarado, 2000), its migration and differentiation into some differentiated cell types tracked, and its contri-

bution to blastema formation explored. The unparalleled renewal system that neoblasts represent warrants further investigation using BrdU labeling in marine microturbellarians. In addition to their good regenerative and growth/degrowth capabilities, marine microturbellarians have over freshwater planarians the advantages of their canonical spiral cleavage and unmodified direct embryonic development.

In this paper we use BrdU labeling to study the distribution, differentiation, and migration of neoblasts in the microturbellarian *Macrostomum* sp. We report the discovery of neoblasts being distributed in two lateral bands and only few, probably gastrodermal, neoblasts in the median area of the animal (see Discussion) and a complete lack of S-phase cells and mitosis in the region anterior to the eyes. We further provide evidence for medially and rostrally migrating BrdU-labeled cells and for differentiation of neoblasts into different somatic cell types and into germ cells. In our view, a detailed analysis of the neoblast pool in platyhelminthes together with a deep knowledge of the origin and fates of these cells in growth, development, cell renewal, and regeneration would promote a better understanding of other stem-cell systems in higher organisms.

MATERIALS AND METHODS

Animals

With over 100 species, the genus *Macrostomum* is a large taxon of the most basal group (Macrostomorpha) within the Rhabdiorhapha, which is in turn the largest of the three monophyletic clades of the Platyhelminthes (Ehlers, 1985). The species used in our study, *Macrostomum* sp. (Fig. 1), was collected in the northern Adriatic Sea in intertidal fine sands of the Lagune of Lignano (45°41'31" N and 13°07'53" E; World Geodesic System 1984), Italy.

Animals of *Macrostomum* sp. are 1.2–1.5 mm long. Two eyes mark the posterior border of the brain; the anterior body region including the area of the brain is referred to as the rostrum. Immediately behind the eyes, the mouth opening leads into a ciliated pharynx tube and further on into the ciliated gut. The animals use a special tale plate ("adhesive plate") for adhering to the substrate. Small duo-gland adhesive organs are concentrated in a horseshoe-shaped area on the ventral side of the adhesive plate. Small rod-shaped gland secretion products (rhabdites) occur in the dorsal and ventrolateral body wall. Similar glands (rhammite glands) in the anterior region of the gut form tracks which penetrate the brain and open at the tip of the rostrum. The animals are hermaphrodites, the paired testes are located anterior to the female gonads and the female pore lies anterior to the male pore. The sclerotized stylet of the male copulatory organ is the most important taxonomic feature: it distinguishes the species as a member of the *Macrostomum tuba* clade. The male copulatory stylet of all these species is characterized by a blunt ending at the distal opening.

The animals were kept in culture as described by Rieger *et al.* (1988). Prior to experiments, *Macrostomum* sp. were starved for 2 days. Hatchlings were collected 1–2 h after they had emerged from the egg capsule. Juveniles could be distinguished from adults by their smaller size and by the lack of gonads and associated structures. Unless otherwise stated, all experiments were run at 20°C.

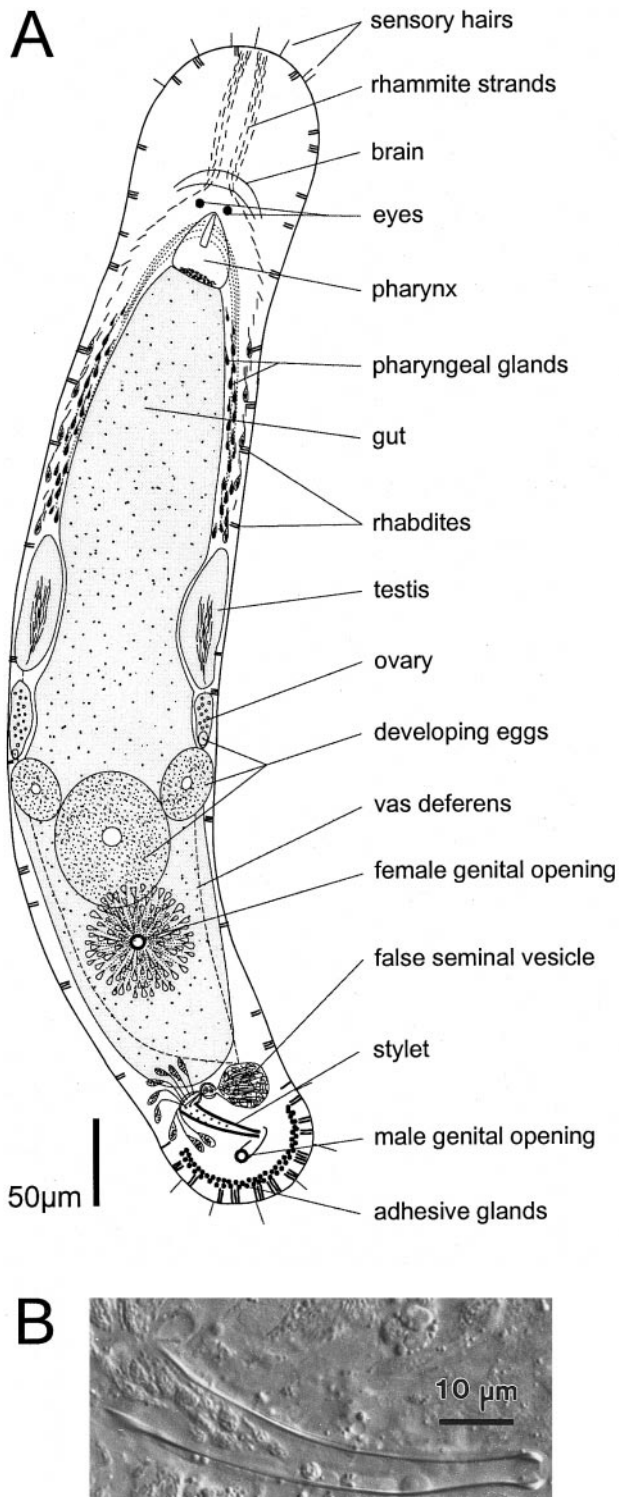


FIG. 1. Diagrammatic ventral view of adult *Macrostomum sp.* Schematic drawing after life specimens (A) and Nomarski interference contrast image of stylet (B).

***BrdU* Labeling and Immunocytochemical Localization**

The thymidine analog 5'-bromo-2'-deoxyuridine (Sigma) is incorporated into nuclei of cells in or entering S phase during pulse periods. The organisms were labeled with BrdU by incubation: (1) 30 min in 5 mM BrdU in artificial sea water (ASW) for short pulse experiments; BrdU-incorporation could be observed already after 5 min pulse (data not shown); (2) in 50 μM BrdU in ASW for continuous labeling experiments; animals were kept in petri dishes in darkness without feeding until the appropriate labeling time; and (3) 30 min in 5 mM BrdU in ASW for pulse-chase experiments followed by a washing series (5×) with ASW and different chase times (4, 6, 10, 12, 20, and 24 h); during chase times organisms were maintained in standard culture conditions without feeding.

Animals were relaxed in 1:1 7.14% MgCl₂:ASW for 5 min and fixed for 60 min in 4% paraformaldehyde (PFA) followed by several rinses with PBS and incubation in PBS-T (PBS plus 0.1% Triton X-100) for 30 min. Specimens were incubated in 0.075 μg/ml Pronase E for 15 min at 37°C, exposed to 0.1 N HCl for 10 min on ice and 2 N HCl for 1 h at 37°C, washed several times with PBS, and blocked in BSA-T (bovine serum albumin plus 0.1% Triton X-100) for 30 min. Incorporated BrdU was localized using a monoclonal mouse anti-BrdU antibody (Sigma) at 1:1000 dilution in BSA-T overnight at 4°C. After being washed with PBS (3 × 10 min) secondary FITC- or TRITC-conjugated goat-anti mouse antibodies (DAKO) were applied for 1 h at room temperature at 1:100 and 1:150 dilution in BSA-T, respectively. After being washed with PBS (3 × 10 min), slides were mounted using Vectashield (Vector Laboratories).

Labeling for Mitosis

To arrest cells in metaphase, organisms were incubated in 0.05% colchicine (Sigma) in ASW for 24 h. After being washed (3×) in ASW, they were relaxed in 1:1 7.14% MgCl₂:ASW for 5 min, fixed in 4% PFA for 1 h, washed 3 × 15 min with PBS-T and blocked with BSA-T for 30 min. The primary antibody, anti-phosphorylated histone H3 (Upstate Biotechnology, Inc., Lake Placid, NY), was diluted 1:300 in BSA-T and applied overnight at 4°C followed by washing 3 × 15 min with PBS. Incubation with the secondary antibody (swine anti-rabbit-FITC, diluted 1:100 in BSA-T; DAKO) for 1 h was followed by a PBS washing series.

Number of Total Cells and Identification of Cell Types by Maceration

The number of total cells was determined in macerated cell preparations following the protocol of Baguña and Romero (1981) for freshwater planarians, slightly modified. Five animals were pooled for each experiment. They were washed in calcium/magnesium-free medium (CMF) and incubated in 100 μl CMF plus 1% trypsin for 1 h at 37°C. One hundred microliters of maceration solution (glacial acetic acid:glycerol:distilled water 1:1:13) was added and tubes were carefully rocked. The animals fell apart into single cells and were either counted using a hemocytometer or centrifuged on poly-L-lysine-coated slides using a cytospin centrifuge. The BrdU staining procedure for macerated cell preparations was identical to that described above for whole-mount preparations, except that the Pronase E and 0.1 N HCl steps were omitted. Cells were identified after maceration following the criteria set in Baguña and Romero (1981). Specifically, neoblasts appeared as

small cells (5–10 μm), rounded, with a large nucleus, and scanty cytoplasm and sometimes bearing a short process of 5–15 μm that may contain a lipid droplet. The number of neoblasts was estimated following the method of Schürmann *et al.* (1998).

Epifluorescence, Nomarski, and phase-contrast microscopy was performed on a Reichert POLYVAR. Selected samples were then further analyzed by confocal microscopy using a Zeiss LSM 510. The confocal images were processed using the LSM 510 software and the Adobe PhotoShop 5.0 program. Total number of cells, the number of S-phase cells labeled with BrdU, and the number of mitotic cells labeled with anti-phosphorylated histone H3 are expressed as means \pm standard deviation.

RESULTS

Adult (1200–1500 μm) *Macrostomum* sp. have a total number of $24,708 \pm 3831$ ($n = 19$) cells. Cell separations according to Schürmann *et al.* (1998) suggest that the total percentage of neoblasts is about 20%. Roughly, 10% of neoblasts are in S phase and 0.5% in mitosis. Hatchlings (240–300 μm) have an average of 5465 ± 761 ($n = 5$) total cells, 164 ± 29 ($n = 9$) are in S-phase and 10.2 ± 4.6 ($n = 59$) in mitosis. The number of neoblasts of hatchlings was not determined.

Spatial Distribution of S-Phase Cells

After 30-min and 4-h (not shown) incubations in BrdU, maceration of organisms into single cells (see below) showed all S-labeled cells to be neoblasts (Fig. 2D). Based on the analysis of macerated cell populations all of the S-phase cells observed in intact preparations were assumed to be neoblasts. S-phase neoblasts are spherical or ovoid, with the characteristic high nuclear cytoplasmic ratio (Fig. 7E).

After 30-min incubations, a total of 164 ± 31 ($n = 19$) S-phase neoblasts are found in the hatchlings and 435 ± 79 ($n = 21$) in adult *Macrostomum* sp. They are distributed in two bands along the lateral sides of the organism (Figs. 2B and 2C for hatchlings-juveniles; Fig. 3B for adults). Within each band in the adults, two clusters of S-phase neoblasts are observed in the gonads (Fig. 3B). The larger anterior group, made by 26.7 ± 7.6 ($n = 21$) S-phase cells, is in the testes, whereas the smaller posterior group, with 14.5 ± 4.3 ($n = 21$) S-phase cells, is in the ovary. S-phase neoblasts were never found in the rostrum, i.e., the region in front of the eyes, and are very rare along the median axis of the body and at the posterior edge of the tail plate. The highest density of somatic S-phase neoblasts along the median axis is found in two small areas: posterior to the mouth opening at the level of the postpharyngeal commissure (for nervous system of macrostomid species see Ladurner *et al.*, 1997) and in the tail plate. Epidermal cells are never labeled. In the gut region only one or two S-phase neoblasts were visible in one-third of all specimens studied.

After 4 h incubation in BrdU (data not shown), the number of S-phase cells increases slightly and pairs of dividing cells appear at the level of the eyes, similar to what happens after a 30-min incubation and a 4-h chase (see Fig.

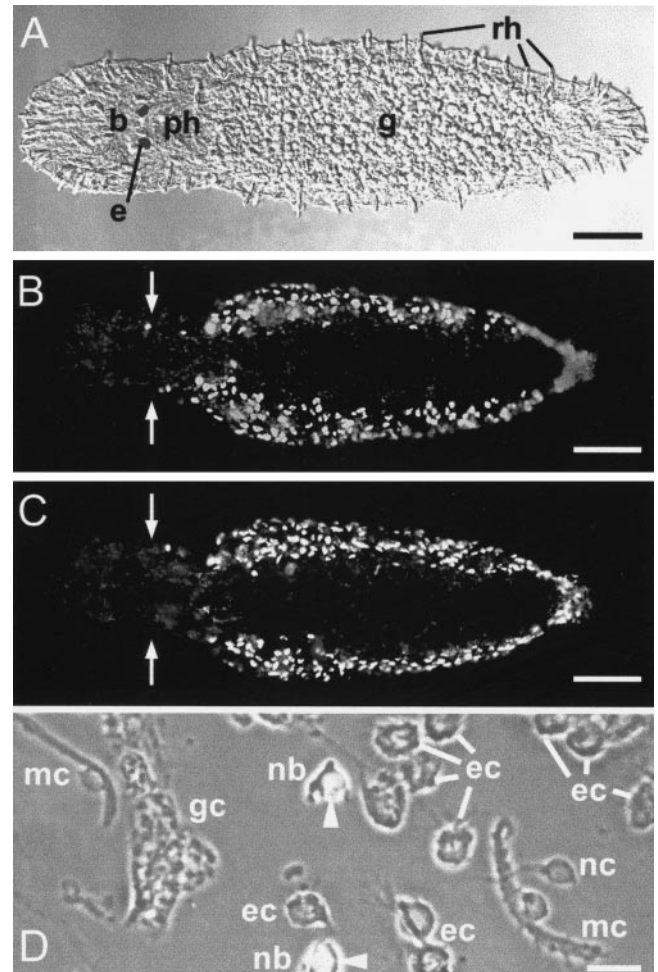


FIG. 2. Labeled S-phase cells in juvenile *Macrostomum* sp. after a 30-min pulse (no chase) with BrdU. (A) Nomarski interference contrast image; b, brain; e, eye; g, gut; ph, pharynx; rh, rhabdites. (B, C) Epifluorescence images of dorsal (B) and ventral (C) sides of juvenile *Macrostomum* sp. after a 30-min BrdU pulse. Labeled S-phase cells are found in two bands along the lateral sides of the animal. No labeled cells are present anterior to the eyes, along the median axis, or at the posterior edge of the tail plate. Arrows indicate level of the eyes. In all images, anterior is to the left. (D) Phase-contrast and fluorescence image of macerated cells of 30-min BrdU-pulsed animals; two labeled S-phase neoblasts (nb) with fluorescent nuclei (arrowheads) are visible; no differentiated cells contained labeled nuclei. ec, epidermal cell; gc, gut cell; mc, muscle cell; nb, neoblast; nc, nerve cell. Scale bars, 50 μm (A–C) and 10 μm (D).

9A and below). All S-phase cells were shown to be neoblasts, and cells other than neoblasts (e.g., epidermis, muscle, gut, . . .) were never labeled.

Spatial Distribution of Mitotic Cells

To examine the distribution of mitotic cells, organisms were incubated in 0.05% colchicine in ASW for 24 h and

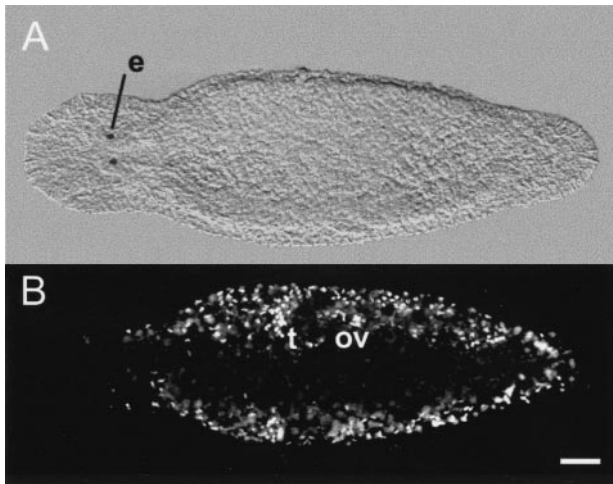


FIG. 3. Labeled S-phase cells in adult *Macrostomum* sp. after 30-min pulse (no chase) with BrdU. (A) Nomarski interference contrast reference image of adult *Macrostomum* sp.; e, eye. (B) Epifluorescence image of BrdU cells after a 30-min pulse. Note bilateral distribution of BrdU cells and clusters of labeled cell in testes (t) and ovaries (ov). In both images, anterior is to the left. Scale bar, 50 μ m.

mitotic cells stained using an antibody against phosphorylated histone H3. A total of 315 ± 135 ($n = 13$) metaphase cells appeared distributed in two bands along the lateral sides of the animal (Fig. 4). No mitoses were found in front of the eyes. Along the median axis single mitotic cells were detected, probably representing neoblasts in the gastrodermis (see Discussion). These data indicate that cell divisions occur mainly along the lateral sides of the animal, as also found for S-phase cells after 30 min BrdU pulse (Figs. 2B, 2C, and 3B) and 4 h pulse (data not shown).

Continuous BrdU Labeling and Identification of Labeled Cell Types by Maceration

Continuous 7 and 14 days exposure to BrdU showed extensive cell differentiation and cell renewal in *Macrostomum* sp. After 7 days continuous exposure to BrdU, at least 10% of epidermal cells were replaced by BrdU-labeled cells. After 14 days exposure, approximately one-third of all epidermal cells were BrdU-labeled cells (Figs. 5B and 5C). From pulse–chase experiments we know that 2 days are needed for labeled cells to be incorporated into the epidermis. This might explain the relatively low number of labeled epidermal cells after 7 days of continuous BrdU exposure (Fig. 6). Differentiation of neoblasts into other cell types was followed after maceration of animals into single cells. Isolated cells were mounted on slides and stained for BrdU. We found a variety of differentiated BrdU-labeled cells including epidermal cells (Fig. 7A), gut cells (Fig. 7B), and muscle cells (Fig. 7C). In addition, labeled spermatogo-

nia and spermatids and sperm cells with their characteristic elongated nucleus were observed after 14 days continuous BrdU exposure (Fig. 7D). Oogonia and oocytes were also labeled (not shown).

Pulse and Chase Experiments and Migration of Cells

To investigate patterns of cell division and cell migration of BrdU-labeled cells, pulse and chase experiments were performed. After a 30-min pulse and 4-h chase, labeled cells are still restricted to the lateral bands as seen after 30-min pulse and no chase (see Fig. 3B). After a 30-min pulse and 12-h chase, some labeled cells are seen medially and in the rostrum (Fig. 8). In maturing individuals, labeled cells were seen to cluster at the developing genital blastema (gb, Fig. 8). After a 4-h pulse–chase, up to 45 pairs of BrdU-labeled cells are seen in adult *Macrostomum* and up to 25 pairs in juveniles (immature individuals). These pairs likely result from divisions of labeled cells. Variation in the number of pairs between individuals was high. In juvenile animals four or more pairs of BrdU cells were detected behind the mouth opening and behind the eyes (Fig. 9A). These pairs were usually oriented along the anterior–posterior body

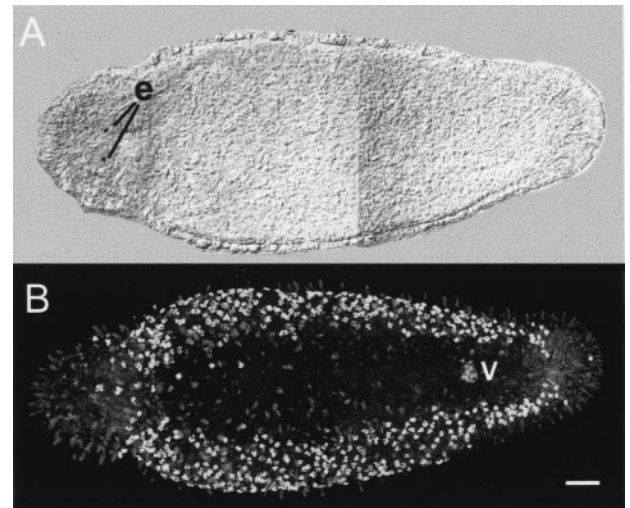


FIG. 4. Immunostaining of subadult (maturing) *Macrostomum* sp. labeled to detect mitotic cells after 24 h colchicine treatment. (A) Nomarski interference contrast image; e, eyes. (B) Confocal projection (ventral view) of *Macrostomum* sp. labeled with an anti-phosphorylated histone H3 antibody (FITC green fluorescence). Labeled cells are distributed in two lateral bands. After this 24 h colchicine treatment the number of mitotic profiles appears to be similar to the number of S-phase cells after 30 min BrdU application (compare Fig. 2). Shorter colchicine treatment results in fewer labeled mitotic cells (image not shown). A cluster of labeled cells occurs at the developing vagina (v). No labeled cells are present in epidermis, gut, or the region anterior to the eyes. In both images, anterior is to the left. Scale bar, 50 μ m.

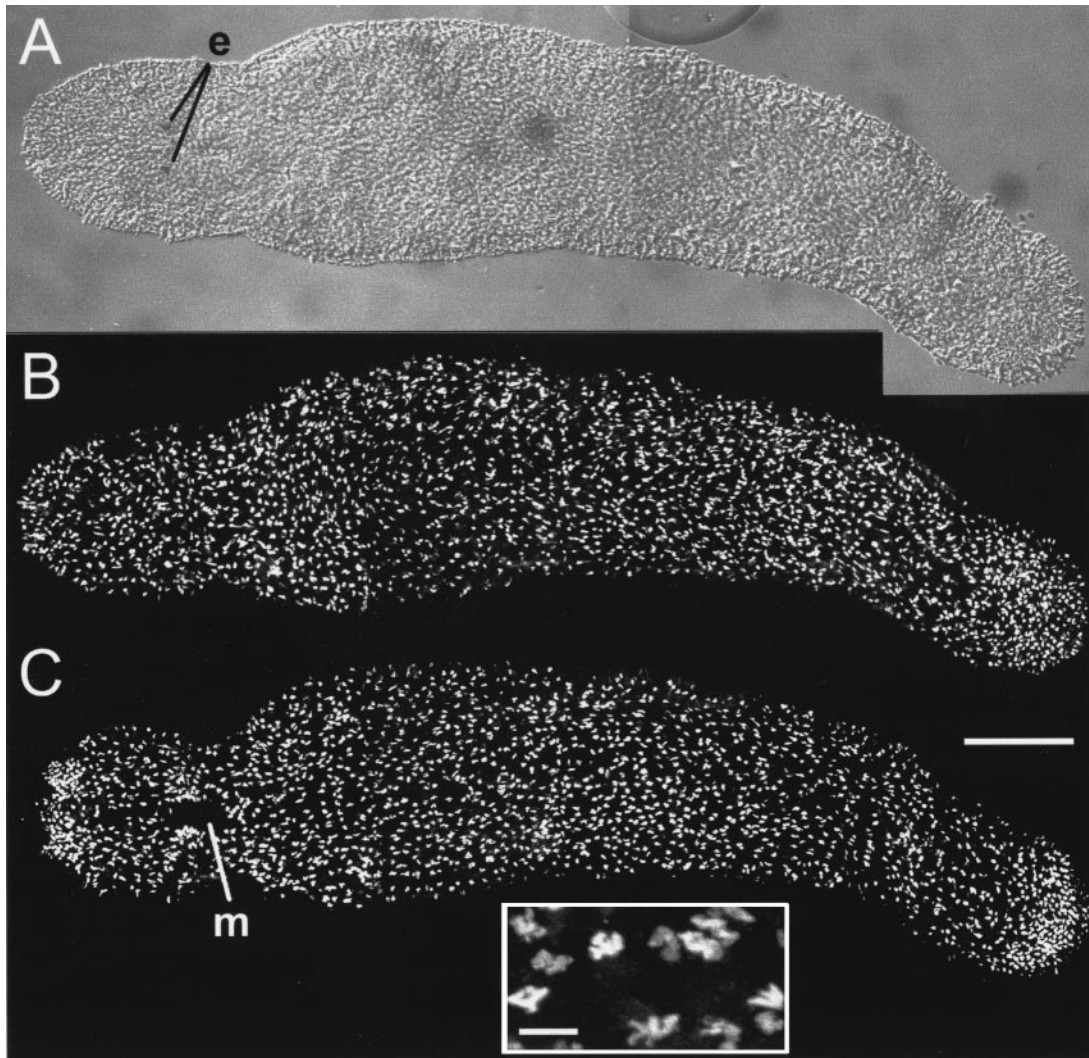


FIG. 5. Labeling of S phase in *Macrostomum* sp. after continuous exposure to BrdU. (A) Nomarski interference contrast reference image; e, eyes. (B, C) Confocal projections of dorsal (B) and ventral (C) layer showing BrdU-labeled epidermal nuclei after 14 days of continuous BrdU exposure. Inset shows lobulated epidermal nuclei. In all images, anterior is to the left. Scale bars, 50 μm (A–C) and 5 μm (inset).

axis. After a 12-h chase, clusters of four cells could be observed at the lateral sides (Fig. 9B). To estimate the rate of cell migration to the rostral area (see Fig. 8) the number of BrdU-labeled cells in front of the eyes was measured after different chase periods. Thirty minutes after BrdU incorporation no S-phase cells were seen in the rostrum. However, after 6 h chase, 1.2 ± 1.1 cells ($n = 17$) were already there, and 11.3 ± 4.4 cells ($n = 11$), 12.5 ± 4.5 cells ($n = 7$), and 13.8 ± 2.3 cells ($n = 9$) were seen at 12, 20, and 24 h chase times, respectively. The first BrdU-labeled cells reached the anterior tip of the rostrum at about 12 h chase time (Fig. 8). This suggests an average migration rate of $6.5 \mu\text{m}/\text{h}$.

DISCUSSION

We present here the incorporation of BrdU into neoblasts of the marine platyhelminth *Macrostomum* sp. and the distribution of labeled cells along the body axis. We also report on the cell migration and cell renewal capabilities of neoblasts and on their differentiation into several somatic cell types and, most importantly, into germ cells. Together with the recent report of BrdU incorporation in freshwater planarians (Newmark and Sanchez Alvarado, 2000), this paper shows incorporation of BrdU into neoblasts of Platyhelminthes. These findings open new avenues of research

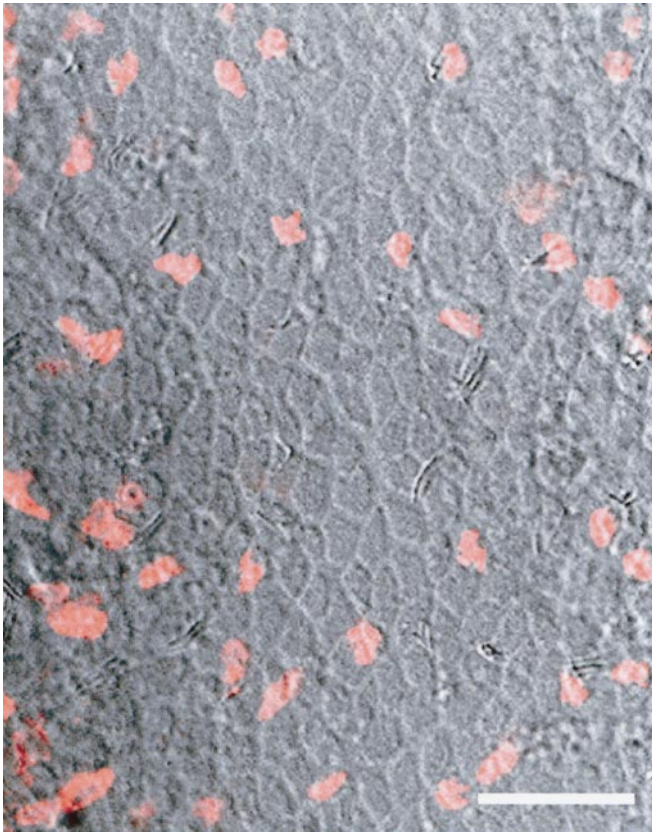


FIG. 6. Cell renewal of the epidermis after 7 days continuous exposure to BrdU. New epidermal cells (pink nuclei, as stained with TRITC-conjugated secondary antibody) intercalate between old cells. Nomarski interference contrast. Scale bar, 20 μm .

into this stem-cell system which has no parallel in the Animal Kingdom.

Distribution of S-Phase Cells and Mitotic Cells in *Macrostomum sp.*

S-phase cells in *Macrostomum sp.* are distributed in two main lateral bands. First evidence for a bilateral distribution of neoblasts in macrostomids was provided by Rieger *et al.* (1994) in an ultrastructural reconstruction of serial sections of the caudal region of *Macrostomum hystricinum marinum*. Neoblasts were identified because of their characteristic pattern of heterochromatin. A clear bilateral distribution was observed for neoblasts in the mesoderm (Rieger *et al.*, 1994; Fig. 9D). The so-called gut neoblasts, fewer in number and identifiable by their heterochromatin pattern, had random positions at the dorsal and lateral sides. The pattern of BrdU labeling reported here supports these ultrastructural findings. The very few BrdU-labeled cells appearing along the median axis of the gut region should correspond to the gut neoblasts. The bulk of labeled cells at the

two lateral edges might correspond to the mesodermal neoblasts.

The concentration of S-phase neoblasts in a zone along the main longitudinal nerve cords has not been observed in planarians (Newmark and Sanchez Alvarado, 2000). The lateral distribution of S-phase labeled cells may be a reflection of the topological constraints imposed upon neoblasts by the distribution of the parenchyma, the mesodermal tissue between epidermis and gut in Platyhelminthes. Cross sections of *Macrostomum* show more space available between the gut and the lateral edges of the epidermis than between the gut and the dorsal epidermis. Even so, other species of macrostomids with fairly similar spatial constraints show different neoblast distributions. A more promising way is to consider a relationship between neoblast proliferation and the topology of the central nervous system in *Macrostomum sp.*, namely with the lateral nerve

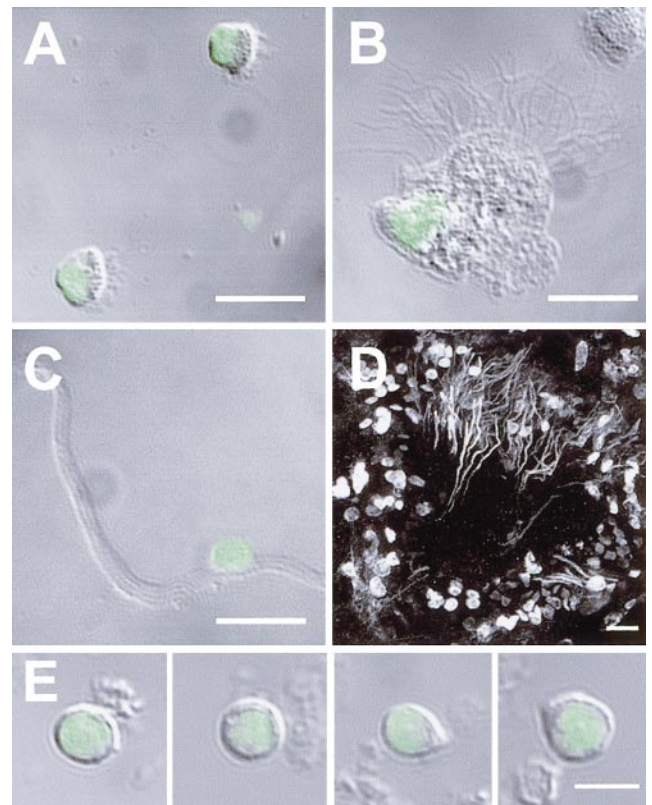


FIG. 7. Superimposition of interference-contrast and fluorescence images of macerated labeled cells; nuclei are stained green (FITC-conjugated secondary antibody). (A–D) Differentiated cells after continuous 14-day exposure to BrdU; (E) neoblasts after 30-min BrdU pulse. (A) Differentiated epidermal cell, (B) gut cell, (C) muscle cell, (D) whole-mount image of a testis showing several stained spermatogonia (round bright nuclei at the periphery) and sperm cells (filiform bright nuclei at top). (E) Four BrdU-labeled neoblasts after 30-min pulse. Scale bars, 10 μm .

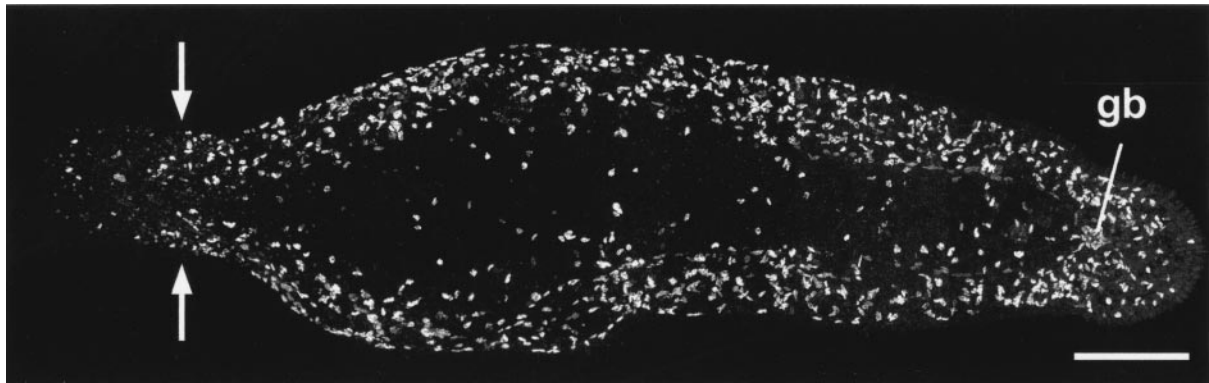


FIG. 8. Evidence for cell migration of S-phase cells in *Macrostomum* sp. after short pulse–long chase experiments. After 30-min pulse–12-h chase, most labeled cells form two bands at the lateral edges of the body (confocal projection). However, some cells have migrated to the median area of the body and to the area in front of the eyes (for comparison, see Fig. 3B). A cluster of labeled cells is also seen at the male genital blastema (gb). Arrows indicate the level of the eyes. Anterior is to the left. Scale bar, 50 μm .

cords (for the organization of the nervous system in two macrostomid species, see Ladurner *et al.*, 1997).

A more intriguing feature is the lack of S-phase cells in the region in front of the eyes (Figs. 2B, 2C, and 3). Such a deficiency of proliferative cells in front of the eyes has been observed also by Newmark and Sanchez Alvarado (2000) for planarians. This parallels the lack, or near lack, of mitotic cells in this region even after long (24 h) exposure to colchicine in *Macrostomum* (Fig. 4). Lack of mitotic figures in front of the eyes has been repeatedly observed in freshwater planarians (e.g., Baguña, 1976). Because neoblasts were also found there, likely involved in renewal of head tissues and cells, they should have migrated from regions behind the eyes and be committed to any of the head-specific cell types either before migration or once they are near the target cell to be replaced. That this migration actually takes place has been shown in freshwater planarians (Newmark and Sanchez Alvarado, 2000). In *Macrostomum*, the finding of BrdU-labeled cells in front of the eyes after pulse and chase (e.g., 12 h; Fig. 8) and the extensive renewal of epidermal cells (some at the very anterior tip of the body) after continuous BrdU labeling (Fig. 5), together with the lack of mitoses in front of the eyes, support extensive cell migration of nonproliferating, and likely determined, neoblasts in lower as well as in higher Platyhelminthes.

Migration of S-Phase Cells

The migration of S-phase cells observed in *Macrostomum* sp. follows two main routes. First, as seen after a 4-h chase period, a few cells move from the lateral sides to the dorsal and ventral areas at the median plane; this phenomenon continues steadily as seen after longer chasing times. Second, BrdU-labeled cells also migrate to the rostrum, as demonstrated with the same techniques for planarians (Newmark and Sanchez Alvarado, 2000). In *Macrostomum*,

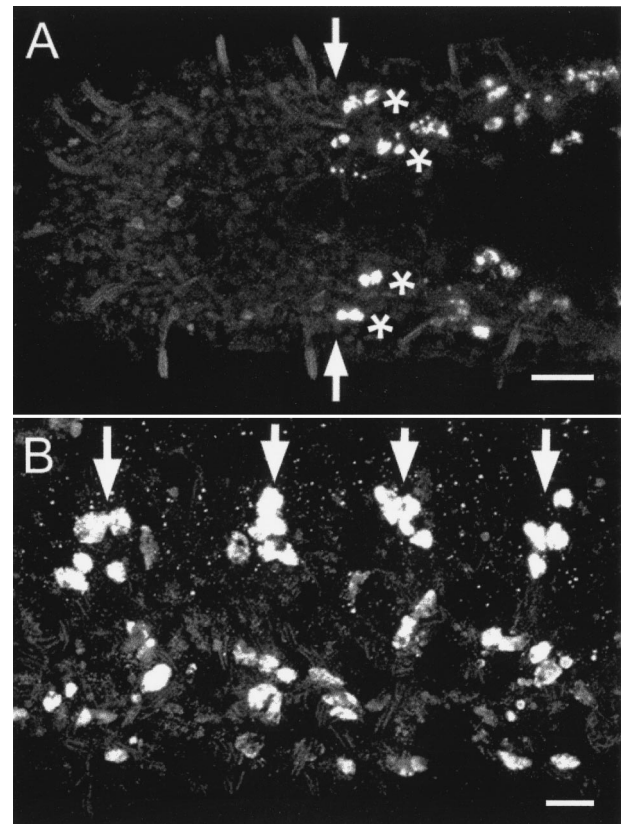


FIG. 9. Clusters of BrdU-labeled cells in *Macrostomum* sp. after short pulses and different chase times. (A) Confocal projections of a 4-h pulse–chase head area with labeled cells in two-cell clusters (asterisks) oriented along the anterior–posterior body axis. Arrows indicate the level of the eyes. (B) Confocal projection of the left edge of the body with labeled cells in four-cell clusters (arrows). In both images, anterior is to the left. Scale bar, 10 μm .

the number of labeled S-phase cells migrating into the rostrum does not increase significantly after 12, 20, and 24 h (30-min pulse). Because the pulse was short there is a limited number of labeled cells becoming determined and migrating to the rostrum. These few cells are distributed within the rostrum already after 12 h and therefore the number of labeled cells does not increase after longer chase times.

The estimated rate of cell migration, 6–7 $\mu\text{m}/\text{h}$, despite being lower than the 40–50 $\mu\text{m}/\text{h}$ of typical migratory cells (e.g., fibroblasts), is likely to result from active cell migration. Previous data on neoblast migration rates in freshwater planarians (40 $\mu\text{m}/\text{day}$ in intact organisms, 120–140 $\mu\text{m}/\text{day}$ in regenerating organisms; Saló and Bagaña, 1985) as measured using chromosomal markers, were suggested to result, with the exception of cells close to the wound during regeneration (Saló and Bagaña, 1989), mainly from the stochastic movements associated with cell division and not from active cell migration. However, the likely cell migration from areas behind the eyes to the anterior body tip could not be measured using these markers.

S-phase cells and mitoses occur at certain positions in the lateral edges of the mouth opening posterior to the eyes, a phenomenon not observed by Newmark and Sanchez Alvarado (2000) for planarians. In addition, after a 4-h pulse-chase (Fig. 7A), pairs of BrdU-labeled cells usually are aligned along the anterior–posterior axis. It might be that these cells are located in a “niche,” such that after cell division one daughter remains in place, whereas the other migrates to the rostrum. Were it so, cell migration in *Macrostomum* sp. poses several interesting questions: (1) Are cell divisions symmetrical or asymmetrical? (2) If asymmetric division holds, are migrating cells already committed to specific cell types? (3) Are there preferred routes of migration and specific substrates upon which cells move? The close proximity of S-phase cells to the main lateral nerve cord may suggest a role for the nervous system in neoblast proliferation and guidance. Indeed, double labeling experiments of mitosis and the serotonergic and FMRF-amidergic part of the nervous system show that 80% of all mitoses are in direct contact with nerve fibers (Ladurner, unpublished).

Are Stem Cells in *Macrostomum* sp. Pluripotent or Totipotent?

The feature that distinguishes the neoblast system in Platyhelminthes as a unique stem-cell system in the Animal Kingdom is that renewal of the 20–30 differentiated cell types (germ cells included) rests upon a single stem cell, the neoblast. The evidence for this notion is based on (1) lack of mitoses in somatic cells (Bagaña, 1981; Ehlers, 1992a); (2) extensive light and electron microscopy data showing differentiation of neoblasts into any differentiated cell type, germ cells included (Sauzin-Monnot, 1973; Hori, 1997); (3) the pluripotency shown by injected purified neoblasts in irradiated intact and regenerating freshwater

planarians (Bagaña *et al.*, 1989); (4) the ability of tiny pieces from any axial level to regenerate a whole individual with the full set of somatic and germ cells; and (5) the scanty but informative data on thymidine labeling and cell differentiation in some microturbellarians (Palmberg, 1990).

Our results add further support to this evidence, with the additional bonus of easily traceable differentiation of S-phase cells to different somatic cells and to germ cells and showing renewal of cells and tissues (e.g., the epidermis) at an unprecedented scale. Three somatic cell types and sperm cells have been presented here (see Fig. 7). Differentiation of BrdU-labeled cells to other somatic cell types and to oögonia has also been observed (data not shown). The finding of BrdU-labeled germ cells is particularly relevant because previous results using thymidine (Palmberg, 1990) were actually obtained from an asexual strain of *M. lineare*. To our knowledge, and with the exception of germ cells derived from interstitial cells in *Hydra* (see Bode, 1996, for review), this is the first time that differentiation of stem cells into germ cells is reported in fully developed adult organisms. Based on our present data, however, we cannot exclude the possibility that germ cells differentiate from a special subset of S-phase cells. Recent reports on specific markers for germ cells and their precursors in freshwater planarians (e.g., the tyrosine kinase gene *DjPTK1*, Ogawa *et al.*, 1998, and the *vasa-like* genes *DjvlgA* and *DjvlgB*, Shibata *et al.*, 1999) will afford the finding of homologous genes in lower Platyhelminthes and the use of them, together with BrdU, in double labeling experiments to work out the pathway of differentiation to germ cells. The fact that BrdU incorporation in *Macrostomum* can be already observed after 5 min makes this microturbellarian better suited to such experiments than planarians that incorporate BrdU only with food or injection. Similar approaches could be applied to study the different pathways of differentiation to somatic cell types, using the increasing number of cell-specific genes reported in freshwater planarians (for recent compilations, see Bagaña, 1998; Agata and Watanabe, 1999).

Altogether, these results suggest that neoblasts (or a subset of them) can apparently give rise to all cell types in *Macrostomum* sp., germ cells included. However, whether all neoblasts, or a specific subset of them, retain their pluripotency is still an open question. In addition, to know whether neoblasts, or again a subset of them, could be regarded as totipotent cells, a single neoblast should be isolated and, either in culture *in vitro* or injected *in vivo*, shown to give rise to all differentiated cell types, including germ cells. However important, such experiments have, to our knowledge, never been attempted (see below).

The finding that almost a third of epidermal cells are replaced after 2 weeks continuous exposure to BrdU is remarkable. A simple extrapolation suggest that 5 weeks are needed to replace all epidermal cells. The fate of worn out cells has, to our knowledge, not been studied in macrostomids. In freshwater triclads epidermal cells are sloughed off (Skaer, 1965), whereas dying or lysing paren-

chyma (mesodermal) cells are phagocytosed by neighboring cells (Bowen *et al.*, 1976). These phenomena increase during degrowth by starvation and during remodeling processes in regenerating organisms (Bowen *et al.*, 1982). In the lower platyhelminth taxon Acoela, lacking a basement membrane, there is some evidence that degenerating epidermal cells (so-called "pulsatile bodies") are withdrawn into subepidermal digestive parenchymal tissues to be phagocytosed (Ehlers, 1992b). Because a complete basement membrane is wanting in the genus *Macrostomum* (Rieger *et al.*, 1991b) we cannot rule out the possibility that epidermal cells may also be withdrawn and phagocytosed in these animals.

Open Questions and Prospects

Because—as in all Platyhelminthes—the tissues and cells in *Macrostomum* sp. are in a steady state, stem cells must have an indefinite capacity for self-renewal. Less clear is the fraction of neoblasts that are "true" stem cells. Were neoblasts comprising only proliferating stem cells and postmitotic committed cells, then more than 50% of them would be stem cells. Should the population contain pluripotent or totipotent stem cells, transit amplifying populations with limited proliferative capacity, and postmitotic committed cells, then the population of stem cells could be considerably smaller. Data on freshwater planarians (Baguña *et al.*, 1990; see Introduction) support the latter model. In addition, the sole study so far using thymidine in the macrostomid *M. lineare* (Palmberg, 1990) supports the presence of transit cells in some somatic cell pathways.

A second set of questions is: (1) Are stem cells located everywhere or are they present at specific places? and (2) Do totipotent neoblasts exist at all or are neoblasts in Platyhelminthes a mixed population of pluripotential cells already committed to give different sets and subsets of somatic cells? Whereas the final answer to the second question must await the *in vitro* cloning of neoblasts from single neoblasts followed by testing their potentialities either *in vitro* or when injected into irradiated hosts, the answer to the first question is that stem cells seem to be everywhere. Pieces as small as a 1/300 of the total body of a planarian (a mere 10,000 cells), isolated anywhere between the head and the tail, will regenerate an entire animal (Montgomery and Coward, 1974). As these animals have all cell types, including germ cells when mature, stem cells must be present throughout the body axes.

A last, very important set of questions refers to how stem cells are committed to particular types of differentiation. Does this occur in a single step, or does it occur in two steps: first to class (e.g., nerve) and then to type (e.g., serotonergic)? Or do these steps refer first to topology (e.g., head versus trunk + tail), then to class (e.g., nerve) and further to type (e.g., serotonergic)? Or, alternatively, do these steps lead first to cell lineage (mesodermal versus endodermal) and then to class and type? And finally, is the commitment of a stem cell in any of these steps influenced

by external or internal factors? These are fascinating questions that the BrdU results here may help to unravel.

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