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Bromodeoxyuridine Specifically Labels the Regenerative Stem Cells of Planarians

Phillip A. Newmark[1] and Alejandro Sánchez Alvarado

Department of Embryology, Carnegie Institution of Washington, 115 West University Parkway, Baltimore, Maryland 21210

The singular regenerative abilities of planarians require a population of stem cells known as neoblasts. In response to wounding, or during the course of cell turnover, neoblasts are signaled to divide and/or differentiate, thereby replacing lost cell types. The study of these pluripotent stem cells and their role in planarian regeneration has been severely hampered by the reported inability of planarians to incorporate exogenous DNA precursors; thus, very little is known about the mechanisms that control proliferation and differentiation of this stem cell population within the planarian. Here we show that planarians are, in fact, capable of incorporating the thymidine analogue bromodeoxyuridine (BrdU), allowing neoblasts to be labeled specifically during the S phase of the cell cycle. We have used BrdU labeling to study the distribution of neoblasts in the intact animal, as well as to directly demonstrate the migration and differentiation of neoblasts. We have examined the proposal that a subset of neoblasts is arrested in the G2 phase of the cell cycle by double-labeling with BrdU and a mitosis-specific marker; we find that the median length of G2 (~6 h) is sufficient to account for the initial mitotic burst observed after feeding or amputation. Continuous BrdU-labeling experiments also suggest that there is not a large, slow-cycling population of neoblasts in the intact animal. The ability to label specifically the regenerative stem cells, combined with the recently described use of double-stranded RNA to inhibit gene expression in the planarian, should serve to reignite interest in the flatworm as an experimental model for studying the problems of metazoan regeneration and the control of stem cell proliferation.

Key Words: **planaria; regeneration; stem cells; neoblasts; flatworms; bromodeoxyuridine.**

INTRODUCTION

Adult freshwater planarians exhibit exceptional developmental plasticity that permits these simple, triploblastic metazoans to regenerate completely, whether cut transversely, longitudinally, or into tiny fragments (Brøndsted, 1969; Baguñà, 1998). Due to their unparalleled regeneration potential Dalyell concluded in 1814 that planarians could "almost be called immortal under the edge of the knife" (cited in Randolph, 1897). This "immortality" derives from a population of stem cells that remains mitotically active throughout adult life. These stem cells, referred to as neoblasts, are the only mitotic cells in the flatworm and they serve to replace cells lost during the course of cell turnover in the intact animal (Baguñà *et al.*, 1990). Following amputation, the neoblasts proliferate; their accumulation beneath the wound epithelium drives the formation of the regeneration blastema, the pigment-free regenerative bud in which postmitotic neoblasts will differentiate to reform the missing structures.

Classic work on planarian regeneration addressed the role of the neoblasts in blastema formation. Utilizing the observation that X-irradiation of an entire planarian eliminates the mitotically active stem cell population, as well as the organism's regenerative abilities (Bardeen and Baetjer, 1904; Curtis and Hickman, 1926), Wolff and Dubois performed a series of partial X-irradiation experiments, in which various portions of the animal were shielded from irradiation (reviewed in Wolff, 1962). If the anterior third of the animal was irradiated and the animal was left intact, the irradiated region became necrotic and died, with the unirradiated region surviving. If, however, the anterior third was irradiated and the head was then amputated, the irradiated region was repaired and a regeneration blastema was formed, albeit with a delay when compared with nonirradiated animals. This delay in blastema formation was proportional to the length of the body that received irradiation. Wolff and

[1] To whom correspondence should be addressed. Fax: (410) 243-6311; E-mail: newmark@mail1.ciwemb.edu.

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Dubois interpreted this delay to reflect the distance that neoblasts had to migrate through the irradiated region in order to reach the wound; wounding somehow served to stimulate this long-range migration and the proliferation of neoblasts. Histological methods and grafts using [^{3}H]UTP-labeled unirradiated tissues were used to confirm the repopulation of the irradiated region by neoblasts (reviewed in Lender, 1962; Lender and Gabriel, 1965).

Saló and Baguñà (1985, 1989) reexamined the issue of neoblast migration using chromosomal markers to distinguish between host- and graft-derived neoblasts. They concluded that movement of neoblasts far from the wound was not directed toward the wound, but was rather a nondirected spreading due "...to random movements occurring during and/or after cell division" (Saló and Baguñà, 1985). Subsequent experiments placed the grafted tissue closer to the amputation site and revealed that cells within 500 μm of the wound move two to three times more rapidly than cells farther from the wound (Saló and Baguñà, 1989).

The mechanism by which the wound signals the proliferation and directed movement of the neoblasts is not known, but there is an early mitotic peak that occurs in the initial 5–12 h of regeneration (Baguñà, 1976b; Saló and Baguñà, 1984). A similar peak is seen in intact animals following feeding (Baguñà, 1974; Baguñà, 1976a). These early mitotic peaks have been interpreted to reflect the existence of a large population of neoblasts in the G2 phase of the cell cycle, awaiting the appropriate signal to undergo division (Baguñà, 1974; Baguñà, 1976a,b; Saló and Baguñà, 1984). This observation has led to the suggestion that G2-arrested neoblasts represent the true stem cells, whereas the remainder of the neoblasts are already fated to give rise to specific differentiated cell types (Baguñà *et al.*, 1990).

Such potential heterogeneity of the neoblast population is important when one considers that injection of cell fractions highly enriched in neoblasts can rescue both the regenerative abilities and the long-term viability of lethally irradiated planarians (Baguñà *et al.*, 1989). Furthermore, injection of neoblasts from the sexual strain of *Schmidtea mediterranea* was capable of transforming lethally irradiated asexual individuals to a sexual mode of reproduction (Baguñà *et al.*, 1989). Thus, neoblasts (or a subset thereof) can apparently give rise to all cell types in the planarian, including the germ line, confirming earlier cytological work (reviewed in Lender, 1962; Fedecka-Bruner, 1965). Whether all neoblasts, or only a subset of them, retain their pluripotentiality is still an open question.

Analysis of this fascinating cell type and its role in regeneration has been hampered by the lack of neoblast-specific markers; thus, the original reports of neoblast localization and migration relied on rather general histochemical stains (Pedersen, 1959; Lender and Gabriel, 1960). As the only mitotic cell population in the planarian, neoblasts should be specifically labeled by exogenous DNA precursors during the S phase of the cell cycle. However, numerous reports in the literature concern the inability of planarians to incorporate (or take up) exogenous thymidine (Best *et al.*, 1965; Coward *et al.*, 1970; Martelly *et al.*, 1981); as recently as last year Kato *et al.* (1999) mentioned their inability to use bromodeoxyuridine to label proliferating cells in planarians. Thus, many workers have relied on much less specific methods for marking neoblasts. The inability to label planarians with exogenous DNA precursors has precluded careful analyses of the planarian cell cycle and led numerous investigators to abandon altogether their studies of planaria.

We have reexplored this issue and found that planarian cells are, in fact, capable of incorporating the thymidine analog bromodeoxyuridine (BrdU) (Gratzner, 1982). Here, we have used BrdU labeling to study the distribution, migration, and differentiation of neoblasts. We have also determined the length of the G2 phase by using BrdU labeling in combination with detection of a mitosis-specific marker (anti-phospho-histone H3; Hendzel *et al.*, 1997). Finally, continuous BrdU labeling has been used to examine the proposal that a subset of neoblasts are arrested in G2 (Baguñà, 1974; Baguñà, 1976a,b; Saló and Baguñà, 1984). Our results suggest that (i) the length of G2 suffices to explain the initial proliferative burst following feeding and amputation and (ii) there is not a large population of neoblasts arrested in G2.

MATERIALS AND METHODS

Organisms. A diploid ($2n = 8$), asexual strain of *Schmidtea* (formerly *Dugesia*) *mediterranea* (Baguñà, 1973; Benazzi *et al.*, 1975) was obtained from Barcelona, Spain. This species was maintained at room temperature (RT; 21–22°C) in dechlorinated tap water supplemented with 2 mM NaCl, 0.1 mM KCl, 0.1 mM $MgSO_4$, 0.12 mM $NaHCO_3$ and fed beef liver paste twice each week. *Girardia* (formerly *Dugesia*) *dorotocephala* and *Phagocata sp.* were obtained from Carolina Biological Supply and were maintained in dechlorinated tap water at RT. Animals were starved for 1 week prior to all labeling experiments.

BrdU labeling. Planarians were fed an artificial food mixture (Romero, 1987) containing BrdU (Sigma) at either 2.5 or 5 mg/ml. The BrdU was dissolved as a 10× stock in 40% EtOH. Twenty microliters was added to 80 μl of beef liver homogenate (~250 μl beef liver paste in H_2O to ~1 ml total volume; homogenized with a Kontes pestle in a 1.5-ml Eppendorf tube) and mixed well. One hundred microliters of 1% ultra-low gelling temperature agarose (Sigma A-5030) kept at 37°C was then added. Red food coloring (Durkee; 1 μl) was often included with the agarose to visualize ingestion of the mixture. After vortexing, the mixture was allowed to solidify on ice and fed to planarians, which commenced eating within a few minutes and finished 10–20 min later. Microinjections were performed as previously described (Sánchez Alvarado and Newmark, 1999).

Immunofluorescent detection of BrdU. At appropriate times following feeding or microinjection, planarians were killed in 2% HCl (30 s to 1 min) and fixed in Carnoy's for 2.5–3 h at RT. After rinsing in MeOH, fixed specimens were bleached overnight in 6% H_2O_2 in MeOH at RT. Planarians were either stored at −20°C in MeOH or immediately rehydrated through a MeOH/PBS+0.3% Triton X-100 (PBTx) series (75%, 50%, 25% MeOH). Following one

5-min wash in PBTx, planarians were rinsed in 2 N HCl (in PBTx) and then incubated for 30 min in 2 N HCl in PBTx. After PBTx rinsing, planarians were washed twice for 5 min in PBTx. In some cases a 30-min treatment in 2 N HCl (in H_2O) was followed by neutralization in 0.1 M borax (Sigma). Planarians were then blocked for 2–4 h in PBTx+0.25% BSA (PBTxB) at RT. Incubation in anti-BrdU was performed overnight at RT. Initial experiments reported here (Figs. 1–5) utilized an anti-BrdU monoclonal (Becton Dickinson) diluted 1:25 in PBTxB. Because of lot-to-lot variablity we subsequently switched to monoclonal G3G4 (George-Weinstein *et al.*, 1993), developed by S. J. Kaufman and obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa (Department of Biological Sciences, Iowa City, IA 52242). For experiments utilizing G3G4, animals were blocked in PBTx+10% nonfat dry milk and the ascites fluid was diluted 1:2000–1:4000 in the same solution including 0.02% sodium azide. Animals were washed for at least 4 h and up to 24 h in numerous changes of PBTx, with the final wash including either 0.25% BSA or 10% nonfat dry milk. Samples were incubated overnight at RT in secondary anti-mouse Alexa 488 (Molecular Probes), diluted 1:400 in PBTx+0.25% BSA or 10% nonfat dry milk, and washed extensively in PBTx. Planarians were mounted in Vectashield (Vector Laboratories, Inc.) and examined using a Leica TCS confocal microscope.

For detection of BrdU in macerates, planarians were placed in a maceration solution (glacial acetic acid:glycerol:water (1:1:13); David, 1973) and left to dissociate at 4°C at least overnight. Samples were gently triturated with a wide bore pipet, spotted onto subbed slides (VWR SuperFrost Plus or Surgipath Snowcoat X-tra), and left to dry 12–24 h at RT. Cells were washed twice for 5 min in PBS+0.5% Triton X-100 (PBTx(0.5)), treated for 30 min at RT in 2 N HCl and 0.5% Triton X-100, and neutralized for 1 min in 0.1 M borax. After two 5-min washes in PBTx(0.5) slides were incubated in anti-BrdU (1:2000 G3G4 in PBTx(0.5)) for 30 min at RT in a humid chamber. Following two 20-min washes in PBTx(0.5) slides were incubated in anti-mouse Alexa 488 diluted 1:100 in PBTx(0.5). Slides were washed twice for 20 min in PBTx(0.5), rinsed for 5 min in PBS, air-dried, and mounted in Vectashield containing 40 ng/ml DAPI (Sigma).

BrdU/anti-phospho-histone H3 double-labeling. To detect mitotic cells, a rabbit anti-phospho histone H3 antibody (anti-H3P; Hendzel *et al.*, 1997; Upstate Biotechnology) was used. Following BrdU incorporation by injection or feeding, animals were fixed and rehydrated as described above. Prior to acid denaturation, planarians were blocked and incubated overnight at RT in anti-H3P diluted 1:500 in PBTx+10% horse serum or 10% nonfat dry milk. After multiple PBTx washes (at least 8 h), samples were postfixed for 15 min at RT in 4% paraformaldehyde in PBS, washed twice in PBTx, and hydrolyzed and processed for BrdU detection as described above. Anti-rabbit Cy3 (Amersham) was diluted 1:1000 in the solution containing anti-mouse Alexa 488.

For the fraction of labeled mitoses time course (Fig. 6), 30 *S. mediterranea* individuals (4–6 mm in length) were fed an artificial food mixture containing either 2.5 or 5 mg/ml BrdU. Beginning 4 h postfeeding, five animals were fixed every 2 h and processed for anti-H3P and BrdU detection as described above. Specimens were examined by confocal microscopy using a 20× Neofluor objective. Optical sections were taken from tail regions of three to five animals per time point. BrdU/anti-H3P staining was counted on projections made from these sections. In a few cases signals were resolved by checking consecutive sections. One hundred forty to two hundred ninety mitotic cells were counted per time point.

Continuous BrdU labeling. To determine the time required for all neoblasts to enter S, *S. mediterranea* (4–6 mm in length; $n = 5$ per time point, totaling 35 animals) were injected every 6–8 h for 4 days with a solution of 10 mg/ml BrdU dissolved in water (prepared fresh for each injection set). Three 32-nl injections were delivered per time point per planarian. Samples were collected at the time points shown in Fig. 7. Planarians were cut into head, trunk, and tail pieces; macerated; and BrdU-detected as described above. BrdU-positive cells were counted using a 40× objective on a Zeiss Axiovert S-100 fluorescent microscope equipped with a SPOT digital camera (Diagnostic Instruments, Inc.). Fields of cells were captured under both phase-contrast and DAPI epifluorescence. All DAPI-labeled nuclei were counted and neoblasts identified based on their morphology [small (~5–10 μm) undifferentiated cells with scant cytoplasm and a prominent nucleus, often containing a distinct nucleolus, occupying most of the cell volume (Betchaku, 1967; Brøndsted, 1969; Baguñà and Romero, 1981)]. An example of a typical neoblast labeled with BrdU is seen in Fig. 3C, upper-right corner. To prevent bias, the BrdU-labeled cells were imaged only under green epifluorescence and the signals counted. The image of BrdU-labeled cells was superposed on the corresponding phase/DAPI image using Adobe Photoshop. Only cells scored independently as neoblasts and containing BrdU signal after superposition were scored as BrdU-positive neoblasts. For each time point 400–500 cells from the tail pieces of at least two different animals were counted.

RESULTS

Distribution of Proliferating Neoblasts in Intact Planarians as Visualized by BrdU Labeling

We were prompted to reexplore the reported inability of planarians to incorporate exogenous thymidine analogs when examining the feeding behavior of *Phagocata* hatchlings after emergence from the egg capsule. The hatchlings emerge with their intestinal tract filled with yolk ingested during embryogenesis. When the hatchlings are fed liver paste, the yolk is "dumped" into the parenchyma (mesenchyme) as the gut fills with liver. Therefore, inclusion of BrdU in the planarians' food seemed like a reasonable approach to deliver BrdU to the mesenchymally localized neoblasts; this approach has been used to label proliferative cells in *Drosophila* larvae (Truman and Bate, 1988).

Phagocata hatchlings ingested an artificial food mixture containing BrdU. Twenty-three hours after feeding, BrdU incorporation was observed in a mesenchymal cell population. Labeled cells were not observed in the pharynx or in the area in front of the photoreceptors (Fig. 1A); these areas have been previously described as devoid of mitotic activity (Baguñà, 1976a). Labeled cells display BrdU signal that either occupies the entire nucleoplasm or is confined to a punctate pattern within the nucleus. The former cells likely represent BrdU incorporation during the replication of euchromatin, whereas the latter likely reflect late-replicating heterochromatin (Dej and Spradling, 1999). Maceration experiments (see below) demonstrated that the first cells incorporating BrdU were neoblasts.

To eliminate the possibility that incorporation of BrdU

was peculiar to this species of *Phagocata*, we also tested the common North American planarian *G. dorotocephala*—the subject of the earlier studies reporting the inability of planarians to incorporate [^{3}H]thymidine (Best *et al.*, 1965; Coward *et al.*, 1970)—and the diploid, asexual strain of *S. mediterranea;* individuals of these species also incorporated BrdU (Figs. 1B and 1C). As in *Phagocata*, the cell population observed in the initial 24 h postfeeding is distributed throughout the mesenchyme (with the exception of the pharynx and the region in front of the photoreceptors). In contrast to earlier reports of neoblast distribution based on histochemical techniques (Brøndsted (1969) and references therein), we see no obvious dorsoventral asymmetry or association of BrdU-labeled cells with the ventral nerve cords; instead, this population of cells surrounds the gut (Fig. 1), from which the BrdU must have been transported.

We also explored other methods for delivering BrdU to planarians. In all three species microinjection into the mesenchyme or gut resulted in BrdU labeling indistinguishable from that observed in fed animals (data not shown). Soaking the planarians in a solution of 5 mg/ml BrdU was effective only on *Phagocata* hatchlings (data not shown); adult *Phagocata*, *G. dorotocephala*, and *S. mediterranea* were not labeled by this method. Addition of 5% DMSO to the BrdU solution did not result in labeling in adults or improved labeling of hatchlings. It is thus likely that differentiation of the adult epithelium contributed to the previously reported impermeability of planarians to exogenous thymidine and its analogs.

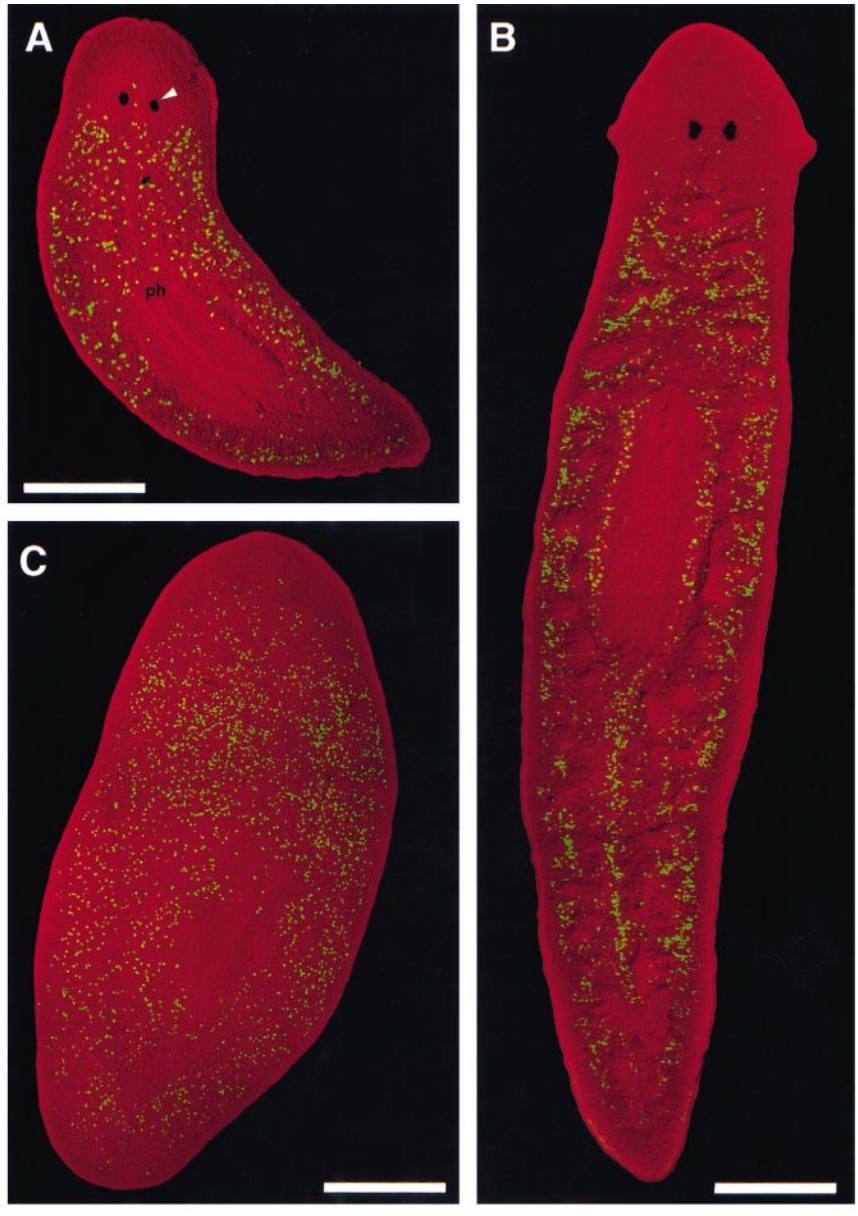
Migration and Differentiation of Neoblasts

Saló and Baguñà (1985) suggested that neoblast movement in planarians is a passive process driven by proliferation, rather than true cell migration. Because neoblasts are the only cells initially labeled by a pulse of BrdU, their movement (and differentiation) may be studied by fixing the flatworms at various times post-BrdU-labeling and detecting the incorporated analog using immunofluorescence. Due to the lack of proliferation anterior to the photoreceptors, this region provides a useful landmark for studying neoblast movement in the absence of proliferation. In all species we examined, during the initial day following a BrdU pulse, BrdU incorporation was detected only behind the photoreceptors (with the exception of a single cell that was occasionally observed anteriorly; Fig. 2A and below). In *S. mediterranea* (the focus of the remainder of this paper), within 2 days of BrdU labeling, BrdU-positive cells are observed in the region anterior to the photoreceptors (Fig. 2B), reaching the anteriormost margin of the animal, just underlying the epithelium in 4–5 days (not shown). By ~6 days postlabeling, BrdU-labeled, differentiated epithelial cells may be observed at the anteriormost margin of the flatworm (Fig. 2C, arrowheads). Thus, neoblasts may migrate and differentiate in a region entirely lacking in cell proliferation, arguing against proliferation as the driving force behind neoblast movement. This movement throughout the anterior region of the flatworm likely reflects physiological cell turnover, in which neoblasts are replacing dead and/or dying cells.

In dissociated cell preparations (or macerates), neoblasts are identified morphologically as small (~5–10 μm) undifferentiated cells with scant cytoplasm and a prominent nucleus (often containing a distinct nucleolus) occupying most of the cell volume (Betchaku, 1967; Brøndsted, 1969; Baguñà and Romero, 1981). Within the first 24 h following labeling, neoblasts are the only BrdU-labeled cells observed in macerated preparations (Fig. 3A). In all experiments (n = 4) in which BrdU-labeled animals were macerated (n = 3–4 animals per time point), the first morphological manifestations of BrdU-labeled neoblasts differentiating to other cell types are apparent within 35 h of BrdU labeling (5 out of 62 labeled cells (8%) in a total of 498 cells counted). Figures 3B–3D show BrdU-positive cells 48 h postlabeling. The cells indicated with arrowheads have larger cytoplasmic volumes and prominent cellular protrusions: morphological changes that are characteristic of cellular differentiation. The number of BrdU-labeled cells displaying these characteristics of differentiation increases with time (25 out of the 119 labeled cells (21%) in 550 total cells scored at 52 h, and 48 out of the 191 labeled cells (26%) in 457 total cells counted at 70 h). Because these cells have yet to attain their final differentiated form, it is not entirely clear what cell types these represent. Baguñà and Romero (1981) have reported that approximately 5% of the cells from *S. mediterranea* are unclassifiable in macerated preparations and likely represent intermediate forms of the differentiation process.

FIG. 1. BrdU labeling of planarians: (A) *Phagocata sp.*, (B) *Girardia dorotocephala*, (C) *Schmidtea mediterranea*. (A) and (B) were fixed 23 h and (C) was fixed 8 h after being fed 5 mg/ml BrdU and then were processed for detection of BrdU. Confocal sections were obtained from whole-mount preparations and the BrdU signal (in green) was projected and superposed upon the bright-field image (in red). Note the absence of proliferation in the regions anterior to the photoreceptors (arrowhead in (A)) and in the central pharyngeal region (indicated by ph in (A)). Animals are oriented anterior toward the top. Scale bars: (A) 150 μm; (B) 450 μm; (C) 300 μm.

FIG. 2. Cell migration visualized by BrdU labeling. Planarians were fixed at (A) 8 h, (B) 48 h, and (C) ~6 days postfeeding with BrdU. (A) In this magnified view of the specimen shown in Fig. 1C, only a single BrdU-positive cell is observed anterior to the photoreceptors at 8 h post-BrdU-labeling. (B) By 48 h numerous cells have moved into the region anterior to the photoreceptors. (C) At 6 days postlabeling, BrdU-labeled epithelial cells are seen (arrowheads). Confocal projections of BrdU-labeling (in green) are superposed upon bright-field images (in red). Anterior is up. Scale bars equal 50 μm.



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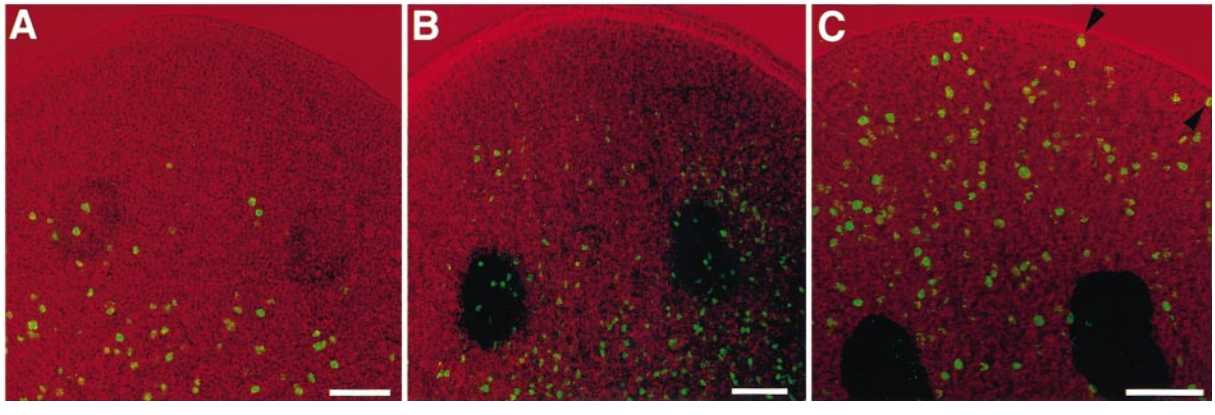

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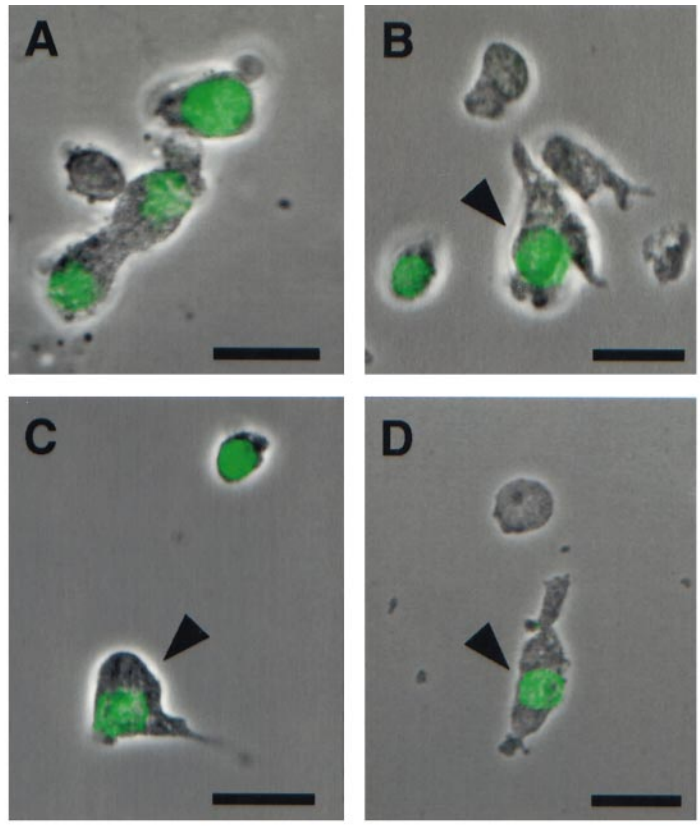

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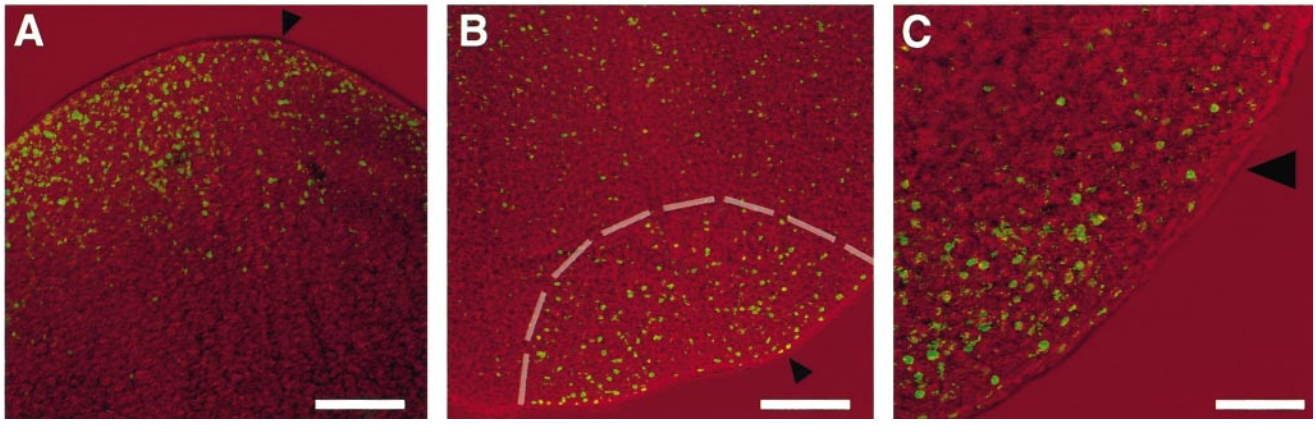

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FIG. 3. BrdU labeling in macerated cell preparations. Planarians were dissociated into single cells at either 17 (A) or 48 h (B–D) post-BrdU-labeling. Cells were spotted onto microscope slides and processed for detection of BrdU. Superpositions of confocal sections of BrdU signal (in green) upon the phase-contrast image (in grey). (A) Neoblasts are the only cells labeled initially by BrdU. This field shows a BrdU-labeled cell with typical neoblast morphology (Betchaku, 1967; Baguñà and Romero, 1981), a BrdU-labeled cell in telophase, and an unlabeled neoblast. (B–D) Various labeled cells in the process of differentiation 48 h post-BrdU-labeling (arrowheads). Scale bars equal 10 μm.

FIG. 4. BrdU-labeled neoblasts contribute to the formation of the regeneration blastema and differentiate into blastemal epithelial cells. (A) Five-day anterior regeneration blastema resulting from amputation 17 h after initial BrdU labeling. Regenerated pigment cups of the photoreceptors are seen within the blastema. (B) A 3.5-day posterior blastema (indicated by the dashed line) resulting from amputation 18 h after BrdU labeling. Note the accumulation of labeled cells within both blastemas and the BrdU-labeled epithelial cells at their edges (arrowheads). (C) Lateral edge of a 4.5-day posterior regeneration blastema. Arrowhead indicates the blastema/stump boundary (the blastema lies below the arrowhead). Accumulation of BrdU-labeled cells within the blastema is apparent. Superpositions of confocal projections of BrdU labeling (in green) upon bright-field images (in red). Scale bars: (A, B) 100 μm; (C) 50 μm.

BrdU-Labeled Neoblasts Contribute to the Formation of the Regeneration Blastema

Neoblast contribution to the formation of the regeneration blastema may be observed directly by labeling planarians with BrdU, transecting them at an appropriate time post-BrdU-labeling, and allowing them to regenerate. Regardless of animal size or level of transection, BrdU-labeled neoblasts were observed to contribute to the regeneration blastema. Representative animals (6–8 mm in length) are shown in Fig. 4, in which planarians were labeled with BrdU for ~18 h prior to amputation and cut pre- and postpharyngeally. Figures 4A and 4C show the anterior and posterior regeneration blastemas that result after a single prepharyngeal transection of the animals. Figure 4B shows a 3.5-day posterior regeneration blastema formed after cutting animals postpharyngeally. BrdU-positive cells can be seen throughout the blastemas and within the wound epithelia (arrowheads), demonstrating that BrdU-labeled neoblasts continue to proliferate and then migrate into the blastema where they can differentiate appropriately. BrdU-labeled cells are observed at a higher density in the blastema than in the stump (in Fig. 4A this difference is exaggerated by the confocal sectioning of the specimen), confirming the directed movement of neoblasts into the blastema. Figure 4C magnifies the blastema/stump boundary (indicated with arrowhead) of a 4.5-day posterior blastema; the accumulation of BrdU-labeled cells within the blastema relative to the stump area is apparent.

Distribution of Mitoses in Intact Planarians Visualized with Anti-phospho Histone H3 Antibodies

Just prior to the initiation of mitosis, serine 10 (ser10) of histone H3 becomes phosphorylated throughout condensing chromatin (Hendzel *et al.*, 1997); this phosphorylation is required for proper chromosome condensation and segregation in *Tetrahymena* (Wei *et al.*, 1999). Histone H3 modification during mitosis is highly conserved; thus, antibodies generated against an H3 oligopeptide phosphorylated at ser10 recognize mitotic cells in a wide range of organisms, from *Tetrahymena* to mammals (Hendzel *et al.*, 1997; Wei *et al.*, 1998). We have used these anti-phosphohistone H3 antibodies (anti-H3P) and confocal microscopy to study the distribution of mitotic cells throughout the planarian. In agreement with the BrdU labeling results and previous studies using classical cytological techniques, mitotic cells are absent from the pharynx and the region in front of the eyes (Fig. 5A). The number of cells labeled with anti-H3P at any given time is much smaller than the number of cells labeled with BrdU, reflecting the longer time required for DNA synthesis than for mitosis (Aherne *et al.*, 1977). In approximately 25% (20/79) of the samples, we observed a single cell that is mitotically active anterior to the photoreceptors.

There appears to be no preferential localization of mitoses near the ventral nerve cords. We do, however, observe the majority of anti-H3P-positive cells in two dorsoventral "domains" that are most easily visualized with a 10× objective under epifluorescence illumination; most mitotic cells appear to be distributed within either of two focal planes, in a rather narrow region of the dorsal or ventral parenchyma. These "domains" may be observed by optically sectioning along the *xz* axis using the confocal microscope (Fig. 5B, arrowheads). When observing specimens double-labeled with anti-H3P and BrdU, the mitotic cells (in red) are largely confined to these dorsal/ventral domains (although they are also visible within more internal regions of the parenchyma), whereas the S-phase neoblasts (in green) are found scattered throughout the entire parenchyma (Fig. 5B). This observation suggests that prior to the M phase, cells migrate to these regions of the parenchyma, where they undergo division. Given that neoblasts ultimately give rise to all differentiated cell types thoughout the animal, the distribution of mitotic cells also argues against proliferation as the driving force behind the movement of neoblasts within the planarian.

Length of G2 Determined with BrdU and Anti-phospho-H3 Double Labeling

In planarians, a rapid burst of proliferation is detectable in the initial 5–12 h postamputation or feeding (Baguñà, 1974; Baguñà, 1976a,b; Saló and Baguñà, 1984). These authors have suggested that this rapid proliferative response is the result of a neoblast population that is arrested in G2. Double-labeling neoblasts with BrdU and the anti-H3P mitotic marker provides a direct method for testing the hypothesis that the initial mitotic peak is due to G2-arrested cells and also allows us to measure the length of G2. If the initial mitotic peak is due to G2-arrested neoblasts that have already traversed S, then the mitotic cells observed in the initial 12 h after BrdU feeding should not be double-labeled with BrdU. Furthermore, the earliest appearance of BrdU-labeled mitotic cells will define the minimum length of G2.

Four hours after BrdU feeding, ~2% of the mitotic cells are labeled with BrdU (Fig. 6A; $n = 211$ mitoses counted in 3 planarians). As early as 6 h postfeeding, ~30% of the mitotic cells are labeled with BrdU ($n = 143$ mitoses; 3 planarians) and by 12 h ~96% of the mitotic cells are labeled with BrdU (Fig. 6C; $n = 171$ mitoses; 4 planarians). When the percentage of BrdU-labeled mitoses is plotted against time, the resulting fraction of labeled mitoses (FLM) curve (Fig. 6C) allows one to calculate the median length of G2 (Aherne *et al.*, 1977). From this curve, 50% of the mitoses are labeled at ~7 h. Given that we have not been able to detect BrdU labeling in the first hour postfeeding (data not shown), it seems likely that the minimum time required for BrdU to be transported from the gastrodermal cells to the neoblasts is about 1 h. Thus, we estimate that the median length of G2 is ~6 h, with a minimum length of ~4 h. Therefore, a G2-arrested population need not be invoked, since the length of G2 suffices to explain the

initial proliferative activity observed after feeding (Baguñà, 1974). Furthermore, if 16–20% of all neoblasts were arrested in G2 (Saló and Baguñà, 1984) the maximal percentage of mitotic cells labeled with BrdU should reflect this population. Thus, only ~80% of the mitotic cells would be labeled with BrdU at the time of maximal labeling. The observation that ~96% of the mitotic cells can be labeled 12 h after feeding suggests that a large population of G2-arrested neoblasts is not likely to exist in *S. mediterranea*.

Continuous BrdU Labeling

To examine more directly whether there is a G2-arrested neoblast population, we performed a continuous BrdU-labeling experiment, which allows the determination of the rate at which any given cell type enters the cell cycle (Aherne *et al.*, 1977; Cheshier *et al.*, 1999). Because only neoblasts undergo mitosis in planarians, continuous labeling experiments permit a delineation of the proliferative kinetics of these stem cells. If there were a slow-cycling or dormant subset of neoblasts, one would expect that after many days of continuous labeling less than 100% of the neoblasts would be labeled with BrdU; the percentage of unlabeled neoblasts would reflect the percentage of this slow-cycling subpopulation (Aherne *et al.*, 1977).

Therefore, we performed a continuous labeling experiment in which animals were injected with BrdU every 6–8 h for 4 days. At appropriate time points, the specimens were macerated into individual cells and processed for BrdU detection. The results are presented in Fig. 7 and clearly show that after 3 days of continuous labeling ~99% of the neoblasts are labeled. These data also indicate that an average of 6% of neoblasts are labeled soon after a single injection of BrdU, suggesting that neoblasts are entering S at a relatively rapid rate. The fact that >99% of neoblasts incorporate BrdU over a 3-day period suggests that no subpopulation of these cells remains quiescent for more than 3 days. Thus, all neoblasts appear to divide regularly and no subset appears to be slow-cycling or arrested in G2.

DISCUSSION

BrdU Labels Planarian Regenerative Stem Cells

One reason that researchers abandoned the study of planarian regeneration in the late 1960s was their inability to specifically label the neoblasts with [^{3}H]thymidine. The basis for this difficulty was likely the impermeability of the adult epidermis to thymidine analogs; newly emerged *Phagocata* hatchlings incorporated BrdU when placed in BrdU-containing water, whereas adults of the same species showed no detectable BrdU incorporation under these conditions. These results are consistent with the electron microscopic observations of Skaer (1965) showing that the thickness of the epidermis in the planarian *Polycelis tenuis* increases throughout embryogenesis and for several weeks after hatching. Best *et al.* (1965) tried to feed the planarians [^{3}H]thymidine as a mixture with heparinized horse blood, but did not observe thymidine incorporation. The reasons for this negative result are unclear given our success with feeding BrdU to planarians as part of an artificial food mixture.

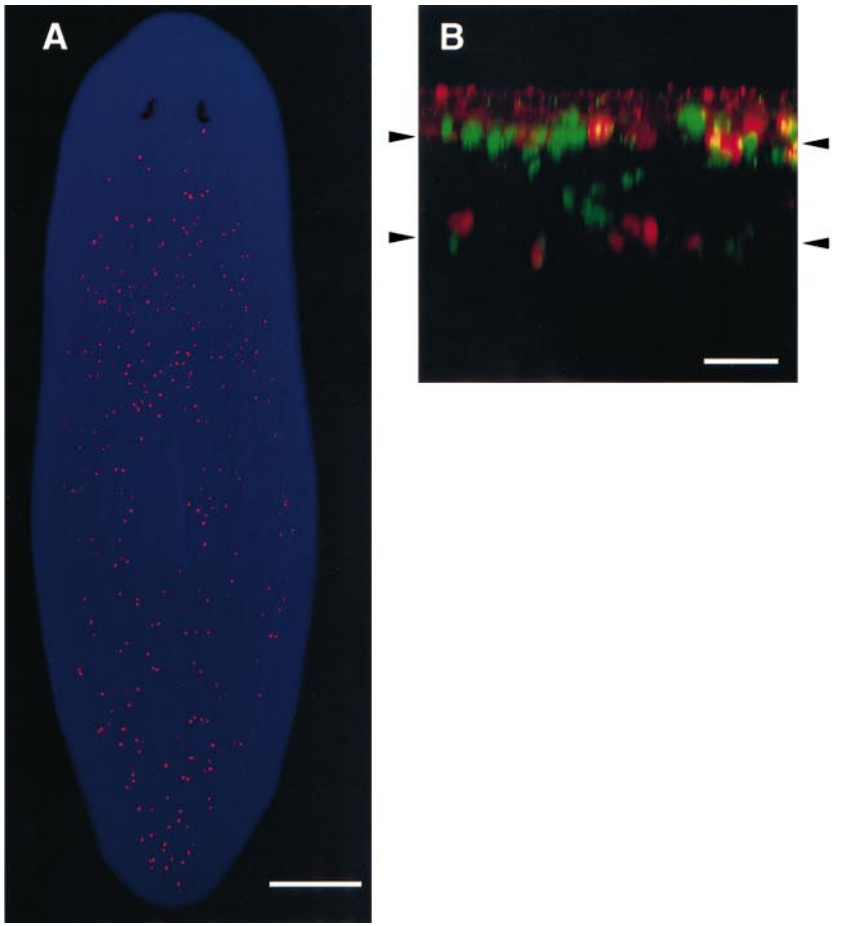
Many important questions relating to the planarian stem cell population and its role in regeneration and tissue renewal can now be addressed by labeling neoblasts with BrdU. For example, we have shown the differentiation of epithelial cells from BrdU-labeled neoblasts in both intact and regenerating planarians (Figs. 2 and 4). To our knowledge, this represents the first direct demonstration of specifically labeled neoblasts giving rise to a differentiated cell type. Extending these experiments using markers for other cell types will permit careful analyses of the processes of differentiation and cell turnover in planarians. Furthermore, based upon our results showing the distribution of BrdU-labeled neoblasts throughout the parenchyma (Fig. 1), the previously reported association of neoblasts with the ventral nerve cords (Brøndsted (1969) and references therein) appears to be incorrect; the histochemical procedures employed in these earlier studies may have labeled cells morphologically similar to neoblasts, but already in the process of forming neurons.

Neoblast Movement Is Not Driven by Cell Proliferation

Based on the results of grafting experiments using a chromosomal translocation to distinguish graft- or host-derived neoblasts, it has been proposed that neoblast movement is the result of passive spreading driven by cell proliferation (Saló and Baguñà, 1985). Our results suggest that neoblasts are, in fact, capable of migrating. BrdU labeling of planarians initially marks neoblasts posterior to the photoreceptors. Over time, BrdU-labeled cells can be observed entering the region anterior to the photoreceptors, a region lacking cell proliferation altogether. BrdU-labeled cells move through this region, eventually giving rise to BrdU-labeled epithelial cells at the anterior tip of the flatworm. Given the absence of cell division in this region (Figs. 1 and 5A), one must conclude that neoblasts may move (i.e., actively migrate) in the absence of proliferation.

The Length of G2 Is Sufficient to Explain the Early Mitotic Peaks Observed after Feeding

We have used BrdU labeling combined with the detection of a mitotic marker (anti-H3P) to examine the proposal that a subset of G2-arrested or slow-cycling neoblasts is responsible for the burst of mitotic activity observed in the initial 5–12 h after amputation (Baguñà, 1976b; Saló and Baguñà, 1984) or feeding (Baguñà, 1974; Baguñà, 1976a). As discussed above, 50% of the mitoses are labeled with BrdU ~7 h postfeeding. If it takes approximately 1 h for the BrdU to be digested and transported, the median length of G2



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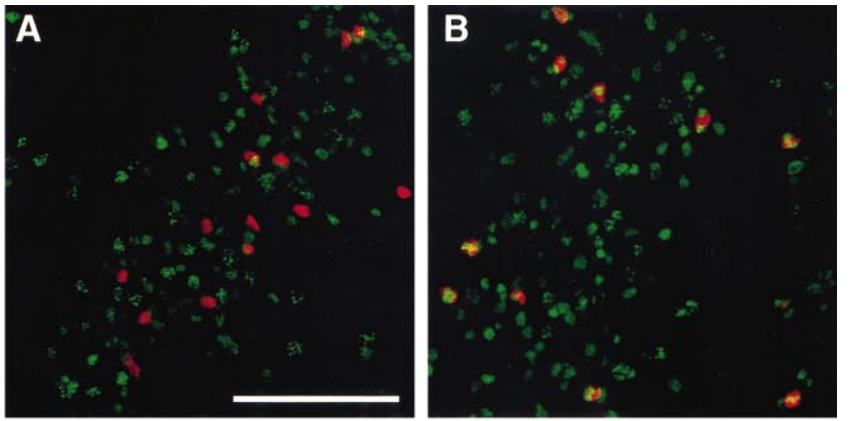

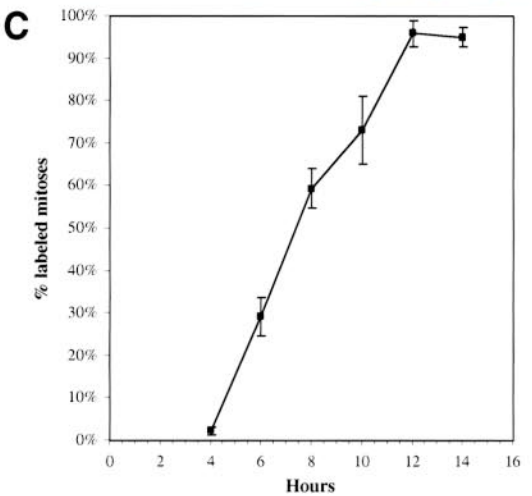

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FIG. 5. (A) Distribution of mitoses in an intact planarian detected by anti-phospho-histone H3. Confocal projections (from eight sections taken at 1.7-μm intervals) of the fluorescent image (in red) were superposed upon a Nomarski image of the entire flatworm (in blue). Mitotic cells are not observed anterior to the photoreceptors or in the pharynx. Dorsal view, anterior up. (B) Dorsoventral "domains" of mitotic cells. Mitotic cells are shown in red and BrdU-labeled cells in green. Projection of a confocal *xz* series (16 sections through 64 μm) through the postpharyngeal area of a planarian fixed 17 h post-BrdU-labeling. Note the relative abundance of mitotic cells within two focal planes (arrowheads) and the more scattered distribution of BrdU-labeled cells. The clarity of this image is reduced due to the poorer resolution of the objective lens in the *z* axis. Dorsal is at the top. Scale bars: (A) 250 μm; (B) 25 μm.

FIG. 6. Fraction of labeled mitoses determined by BrdU and anti-phospho-histone H3 double-labeling. Four hours postfeeding (5 mg/ml BrdU), five planarians were fixed every 2 h and processed for detection of both BrdU (in green) and anti-phospho-histone H3 (in red). Representative fields of confocal projections from tail regions of planarians fixed (A) 4 h or (B) 14 h post-BrdU-labeling. Double-labeling appears yellow. Under these fixation conditions, the chromatin appears to swell, giving a rather diffuse pattern to the anti-phospho-histone H3 staining. Scale bar in (A) is 100 μm. (C) Plot of the fraction of labeled mitoses vs time. Each time point represents measurements (±SD) from three to five planarians. Similar data were obtained in experiments using 2.5 mg/ml BrdU.

measured in these experiments would therefore be ~6 h, with a minimum of ~4 h. These estimated lengths of G2 suffice to account for the early mitotic peaks observed after feeding; thus, a slow-cycling neoblast population is not needed to explain the initial period of proliferation. Instead, because a large number of neoblasts are undergoing DNA synthesis at any given moment, a sizable percentage of these S-phase cells (reflecting the percentage of S occupied by replication of heterochromatic regions) will be either undergoing or completing late S at the time of feeding. Therefore, it seems likely that feeding stimulates the G2/M transition in cells that have recently completed DNA replication. One prediction of this model is that the length of G2 should be longer in animals that have been starved.

Is There a Subpopulation of G2-Arrested or Slow-Cycling Neoblasts?

Two other lines of evidence have been used to support the existence of slow-cycling neoblasts: hydroxyurea treatments and Feulgen cytospectrophotometry (Saló and Baguñà, 1984). Five-day treatment with the S-phase inhibitor hydroxyurea was required to eliminate mitoses both in the intact animal and after amputation, suggesting that some neoblasts were capable of entering mitosis 5 days after completion of S. Given that we observe essentially complete labeling of neoblasts in 3 days, our results are not consistent with this observation. Whether this discrepancy reflects the use of suboptimal doses of hydroxyurea, a lag in the entry of hydroxyurea through the relatively impermeable planarian epithelium, or real biological differences between the different species employed remains unclear.

Feulgen cytospectrophotometry also indicated that approximately 16–20% of neoblasts were in G2 (Saló and Baguñà, 1984). This number was interpreted to reflect the percentage of slow-cycling cells in the neoblast population. A more plausible explanation is simply that this percentage reflects the fraction of the neoblast cell cycle occupied by G2. The continuous BrdU labeling experiment provides a direct test of the proposed slow-cycling neoblast population. If there were a slow-cycling population corresponding to 16–20% of the neoblasts, then the maximum percentage of labeled neoblasts should plateau at approximately 80%. As described above, ~99% of the neoblasts are labeled with BrdU after 3 days of continuous BrdU labeling. Thus, we do not see evidence supporting a large, slow-cycling population.

Analysis of the Planarian Cell Cycle

Because of the previous inability to specifically label neoblasts during the S phase, there is a large gap in our knowledge related to the proliferative kinetics of neoblasts; one searches the planarian literature in vain for reasonable estimates of various cell cycle parameters. We have used the FLM technique (Quastler and Sherman, 1959; Aherne *et al.*, 1977) to suggest that the median length of G2 in

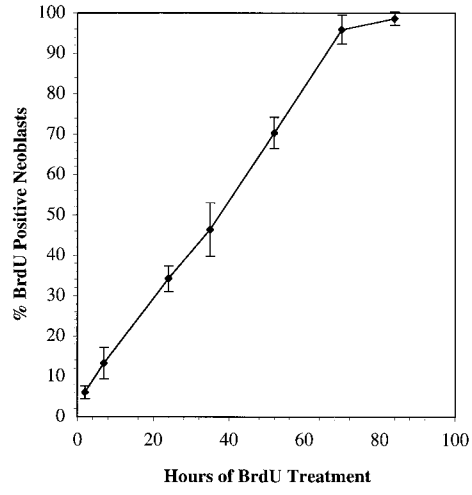

FIG. 7. Continuous BrdU labeling. Each data point represents the mean percentages of BrdU-positive neoblasts (±SD) obtained after scoring 400 or more cells from at least two animals. Except for the first time point, samples were collected 1 h after each injection to allow BrdU uptake into the parenchyma.

recently fed animals is ~6 h. Here we must add the caveat that large doses of BrdU have been shown to slow G2 and very high doses may result in G2 arrest (Rabinovitch, 1983). The fact that within 12 h of BrdU feeding, ~96% of mitoses are labeled suggests that the doses of BrdU employed are not causing G2 arrest. Furthermore, our ability to observe BrdU-labeled differentiated cell types also suggests that these doses do not have inhibitory effects on differentiation. However, it is more difficult to say with certainty that these doses have no effect upon the parameters that we are trying to measure, and it seems reasonable to conclude that we may have slightly overestimated the length of G2.

These types of difficulties are exacerbated in the continuous labeling experiment, in which planarians are exposed continuously to BrdU for many days. BrdU has a variety of toxic effects on cells in culture, not necessarily related to BrdU incorporation within DNA (Davidson *et al.*, 1980), and one could imagine that continuous treatment might seriously perturb some cell cycle parameters. Thus, it is possible that the 3 days required to achieve >99% labeling of neoblasts actually overestimates the time required for all neoblasts to enter S in an unmanipulated planarian.

Because of the caveats of interpeting cell cycle kinetics from continuous labeling experiments (Aherne *et al.*, 1977), accurate cell cycle analysis will likely require long-term FLM experiments and/or flow cytometry. Our future work will utilize clonally derived lines that we have developed to minimize variability due to genetic differences within the population. By performing FLM experiments in starved, fed, and regenerating animals, we will be able to measure differences in cell cycle parameters between shrinking, growing, and regenerating planarians. Such experiments

could provide an indication of which cell cycle regulatory networks are involved in activating proliferation during regeneration and growth.

In summary we have shown that planarian neoblasts can be labeled with the thymidine analog BrdU. This observation contradicts reports dating back well over 30 years suggesting that planarians were incapable of incorporating exogenous thymidine. The ability to label neoblasts specifically provides a critical tool for studying how the proliferation of this stem cell population is controlled in intact and regenerating planarians and, like the use of double-stranded RNA-mediated genetic interference (Sánchez Alvarado and Newmark, 1999), will be a boon to researchers interested in dissecting the problem of regeneration in these relatively simple triploblasts.

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