Terminal Differentiation of Ectodermal Epithelial Stem Cells of *Hydra* Can Occur in G2 without Requiring Mitosis or S Phase

Stefan Dübel and H. Chica Schaller
Zentrum für Molekulare Biologie Heidelberg, 6900 Heidelberg, FRG.

**Abstract.** Using bromodeoxyuridine incorporation to label cells in S phase we found that ectodermal epithelial cells of *Hydra* can start and complete their terminal differentiation in the G2 phase of the cell cycle. Most of the cells traversed their last S phase before the signal for differentiation, namely excision of head or foot, was given. The S phase inhibitor aphidicolin accordingly did not inhibit head or foot specific differentiation. The results show that differentiation to either head- or foot-specific ectodermal epithelial cells can start and is completed within the same G2 phase. This is therefore the first description of a complete differentiation from a population of proliferating cells to terminally differentiated, cell cycle-arrested cells without the necessity of passing through an S phase or mitosis.

For a long time, cell cycle regulation was thought to be restricted to the G0/G1 phase. The discovery of M phase promoting factor and related kinases from yeast to man emphasized the importance of mechanisms regulating the cell cycle in the G2 phase (for review, see Fantes, 1988). Recently, evidence for regulation of proliferation in the G2 phase was found in a number of different organisms. In the flatworm *dugesia*, wounding of the animal induces neoblasts present in G2 to divide and to form a regeneration blastema (Saló and Baguñà, 1984). A similar behavior was shown for mouse mammary stem cells (Smith and Medina, 1988). Mouse B lymphocytes in G2 can be stimulated to undergo mitosis by β-B cell growth factor (Melchers and Lernhard, 1985). The neural cell line NH15-C2A is stimulated in the G2 phase to undergo mitosis by the neuropeptide head activator (Schaller and Hoffmeister, 1989; Schaller et al., 1989). In contrast to the differentiated cells of other organisms as mentioned above, which were present in G1, all terminally differentiated ectodermal epithelial cells of *Hydra* are present in G2 (Dübel et al., 1987; Dübel, 1989). We used the regeneration capacity of *Hydra* and the possibility of removing all differentiated ectodermal epithelial cells by cutting off the extremities to study new differentiation of terminally cell cycle arrested ectodermal epithelial cells from gastric column tissue.

We asked whether the differentiation process is cell cycle dependent, and, if so, in which part of the cell cycle this process is initiated. The epithelial cell cycle consists of 1.5 h mitosis, almost no G1 phase, an S phase of 12-15 h, and a variable G2 phase of 24-72 h (David and Campbell, 1972). If the induction of terminal differentiation is cell cycle dependent, three possibilities exist which result within 24-28 h in a G2 arrest. First, the cells could be induced in G2, divide, and complete an S phase to be arrested in the subsequent G2 phase. Second, the cells could be determined in the S phase, complete it and stay in G2. A third possibility is the direct differentiation of cells in G2 without mitosis or S phase. If ectodermal epithelial cells could be determined to terminal differentiation throughout the whole cell cycle, one should find a mixture of these possibilities according to the respective location in the cell cycle.

In *Hydra*, the terminal differentiation of ectodermal epithelial cells occurs within one cell cycle (Hoffmeister and Schaller, 1985; Dübel et al., 1987). This terminal differentiation starts from the ectodermal epithelial cells of the gastric column. These cells are unusual because they act as differentiated epithelial cells forming the outer layer of *Hydra* tissue. Nevertheless, they are able to proliferate and function as stem cells for the terminal differentiation at the body poles. In contrast to the differentiated cells of other organisms as mentioned above, which were present in G1, all terminally differentiated ectodermal epithelial cells of *Hydra* are present in G2 (Dübel et al., 1987; Dübel, 1989). We used the regeneration capacity of *Hydra* and the possibility of removing all differentiated ectodermal epithelial cells by cutting off the extremities to study new differentiation of terminally cell cycle arrested ectodermal epithelial cells from gastric column tissue.
ing precursor cells can occur in the G2 phase without requiring a mitosis or an S phase.

**Materials and Methods**

*Hydra oligactis* (Zürich strain) and *Hydra attenuata* were cultured as described (Hoffmeister and Schaller, 1985). In regeneration experiments, the last feeding occurred 24 h before sectioning the animals. For aphidicolin treatments, a stock solution of 10 mg/ml aphidicolin (Sigma Chemical Co., St. Louis, MO) was prepared in DMSO. It was added between 8 and 10 a.m. to the culture medium. The final concentration was 590 μM. Control media contained the respective concentration of DMSO (final 0.6%) only. The demonstration of peroxidase activity in foot mucus cells and the footprint assay were done as described by Hoffmeister and Schaller (1985).

Quantitative DNA microcytofluorometry was done on cell preparations (David, 1973) according to Cowell and Franks (1980) as described by Dübel et al. (1987).

**S Phase Labeling**

*Hydra* were incubated in culture medium containing 5 mM 5-bromo-2'-deoxyuridine (BrdU, Sigma Chemical Co., München, FRG) for 1 h. The culture density did not exceed two animals/ml of medium. BrdU was removed by washing the *Hydra* two times for 30 min in an excess of culture medium. Heads were isolated, macerated according to David (1973) and dried onto microscope slides. Feet were isolated, stained for peroxidase activity, and dissociated into cells as described (Dübel et al., 1987). After washing for 10 min in PBS, slides were incubated for 50 min in 0.4 M glycine/HCl, pH 7.2 and for 4 h in 2 M HCl (Plickert and Krohier, 1988). After equilibration in PBS slides were incubated in 1% casein (purified powder; Sigma Chemical Co.) in PBS for 30 min at room temperature to block unspecific binding. The monoclonal anti-BrdU antibody (Becton Dickinson, Mountain View, CA) was used at a dilution of 1:70 in 1% casein in PBS and incubated at 4°C for 16 h. After washing three times for 15 min in PBS, the slides were incubated for 3 h at room temperature with an alkaline phosphatase labeled anti-mouse IgX (Sigma Chemical Co.) at a dilution of 1:50 in 1% casein in PBS. After washing three times for 10 min in PBS the slides were incubated for 30 min at room temperature in a substrate solution containing 0.33 g/l nitroblue-tetrazolium-chloride, 0.17 g/l 5-bromo-4-chloro-3-indolylphosphate (Sigma Chemical Co.), 100 mM NaCl, 5 mM MgCl₂, 100 mM Tris-HCl (pH 9.5). The enzyme reaction was stopped by a short incubation in 20 mM EDTA.

Alternatively, *Hydra* were injected with 20 μCl/ml methyl-[^3]H]thymidine (Amersham Corp., Braunschweig, FRG) and analyzed by autoradiography as described (David and Campbell, 1972).

**Results**

**Daily Rhythm of Ectodermal Epithelial Stem Cell Proliferation**

To interpret S phase labeling data, it is necessary to know whether the proliferative activity of the cells of interest is constant. To test this, the number of cells in S phase was determined in steady state cultures of *Hydra* at various times of the day by incorporation of methyl-[^3]H]thymidine and subsequent autoradiography as described (David and Campbell, 1972). A drastic decrease of cells in S phase was found in the afternoon after the feeding (Fig. 1). In the night, the labeling index increased again to reach the maximum in the morning. If feeding was stopped, the decrease of labeling index during the first day of starvation was less pronounced. Based on these results, all of the following regeneration experiments were started at a defined time of the day: at 10 a.m. ± 1 h.

**Differentiation of Ectodermal Epithelial Cells into Battery Cells**

We have shown previously that the battery cells of the tenta-

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1. Abbreviation used in this paper: BrdU, 5-bromo-2'-deoxyuridine.
after decapitation, the labeling index was determined after a regeneration time of 28 h. The x-axis shows the time of the BrdU pulse with respect to the decapitation (t = 0). Error bars represent the standard deviation of triplicates.

c, BrdU incorporation in head regenerating Hydra oligactis. BrdU pulses of 1 h were given at various periods of time before or after decapitation, the labeling index was determined after a regeneration time of 28 h. The y-axis shows the time of the BrdU pulse with respect to the decapitation (t = 0). Open circles, ectodermal epithelial cells; closed circles, battery cells. The labeling index was calculated as described in Fig. 1. Error bars represent the standard deviation of triplicates.

have been in the S phase, but a large fraction may not be labeled because their S phase was shorter than the time between two BrdU pulses. To discriminate between the two possibilities, the same set of Hydra was consecutively labeled by three 1-h BrdU pulses 20, 10, and 1 h before decapitation. Only 32.2 ± 4.0% (mean value of 3 experiments) of the regenerated battery cells were found to be labeled, indicating that the lack of labeling in two-thirds of the cells is not because of a shortening of S phases. The length of the final S phase seemed to be comparable to that of the proliferating precursor cells. Of the nuclei of undifferentiated ectodermal epithelial cells, 39.0 ± 2.0% had incorporated BrdU.

To further confirm the independence of the differentiation from ectodermal epithelial cells to battery cells from the S phase, regeneration was studied in the presence of an inhibitor of S phases. Aphidicolin, shown to inhibit DNA replication in eukaryotic organisms, was used (Ikegami et al., 1978). Hydra were transferred into medium containing 590 μM aphidicolin. After a 1-h incubation period, the animals were decapitated and transferred to fresh aphidicolin containing medium. No morphological difference between animals regenerated in the presence of aphidicolin and control animals was found within the first 36 h of regeneration. After 48 h of regeneration, the tentacles appeared to be slightly shorter than those of the control animals (Fig. 3, a and b).

To quantify an inhibitory effect of aphidicolin on head regeneration, decapitated Hydra were allowed to regenerate in 590 μM aphidicolin. After various periods of time, the number of regenerated tentacles was determined. Neither the time point of the first visible appearance of tentacle buds nor the total number of tentacles regenerated differed between treated animals and controls (Fig. 3 c). Regenerated battery cells were detected in aphidicolin treated as well as in control regenerates after 28 h. 590 μM aphidicolin, therefore, had no inhibiting effect on head regeneration within the time needed for the reappearance of new battery cells.

To find out whether aphidicolin indeed affected the DNA replication in Hydra, the ability to synthesize DNA was measured by BrdU incorporation just before the beginning and at the end of the regeneration. For this purpose, Hydra were transferred in medium containing 590 μM aphidicolin. After 1 h, BrdU was added. After another hour, the BrdU was washed out and the Hydra were decapitated. The regeneration medium again contained 590 μM aphidicolin. At 47 h of regeneration, a second 1-h pulse of BrdU was given, and then the regenerated head regions were isolated, dissociated into single cells, and analyzed for cells that had incorporated BrdU into the nuclear DNA as described above. Incorporated BrdU was detected in none of the cells isolated from aphidicolin treated Hydra, whereas a large number of nuclei were labeled in cells of the control regenerates. This indicates that the S phase was effectively inhibited during the whole regeneration period.

To determine whether the battery cells regenerated in the presence of 590 μM aphidicolin (i.e., without an S phase) were arrested in the same phase of the cell cycle as the normal battery cells, their nuclear DNA content was determined by quantitative microcytofluorometry (Cowell and Franks, 1980). Compared to the DNA content of nerve cells that are arrested in the G1 phase, containing a 2C genome (David and Gierer, 1974), the battery cells of aphidicolin-treated animals were shown to be arrested in the G2 phase of the cell cycle (Fig. 3 d). These data strongly suggest that for the differentiation of ectodermal epithelial cells to battery cells no S phase was necessary.

**Differentiation into Foot Mucus Cells**

Every ectodermal epithelial cell below a distinct point of the body axis terminally differentiates into a foot mucus cell, which is cell cycle–arrested in G2 (Dübel et al., 1987). Foot mucus cells express a specific peroxidase activity (Hoffmeister and Schaller, 1985). This peroxidase activity was therefore used as a marker for the terminally differentiated ectodermal epithelial cells of the foot. Similar experiments as described for the head were carried out with Hydra attenuata to analyze the process of differentiation of ectodermal epithelial cells in the foot.

The minimal time required for the terminal differentiation of ectodermal epithelial cells into foot mucus cells is 22–24 h.
Figure 3. *Hydra oligactis* treated with aphidicolin during head regeneration: *a*, control regenerate after 2 d; *b*, regenerates treated with 590 μM aphidicolin after 2 d. *c*, Effect of aphidicolin on head regeneration in *Hydra oligactis*. Squares, untreated controls; circles, regeneration in the presence of 590 μM aphidicolin. Error bars represent the standard deviations of >20 determinations. *d*, Nuclear DNA content of battery cells regenerated in the presence of 590 μM aphidicolin. Single nuclei were measured by quantitative microcytofluorometry in cell preparations stained with Hoechst 33258 (Dübel et al., 1987). 2C represents the DNA content of nerve cells as a standard for a G1 genome (David and Gierer, 1974). Bar, 500 μm.

(Hoffmeister and Schaller, 1985). To determine the time between the final S phase and the appearance of the foot specific marker, animals were pulse labeled with BrdU. After various time periods, feet were stained for peroxidase activity and analyzed for labeled foot mucus cells as described above. In these steady state animals, the minimal time needed to obtain foot mucus cells with labeled nuclei was 20 h (Fig. 4 *a*).

To find out in which part of the cell cycle the differentiation starts, pulse labeling of S phases as described above for the battery cell differentiation was performed in foot regenerating animals. The BrdU pulses were given at various times before or after the excision of the foot, the regeneration time was kept constant (32 h). If traverse of S phase is essential for the differentiation process, a dramatic increase of labeled cells has to be expected after the excision of the foot. This was not the case (Fig. 4 *b*). Instead, most of the foot mucus cells were present in the S phase before the differentiation signal was given by the cut (Fig. 4 *b*).

As found for the battery cells, also only a fraction of the foot mucus cells were labeled during the whole experiment. As was the case with the battery cells, two possibilities exist to explain this incomplete labeling of the cells. Either not all of the cells have replicated their genome within the entire period of the experiment, or the length of their S phase is significantly shorter than that of the proliferating ectodermal epithelial cells. To test this, the same set of *Hydra* was consecutively labeled by BrdU at three times: 20, 10, and 1 h before excision of the foot. 71.4 ± 2.7% (n=3) of the regenered foot mucus cells were labeled. Therefore, as in the
after the excision of the foot, the labeling index was determined after a regeneration time of 32 h. The abscissa shows the time of the BrdU pulse with respect to the excision of the foot (t = 0). Error bars represent the standard deviation of triplicates, b. BrdU incorporation in foot regenerating *Hydra attenuata*. BrdU pulses of 1 h were given at various periods of time before or after the excision of the foot, the labeling index was determined after a regeneration time of 32 h. The abscissa shows the time of the BrdU pulse with respect to the excision of the foot (t = 0). Error bars represent the standard deviation of triplicates.

To further confirm the independence of the differentiation from ectodermal epithelial cells to foot mucus cells from the S phase, aphidicolin was used in experiments similar to those described for head regeneration. *Hydra* were transferred in medium containing 500 μM aphidicolin. After 1 h, the feet were excised. The regenerates were stained to detect the peroxidase activity typical for foot mucus cells. 28 h after excision of the foot, the typical pattern of foot mucus cells containing peroxidase activity was detected in the aphidicolin-treated animals as well as in the controls (Fig. 5, a and b). No difference between aphidicolin-treated and control animals was found in the morphology of the basal discs. To quantify a possible inhibitory effect of aphidicolin on the foot regeneration, it was tested in a quantitative assay for foot regeneration (Hoffmeister and Schaller, 1985). The regenerated peroxidase activity was not significantly different from that of the controls (Fig. 5 c). The concentration of aphidicolin used therefore had no inhibiting effect on foot regeneration.

To test whether aphidicolin inhibited the S phases in *Hydra attenuata*, the rate of BrdU incorporation was measured just before the beginning and at the end of the regeneration as described above. As in *Hydra oligactis*, in this species no incorporation of BrdU was detected in any of the cells isolated from aphidicolin-treated *Hydra attenuata*. As in the head regeneration of *Hydra oligactis*, aphidicolin totally inhibited DNA synthesis.

To determine whether the foot mucus cells that regenerated without an S phase were arrested in the same phase of the cell cycle as the normal foot mucus cells, their nuclear DNA content was measured. Compared to the DNA content of nerve cells representing a G1 genome, the foot mucus cells of aphidicolin-treated animals were arrested in the G2 phase of the cell cycle (Fig. 5 d).

**Discussion**

The almost complete lack of a significant increase of the fraction of cells in S phase in ectodermal epithelial cells during the whole process of their terminal differentiation demonstrated the independence of this process from the S phase. Since the terminal differentiation products were found in G2, the independence from the S phase also rules out the need for a mitosis in this process. This conclusion is in accordance with the drastic drop of the mitotic index of epithelial cells within the first day of regeneration, as observed by Berkling (1974).

The question whether the differentiation can start not only in G2, but also in the S phase cannot be answered from the labeling data alone. A small fraction of the cells completed its S phase after the excision of the head or foot. However, this does not rule out the possibility that the differentiation starts only in G2 since it was shown that the "head activator," an endogenous peptide that triggers differentiation in these ectodermal epithelial cells, needs several hours to be released in response to the injury and persists for several hours (Schaller, 1976). The endogenous signal for terminal differentiation may therefore act also on cells that reach the G2 phase several hours after the injury. The finding that the S phase inhibitor aphidicolin did not influence the terminal differentiation of ectodermal epithelial cells may provide an answer to this problem. Studies on S phase inhibition during *Hydra* regeneration using x-ray or γ irradiation yielded inhibition of DNA synthesis, but also a partial inhibition of regeneration (Park, 1958; Hicklin and Wolpert, 1973). *Hydra* treated with hydroxyurea were also able to regenerate or to proceed with bud formation though head formation was in some cases retarded (Clarkson and Wolpert, 1967; Clarkson, 1969; Webster and Hamilton, 1972; Cummings and Bode, 1984). These results indicated that the S phase is not essential for terminal differentiation. In contrast, also the total inhibition of tentacle formation by hydroxyurea was reported (Lesh-Laurie et al., 1976). It was not clear from these experiments, however, whether the observed inhibition of regeneration was a result of specific action on the cell cycle or of an unspecific toxin effect. The S phase inhibitor aphidicolin used in this study was shown to overcome this drawback. Within the first day of regeneration, it had no significant inhibiting effect on terminal differentiation of ectodermal epithelial cells, indicating a minor contribution of cells in S phase. However, we have to note that the experimental errors of regeneration assays and DNA determinations allow also the interpretation that a maximum ~10% of the cells differentiate from the S phase. In comparing the circadian rhythm of the proliferation of the precursor population (Fig. 1) with the regeneration data, it is possible that the fraction size of cells which differentiate from S phase may simply follow the circadian rhythm of proliferation of ectodermal epithelial cells. Nevertheless it is evident that the major fraction of the cells started their terminal differentiation in the G2 phase.

This is therefore the first description of a complete differentiation process from proliferating cells to terminally differen-
tiated, cell cycle-arrested cell types which does not include mitosis or an S phase.

After one day of regeneration in the presence of aphidicolin, a substantial reduction of regenerated tentacle tissue was observed. This was probably because of the inhibition of stem cell proliferation that may supply cells the differentiation of which starts when the first wave of newly differentiating cells is completed. The steep increase of ectodermal epithelial stem cells in S phase measured ~1 d after the start of the regeneration provides support for this idea. Accordingly, Berking (1974) reported a drastic increase of mitoses in epithelial cells at about the same time of regeneration.

Since not all of the total terminally differentiated cells were detected to go through an S phase during the whole 2 d of the pulse labeling experiments, the other part of the cells must have finished their last S phase even before the first BrdU pulse; that is, more than 18 h before the induction of differentiation. This indicates that the induction of differentiation is not restricted to a short period in G2. Ectodermal epithelial cells cannot move as single cells through the Hydra tissue (Campbell, 1967). Their ability to start their differentiation from a large part or from the whole cell cycle may therefore be of advantage to accelerate the restoration of the complete animal after an injury in its natural surroundings, since most or all of the ectodermal epithelial cells near the wound can instantly start to differentiate. The homogenous reappearance of an epitope specific for ectodermal epithelial cells for the head on the entire surface of the regenerating head (Javios et al., 1986) suggests a simultaneous start of differentiation in all ectodermal epithelial cells in this area. It also would explain why in normal steady state animals the peroxidase expression is switched on beneath a strict border line that does not significantly exceed one cell layer (Dübel et al., 1987). Every cell that passes this border line starts to express
the peroxidase activity. Since it is unlikely that all cells of this one cell layer are present at the same point of the cell cycle, a more likely explanation is that the cells can start their differentiation from the whole cell cycle or at least from a large part of it. The described effects of the *Hydra* morphogenes on epithelial cell differentiation (Hoffmeister and Schaller, 1985) are in accordance with the idea that this border line is generated by a threshold response to these factors.

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