DNA synthesis during the first stages of anterior regeneration in the polychaete annelid *Owenia fusiformis* (dedifferentiation and early phases of differentiation)

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SUMMARY

We have analysed DNA synthesis in early phases of regeneration in a marine Polychaete Annelid, *Owenia fusiformis*. The length and efficiency of the prereplicative phase was found to vary with the diurnal rhythm of activity of the animal; that is, it depends on the initial state of the cell population at the moment of the onset of proliferative stimulation. When animals were operated on at 12 a.m., the duration of the prereplicative phase of the first cells stimulated to proliferate was found to be 12 h. The remaining cells entered the S-phase progressively in waves until the 3rd day following amputation when nearly 100 % of the blastema cells were stimulated. At that time the cell-cycles of these dividing cells were found to be highly synchronized. Blastema differentiation takes place on the 4th day and is initiated by stomodeum formation. During the differentiation phase, DNA synthesis is restricted to small areas of the regenerating part. The system described is viewed as a new instrument for investigating the control of the cell cycle in synchronized and subsequently differentiating tissue cells.

INTRODUCTION

In trauma-induced cephalic regeneration of the marine Polychaete *Owenia fusiformis*, expression of histogenetic capabilities involves renewal of cell proliferation in the zone of the amputation.

Study of this process thus presents most of the major problems of developmental biology today: What triggers duplication of genetic material in quiescent cells? How is the cell-cycle controlled? How are the differentiated state and the stage of cellular multiplication related?

Regeneration in *Owenia fusiformis* has the advantage of following a clear-cut sequential pattern of events which may offer an approach to different aspects of these fundamental problems.

Earlier works established that events take place in the following order. (1) By 24 h after amputation the wound is closed off by formation of a cicatricial plug.

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(2) 48 h after amputation the blastema is formed by morphologically dedifferentiated cells. These cells are more or less dedifferentiated depending on the cell type (Thouveny 1967; Coulon & Thouveny, 1974). This blastema grows by cell proliferation. (3) Differentiation in the regenerating part begins on the 4th day. It is practically ended on the 7th day after amputation.

A quantitative study of tritiated thymidine ([³H]T.d.R.) incorporation during these stages (Marilley & Thouveny, 1975) showed the cyclic character of incorporation of the DNA precursor. The amplitude of the labelling peaks increases to a maximum on the 3rd day after amputation. Then labelling sharply decreases but maintains its periodicity.

In this paper we report on studies of the triggering phase in which previously quiescent cells start multiplying. We also describe the timing of the stimulation to proliferate in epidermal and mesodermal tissues with respect to DNA synthesis, and the synchronous nature of the cell cycle occurring at the maximum of [³H]T.d.R. incorporation. The first step in differentiation in the regenerating part takes place immediately after the maximum incorporation.

**MATERIAL AND METHODS**

**Animals**

The Annelids *Owenia fusiformis* are from one batch that were collected near Marseille. All had the same physiological activity (sexual maturity, size, . . .).

**Operational conditions**

All experiments were performed with animals undergoing cephalic regeneration. Since the regenerative ability varies on the basis of an antero-posterior gradient, all amputations were performed in the anterior part of the first abdominal segment. Under these conditions regeneration occurs in 100 % of the individuals.

Animals were amputated at specified hours of the day (varying with the experiment) and were placed in filtered sea water at room temperature (about 18 °C). The animals were then left for varying lengths of time to regenerate until they reached the different stages of development used in the experiments.

**Quantitative study**

When they reached the required stage of regeneration, the animals were incubated in sterile sea water containing tritiated thymidine. The optimal incubation time and concentration of [³H]T.d.R. were determined in a preliminary experiment (data not shown). The specific activity of the [³H]T.d.R. was 26 Ci/mm. The radioactive precursor was diluted in sea water to 2 μCi/ml. The incubation time was 2 h. At the end of that time, the regenerating tissues plus a small piece of stump were cut off. The incorporation of [³H]thymidine into
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Fig. 1. Autoradiographs of early stages of *O. fusiformis* regeneration after incubation with [*H*]thymidine (26 Ci/mM) during 2 h. × 250.

(A, B) 1st day after amputation; the mesodermal cells are labelled. Dark field illumination (B) clearly shows all the labelled nuclei.

(C, D) 2nd day after amputation; about 60% of the epidermal and mesodermal cells are labelled (D, dark-field illumination). *ce*, Cicatricial epiderm; *cp*, cicatricial plug (mesoderm); *d*, digestive duct.

DNA was measured on the acid-insoluble material and visualized by autoradiographs. Both experiments were monitored every 2 h of regeneration time.

DNA synthesis in the first abdominal segment of normal (non-amputated) animals was used as control.

[*H*]thymidine incorporation into acid-insoluble material

For radioactive determinations see the earlier paper Marilley and Thouveny, 1975.

Light-microscope autoradiography

To allow a comparison between our present observations and previous studies (Marilley & Thouveny, 1975) each incorporation measurement was correlated with the autoradiogram of a duplicate sample, i.e. every 2 h during the first 100 h of regeneration.

Samples washed with ‘cold’ medium were fixed for 30 min in a mixture of
alcohol, acetic acid, chloroform and formol (10:2:2:1). They were dehydrated in alcohol, embedded in paraffin and serially sectioned at a thickness of 7 μm. Ilford L 4 emulsion applied to these sections was exposed for 4–5 days at 5 °C, and developed with Kodak D 19. The sections were stained with May-Grünwald/Giemsa.

In double labelling experiments the two different pulses were performed at 24 h interval. Tritiated thymidine was always used for the first pulse and [14C]thymidine for the second. The concentration and activity of [3H]thymidine were the same as mentioned above. The [14C]thymidine specific activity was 59 m Ci/mM.

RESULTS

(1) The prereplicative phase

Autoradiographs taken at the very beginning of the regeneration process show that, near the cut, DNA synthesis occurs after a lag phase. This was not explicit in our earlier results of [3H]T.d.R. incorporation into acid-insoluble material since this technique did not distinguish between labellings localized near the cut and labellings in the stump. The labellings in the stump are due to DNA synthesis in rapidly renewing cell populations, particularly in the ventral zone of the digestive duct (Buongiorno-Nardelli & Thouveny, 1966).
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Fig. 3. Percentage of DNA synthesized in regenerating animals relative to the synthesis occurring at the same time in normal (non-amputated) animals. The animals were amputated at different times during the day. The incubation of regenerating animals with [3H]thymidine was carried immediately after the amputation until the 14th h after the trauma. The normal animals were treated in the same way. Each determination represents the mean value for four animals.

Duration of the prereplicative phase (in vivo and in vitro)

In vivo, DNA synthesis first appears in the mesodermal cells near the section (Fig. 1A, B). Sometimes it occurs simultaneously in the epiderm and in the mesoderm in which cases the number of stimulated epidermal cells is very low. The mean ratio of labelled cells determined on sagittal sections of the regenerate in four different experiments was found to be one epidermal cell to 18 mesodermal cells. In these cells the prereplicative phase lasted 12 h as measured under standard conditions (see Material and Methods), the animals being amputated at 12 a.m.

In vitro, the duration of the lag phase was measured in tissue cultures. The aim of this experiment was to determine the length of the presynthetic phase in cells in their tissue-environment, thus excluding the integration of the complete organism.

Fragments containing epidermal and mesodermal tissues were cut off on either the ventral or dorsal side of the first abdominal segment of the Annelid at 12 a.m. and then placed in a suitable culture medium (modified 199 medium from Institut Pasteur in sterile sea water).

Fig. 2 shows the sequential pattern of [3H]T.d.R. incorporation into dorsal and ventral tissues during the first 16 h of culture. The prereplication period is found to last 12 h in both ventral and dorsal tissues. This is exactly the result obtained with animals operated on at 12 a.m.

In these experiments where the calculated total uptake of thymidine was
found to be very similar in both ventral and dorsal tissues (respectively 51 and 58 cpm/protein), the quantity of $[^3]H$thymidine incorporated into DNA at the start of DNA synthesis is twice as high in the ventral fragment as in the dorsal fragment.

We conclude that the prereplicative phase lasts as long in vivo as in vitro the only difference being in the level of the efficiency of the stimulation. The mesodermal tissue is more stimulated than the epidermal tissue. In vitro, the ventral tissues are more stimulated than the dorsal tissues.

**Effect of initial cell population conditions on duration and efficiency of the prereplicative phase**

It has been noted previously (Marilley & Thouveny, 1975) that in untreated animals, the DNA synthesis corresponding to replacement growth follows a circadian rhythm. Moreover, other diurnal variations were found in these normal animals, such as quantitative variations in neuro-secretory products (Coulon & Marilley, 1976) and fluctuations of $[^3]H$uridine incorporation (with a maximum at 10 a.m., data not shown).

All these results indicate that the initial state of the cells may be different according to the time of the amputation and may exert an influence on the proliferative response to that stimulus.

To test this possibility we operated on animals in the same conditions, the only change in parameter being in the time of amputation. The experiments were performed at 7, 9, 12, 14, 16, 18, 19.30, 22 and 24 h of the day. For each series the total DNA synthesized during the first 14 h after amputation was determined. This duration, 2 h longer than the length of the prereplicative phase previously determined, was chosen in order to measure the start of DNA synthesis.

To discriminate what portion of newly synthesized DNA was a specific response to the stimulus produced by the trauma, we measured the quantity of
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DNA synthesized in the first abdominal segment in control animals at the time of the experiment and for the same duration. These results allowed us to calculate the percentage of DNA corresponding to a response to amputation when the time of amputation varies. The results were plotted on Fig. 3. They clearly indicate a maximum of stimulation at about 10 a.m. From that time, there is a sharp decrease with a minimum at about 20 h.

Since the period of 14 h of T.d.R. labelling includes a lag phase plus the start of the S-phase, the total incorporation depends on the exact length of the S-phase and on the replicative activity in the first part of the S-phase. Moreover, in the regenerating part the incorporation measured results from the number of stimulated cells, and/or the rate of DNA synthesis at the beginning of the S-phase.

Thus, we tried to analyse the results reported in Fig. 3 by following the quantity of DNA synthesized in samples withdrawn at intervals of 2 h up to about the 14th h after amputation. Thereby we can estimate the length of the lag phase and the quantity of DNA synthesized at the start of the replicative phase in response to the trauma, provided we also take into account synthesis occurring in surrounding cells since some of them may synthesize DNA (normal replacement growth). The analysis was accomplished for animals amputated at 7 h, 12 and 20, i.e. just before and immediately after the maximum of stimulation (10 h) as shown in Fig. 3, and at the minimum (20 h). The results are plotted in Fig. 4.

In the case of 7 h amputation, DNA synthesis occurred at the same time as in non-amputated animals (i.e. 18–20 h as demonstrated elsewhere; Marilley & Thouveny, 1975) but the level was higher than in untreated animals. For the 12 h amputation, DNA synthesis was somewhat delayed and the level obtained was the same as in the 7 h case.

Thus we can see that the first group of animals had a longer lag phase than the second one, the lag phase lasting 12 to 14 h (animals amputated at 7 h) instead of 10–12 h (animals amputated at 12 h). In spite of this longer lag phase the percentage of DNA synthesized, related to the quantity of DNA synthesized at the same time in untreated animals, was higher for animals amputated at 7 h than 12 h (Fig. 3). We can see therefore that the efficiency of the stimulation is higher for an amputation occurring before 10 h than immediately after that time.

In the case of 20 h amputation, the results reported in Fig. 4 show that T.d.R. incorporation is low at about 20 h (i.e. at a time when DNA synthesis occurs in untreated control animals, and, 8–10 h later (6 h clock time), the observed incorporation coincides, in quantity and time of day, with the one occurring in non-amputated animals. This incorporation, very narrowly localized in time, does not seem to be the response to the experimental trauma; this latter response seems to be delayed.
2. DNA synthesis during growth of blastema and early phases of regenerate differentiation

**DNA synthesis during growth of blastema**

Double-labelled autoradiographs were performed by incubating the animals in \[^3H\]thymidine during 2 h at the time of the first initiation wave, followed 24 h later by an incubation of 2 h in \[^14C\]thymidine. The experiment shows that 100% of the cells stimulated during the first initiation wave are labelled by both isotopes. This indicates that at the time of the second labelling these cells are all in a new cycle of DNA synthesis, since preliminary observations have shown that the S-phase lasts only about 8 h. Simultaneously, as far as we could observe, the remaining cells were \[^14C\]-labelled only. These results are a good indication that the synchrony of the cells stimulated first is well conserved, and that the newly stimulated cells synthesize DNA at the same time of day as the first groups of cells.

To complete this experiment we calculated the percentage of labelled nuclei after \[^3H\]thymidine pulse at a maximum incorporation-time each day during the first 3 days of regeneration. The results are reported in Fig. 5 and are illustrated by the photos 1A–D and 6A.

The results concerning the 3rd day of regeneration, in which it was shown

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**Figure 6**

Autoradiographs of blastemas in *O. fusiformis* after incubation with \[^3H\]thymidine for 2 h.

- (A) 3rd day after amputation, maximum of labelling. Dark-field illumination. \(\times 250\).
- (B) 3rd day regenerating animals, end of the S-phase. \(\times 250\).
- (C, D) Mitosis phase. \((C) \times 250, (D) \times 800\).
- (E) Stomodeum formation on the 4th day. \(\times 250\).
- (F) Differentiation of the regenerate, formation of coelomic cavity. \(\times 250\). *e*, Epiderm; *b*, mesodermal blastema; *cp*, cicatricial plug; *d*, digestive duct; *coe*, coelomic cavity.
Fig. 6. For legend see opposite.
that nearly 100% of the blastema cells were simultaneously labelled, led us to think that the corresponding cell cycle was well synchronized. However, the S-phase is a long phase with respect to the total length of the cell cycle; thus it is possible that we are dealing at the same time with early S, mid S and late S-phase cells, i.e. parasychronous synthesizing cells. This objection may be answered by two observations. Firstly, if the cells are synchronous whatever the time of marking there will be no variation throughout the S-phase in the number of cells undergoing DNA synthesis. This is indicated by Fig. 6B, since the autoradiography shows that the maximum number of cells has been maintained until the end of the replicative phase. Secondly, about 2 h after this stage the blastema is very poorly labelled, there is no labelling in the sagittal section shown in Fig. 6C, and Fig. 6D (magnification of Fig. 6C) illustrates well the mitotic wave that occurs at that time. Therefore, from the previous results and since mitosis is a rapid phase in the cell cycle (about 1 h, Mitchison 1971), we can at least conclude that this cycle, concerning nearly 100% of the blastema cells, is well synchronized.

**DNA synthesis during regenerate differentiation**

The first evidence of differentiation of the regenerate appears to be the stomodeum formation which is accompanied by a general decrease in the labelling as determined by TCA-insoluble thymidine incorporation. We can see in Fig. 6E that the labelling is no longer generalized and is located only at the level of stomodeal invagination. The labelled cells are mainly epidermal cells from the apical part of the blastema and endodermal cells located at the ventral part of the digestive duct.

Further waves of DNA synthesis are all very narrowly localized in the regenerate (Fig. 6F). These labellings correspond to small peaks of DNA synthesis taking place after the 3rd day of regeneration.

**DISCUSSION**

In this study of the phases of trauma-induced regeneration in the Polychaete Annelid *Owenia fusiformis* we have obtained the following results. (1) Nearly 100% of the blastema cells are able to synthesize DNA. (2) The quantity of the [H3]-T.d.R.-labelled cells begins to increase about 12 h after the trauma. This increase is followed by successive waves of labelling comprising cells that apparently enter the S-phase at the same time as the previously stimulated cells. A maximum of labelling is obtained on the 3rd day. A sharp decrease in DNA synthesis coincides with the beginning of morphogenesis. DNA synthesis is then restricted to small areas of the regenerate.

The cellular kinetics of the dividing cells during *O. fusiformis* regeneration, although not yet completely studied in the present work, appear to be very interesting for further studies of the control of DNA synthesis in a regenerating
system undergoing morphogenesis. Indeed, there seems to be a naturally occurring cell synchronization, since the cells enter the S-phase always at the same time of day, and the synchrony does not seem to be lost during the following $G_2$ and M phases. Finally the stimulation concerns nearly 100% of the blastema cells. Moreover, in spite of individual variations, we found that the timing of the events may be precisely predicted when animals are operated on under standard conditions.

This system, like the stimulated cell systems classically studied, includes, during the prereplicative phase, the main biochemical events reported by Baserga (1968), Allfrey (1969), Cooper (1971), Stein & Baserga (1972). For instance, changes in the structure of genetic material of *O. fusiformis* were indicated by the variations of accessibility of DNA in chromatin tested under the conditions of mild digestion by DNase II (increase of accessibility). This change is associated with morphological transformation (decondensation) as observed by electron microscopy (Fontes, Marilley, Le Parco & Thouveny, 1978). The transcription, *in vivo*, was also increased (Fontes, 1974) as well as the *in vitro* template activity measured with *E. coli* RNA polymerase (Fontes et al. 1978). Furthermore, early synthesis of non histone nuclear protein was demonstrated (Thouveny, 1976). However, all these events are not necessarily related exclusively to the stimulation of DNA synthesis, since the transcription of mRNA may also be implicated in redifferentiation (Thouveny, 1976). Hence, the phenomenon studied differs considerably from the classical stimulated cell-system because it leads to a new morphogenesis.

However, it may be useful to compare our results concerning a further redifferentiating cell system (*O. fusiformis* regeneration) with data concerning the regenerating mammalian liver after partial hepatectomy. This sort of regeneration consists only of compensatory hyperplasia in which the normal gross morphology of the liver is not regained (Bucher, 1967).

Recently, the mathematical analysis by Yakovlev, Zorin, Isanin (1977) of the results obtained by Fabrikant (1968a, b, 1969) on regenerating rat liver, has shown that the phenomenon may comprise two parts: (1) 60% of the initial number of hepatocytes rapidly enter the mitotic cycle in response to partial hepatectomy (parasynchronous initiation); (2) 30% of the parenchymal cell population passes through an additional period of transformation (dedifferentiation) with a minimal duration of more than 25 h.

In the case of *O. fusiformis* regeneration, the kinetics of DNA synthesis stimulation differ considerably, since we have seen that: (1) only a small quantity of cells were able to enter the S-phase during the 1st day (about 6% of the mesodermal cells, and about 0.5% of the epidermal cells); (2) the major part of cell population (about 90%) needed a longer phase of dedifferentiation, the maximum bursts of initiation taking place on the 2nd and 3rd days after amputation.

The results reported in the present paper lead us now to investigate at the
molecular level how DNA synthesis is controlled in the system. Are the variations described due to changes in activity of enzymes implicated in DNA replication (particularly DNA polymerases), and/or due to variations at the template level?

REFERENCES


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