Genetic analysis of developmental mechanisms in hydra

III. Characterization of a regeneration deficient strain

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SUMMARY

Mutant hydra strains showing abnormal development can be isolated through sexual inbreeding of wild hydra. One such mutant strain, called reg-16, regenerates tentacles very poorly following amputation of the head and foot. Tentacle regeneration, however, is significantly enhanced by subdividing the regenerating fragment longitudinally.

Lateral tissue implants that induce head formation in wild-type hydra either regress or induce foot formation in reg-16 polyps. These results suggest that regeneration deficiency in reg-16 is due to a defective polarity gradient.

A chimaeric strain of hydra was produced by combining interstitial cells (and thus their differentiation products, nerve cells and nematocytes) of reg-16 hydra with epithelial cells of another strain which is capable of normal regeneration. The chimaeras regenerate normally, suggesting that the defect of reg-16 is not located in the interstitial or nerve cells.

INTRODUCTION

Hydra is known for its regenerative capacity. Numerous investigators have studied various aspects of hydra regeneration, such as histological changes during regeneration, capacity of different body parts to regenerate, roles of interstitial and other cells in regeneration, polarity and metabolic gradients in regeneration, effects of various chemicals on regeneration, etc. (for review see Kanaev, 1952; Tardent, 1963; Webster, 1971; Burnett, Lowell & Cyrlin, 1973).

However, genetic aspects of hydra regeneration have received only scant attention. Spangenberg & Eakin (1961) examined variation in regenerative capacity among different hydra species and strains. Lenhoff (1965) isolated a mutant strain which showed abnormal patterns of budding and regeneration, and Moore & Campbell (1973 a, b) later studied the same or similar strains.

We thought that a genetic approach might be useful in hydra regeneration studies, and carried out a screening programme to isolate hydra mutants. In a previous paper (Sugiyama & Fujisawa, 1977 a), we reported the isolation of
several strains of *Hydra magnipapillata* which showed greatly reduced regeneration capacities or abnormal regeneration patterns.

In the present work, we subjected one of them (reg-16) to detailed analysis. The results indicated that the reduced regeneration capacity of this strain might be due to the defective polarity gradient(s), and that this defect was not located in the nerve cells.

**MATERIALS AND METHODS**

*Hydra strains and culture*

All strains used belonged to *Hydra magnipapillata*. Strain 105 is a wild-type male strain used as standard in our laboratory. Isolation of the regeneration-deficient strain, reg-16, was previously described (Sugiyama & Fujisawa, 1977a). It was obtained by sexual crosses of wild-type strains originally collected from a pond in Ugowada, Akita, Japan (see Fig. 1). The origin and properties of an interstitial cell-deficient strain, nf-1, were previously described (Sugiyama & Fujisawa, 1977b).

Animals were cultured according to Loomis & Lenhoff (1956) in the modified 'M' solution described by Muscatine & Lenhoff (1965) (1 mM-NaCl, 1 mM-CaCl₂, 0·1 mM-KCl, 0·1 mM-MgSO₄ and 1 mM Tris (hydroxymethyl) aminomethane, pH 7·6). Freshly hatched *Artemia salina* nauplii were used as food. All experiments were done at 18 ± 1 °C.

*Tentacle regeneration assay*

Prior to experiment, animals were heavily fed once daily for at least 2 weeks. One day after the last feeding, polyps bearing no buds were selected from a mass culture and they were dissected under a dissecting microscope. For standard
Hydra regeneration mutant

Fig. 2. Tentacle regeneration after head and foot amputation (standard regeneration assay, see Materials and Methods). Averages of the pooled results of four separate experiments using 20 (reg-16) and 35 (strain 105) animals are shown. Vertical bars show standard deviation. ●, Strain 105; ○, reg-16.

Regenerative capacity of reg-16

Fig. 2 shows the tentacle regenerative capacity of reg-16 (regeneration-deficient strain) and strain 105 (wild-type standard) polyps as examined by the standard
regeneration assay procedure described in Materials and Methods. Strain 105 had originally an average of 6.1 tentacles per animal. After head and foot amputation, animals started to produce visible tentacles by the third day and thereafter tentacle numbers increased rapidly to reach the original level of untreated animals by the fifth day. No feeding was done during this time. Experiments with several other wild-type strains, including one of the original grand-parents of reg-16 (Fig. 1), all produced essentially the same results.

In contrast, tentacle regeneration after head and foot amputation of reg-16 was very poor. This strain had originally about 5.4 tentacles per polyp, and, after 8 days of regeneration, had restored only about one-quarter of the lost tentacles (1.3 per animal). When examined individually the majority of reg-16 animals (67%) did not have any tentacles at all after 8 days of regeneration, and the rest had slightly lower than normal numbers of tentacles (Fig. 3b). When allowed to regenerate for a longer period of time (without feeding), those animals that had fewer than normal tentacles usually restored the normal numbers during the next few days. The fate of those animals that failed to regenerate any tentacles during the first 7–8 days was multifold; some regenerated tentacles very slowly, some produced buds (instead of regenerating), but most accumulated brownish debris in the gastric cavity, contracted fungal infection and eventually died.

In the next experiment animals were dissected in various ways, as shown diagrammatically in Fig. 4, and the resultant tissue fragments, which had different sizes and origins, were examined for their ability to regenerate tentacles. Fig. 5 shows the results. Tissues from the wild-type strain 105 regenerated well
regardless of the dissection used. In contrast, regeneration of reg-16 tissue was markedly affected by the dissection. Regeneration was poor with the first three dissections, but moderately good with the last dissection method described in Fig. 4. This finding was rather surprising because, of the two types of pieces obtained from the same body region (II and V in Fig. 4), the one having smaller size and more wounded surfaces (II–V) regenerated better.

In all the regeneration experiments described above, reg-16 always regenerated foot tissue well, although a quantitative assay of this process was difficult and therefore not attempted.

**Secondary head formation by lateral grafting**

Hydra produces head structures under three different situations: (1) head formation during normal budding, (2) head regeneration after removal of the original one, and (3) secondary head formation upon lateral grafting of tissue from another animal (see Webster, 1971). Head production of reg-16 was normal under situation (1) but deficient under situation (2). We therefore examined in the next experiment the head-producing capacity of reg-16 under situation (3). Two types of grafting were carried out.
Fig. 5. Regeneration capacity of tissues obtained from various body regions shown in Fig. 4. (a–d) Results obtained with tissue pieces designated I, III, IV and V, respectively, in Fig. 4. The result with II has been already shown in Fig. 2. Averages of the pooled results of two separate experiments using at least ten specimens per tissue type are shown. ■, Strain 105; ○, reg-16.

In the first type we took donor tissues from normal animals and transplanted them to hosts as indicated in Fig. 6. Donor and recipient animals belonging to the same strain were used. When this experiment was done with the wild-type strain 105, the results obtained (Table 1) were in good agreement with the previously reported results (e.g. Webster & Wolpert, 1966; Hicklin & Wolpert, 1973). Namely, upper gastric tissues grafted into lower gastric regions induced head structures on the host, while grafts made in the opposite direction induced foot structures. Grafts made between the same regions (upper gastric to upper gastric or lower gastric to lower gastric) mostly regressed and were absorbed by hosts.

In contrast, grafts using reg-16 produced markedly different results in two cases (Table 1). Upper gastric tissues transplanted into the lower gastric regions regressed instead of inducing heads. In addition, upper gastric tissues grafted
onto upper gastric regions sometimes induced foot formation instead of regressing. These observations suggest the possibility that the polarity gradient(s) in reg-16 is abnormal since the fate of the grafted tissues is thought to be determined by the relative polarity gradient levels of donor tissues and recipient sites (Webster, 1971; Wolpert, Hornbruch & Clarke, 1974).

In the second type of lateral grafting experiment, distal tissues of regenerating animals were used as donor tissues and they were grafted into the upper gastric region of the host animals. Webster & Wolpert (1966) showed that distal tissues acquired increased head-inducing capacity during regeneration. Our present experiment with strain 105 confirmed their results (Table 2). Tissues removed from distal ends of regenerating animals and grafted into hosts all failed to induce head formation when done immediately after head and foot amputation of the donor animals, but they all succeeded when grafted after 1 day of regeneration.

Experiments with reg-16 showed that this acquisition of head-inducing capacity during regeneration was absent from these hydra (Table 2). Even distal
Table 1. Structures induced by lateral grafting. 
I. Grafting of tissues from uninjured animals

<table>
<thead>
<tr>
<th>Strain</th>
<th>Donor tissue</th>
<th>Recipient site</th>
<th>No. of grafts made</th>
<th>Head (%)</th>
<th>None (regression) (%)</th>
<th>Foot (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>105</td>
<td>Upper gastric</td>
<td>Upper gastric</td>
<td>26</td>
<td>1</td>
<td>24</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Upper gastric</td>
<td>Lower gastric</td>
<td>14</td>
<td>14</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Lower gastric</td>
<td>Upper gastric</td>
<td>21</td>
<td>0</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Lower gastric</td>
<td>Lower gastric</td>
<td>8</td>
<td>1</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>reg-16</td>
<td>Upper gastric</td>
<td>Upper gastric</td>
<td>31</td>
<td>0</td>
<td>20</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Upper gastric</td>
<td>Lower gastric</td>
<td>16</td>
<td>0</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Lower gastric</td>
<td>Upper gastric</td>
<td>17</td>
<td>0</td>
<td>16</td>
<td>17</td>
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<tr>
<td></td>
<td>Lower gastric</td>
<td>Lower gastric</td>
<td>10</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2. Structures induced by lateral grafting. II. Grafting of distal end tissues of regenerating animals to the upper gastric region of recipients*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Type of amputation</th>
<th>Regeneration time (days)</th>
<th>No. of grafts made</th>
<th>No. of head induced (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>105</td>
<td>Head and foot removal†</td>
<td>0</td>
<td>26</td>
<td>1 (4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>14</td>
<td>14 (100)</td>
</tr>
<tr>
<td>reg-16</td>
<td>Head and foot removal†</td>
<td>0</td>
<td>31</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>24</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>Head and foot removal‡ with longitudinal slicing</td>
<td>1</td>
<td>14</td>
<td>2 (14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>16</td>
<td>2 (13)</td>
</tr>
</tbody>
</table>

* Distal end tissues of regenerating animals correspond to the upper gastric region of the original animal before amputation.
†, ‡ Structures II and V, respectively, in Fig. 4.

tissue allowed to regenerate for 2 days did not affect induction. Earlier, we observed moderately restored tentacle regeneration in strain reg-16 (Fig. 5d) when animals were dissected into small pieces by the last dissection method shown in Fig. 4. When the distal tissues of such pieces were used for grafting, some head-inducing capacity was observed with reg-16, after one and two days of regeneration (Table 2).

Regenerative capacity of chimaera hydra

Hydra is made up of six basic types of cells; epitheliomuscular, digestive, gland, interstitial, nerve cells and nematocytes (David, 1973). We have previously described the production of a chimaeric strain that contained the first three cell
types derived from one donor strain and the last three cell types from another (Sugiyama & Fujisawa, 1977b). It was possible to produce such a chimaera because of the availability of a mutant strain named nf-1. This strain, like a similar strain produced by Campbell (1976) using colchicine treatment, contained no interstitial cells, nerve cells or nematocytes. Nf-1 arose by spontaneous loss of interstitial cells from its parental strain sf-1. We previously showed that nf-1 and sf-1 had normal tentacle-regenerative capacity (Sugiyama & Fujisawa, 1977b). The chimaera was produced by making a temporary graft between a polyp of strain nf-1 and of another strain for 24 h. During this time only interstitial cells and their derivatives (nerves and nematocytes) migrated into nf-1 tissue (Sugiyama & Fujisawa, 1977b).

Using the same procedure, we produced a new chimaeric strain (named chim-3) that consisted of epithelial cells of strain nf-1 and interstitial (and hence nerve and nematocyte) cells of strain reg-16. Starting from a single polyp, chim-3 multiplied by budding to exceed well over 50 animals in about 2 months. At that time, chim-3 hydra were subjected to the standard tentacle regeneration assay described in Materials and Methods, and the result is shown in Fig. 7. Chim-3 hydra had originally about nine tentacles per polyp and about the same number was restored upon regeneration, demonstrating that the regenerative capacity of chim-3 was comparable to that of the wild-type strains. The same experiment
repeated two months later with the same chimaera strain gave nearly the identical result. These observations show that the regeneration deficiency of reg-16 did not accompany interstitial cells and their derivatives into the chimaera strain.

**DISCUSSION**

*Regenerative capacity*

After head and foot removal, reg-16 hydra regenerated tentacles very poorly. This, however, was not due to the generally reduced morphogenetic activity since foot regeneration appeared to be normal, and since asexual multiplication by budding was quite vigorous in this strain. The doubling time in a mass culture was about 5 days for reg-16 and about 3 days for strain 105. There were many sexually produced F₁ and F₂ strains that multiplied slower than reg-16 but had perfectly normal tentacle-regenerative capacities.

One of the interesting findings with reg-16 was the observation that regeneration efficiency of this strain was strongly influenced by how animals were dissected. A long cylinder of body column obtained by amputation of head and foot (structure II in Fig. 4) regenerated tentacles very poorly, and additional transverse dissection of the long cylinder into two shorter ones (structures III and IV) had little effect. However, splitting the long cylinder longitudinally into two open pieces (structure V in Fig. 4) greatly enhanced the regeneration of tentacles (Fig. 5). At first we thought that this might be due to the increased leakage of substances such as morphogens through the wounded surfaces. However, many small incisions made on the long cylinder failed to stimulate its regeneration. At present we have no good explanation for these observations.

*Polarity gradients*

According to current theories, polarity gradients play vital roles in hydra morphogenesis (Wolpert *et al.* 1974; Meinhardt & Gierer, 1974). We thought that defective polarity gradients might be responsible for the regeneration deficiency of reg-16, and examined this possibility by using lateral grafting of tissues. This technique was elegantly used by Wolpert and his coworkers to analyse the process of head or foot determination during regeneration (see Wolpert *et al.* 1974). In the present study, however, this technique had one serious limitation in that the technique used regeneration (or related phenomena) as a means of assaying the underlying mechanisms of regeneration. Nevertheless, experiments provided some interesting results that can be discussed in the light of positional informational theory (Wolpert, 1971; Wolpert *et al.* 1974) and the lateral inhibition theory (Gierer & Meinhardt, 1972; Meinhardt & Gierer, 1974).

Although they differ in some important ways, the two theories are similar in aspects essential for the present discussion. According to them, polarity in hydra is primarily maintained by two gradients; one is a stable (non-diffusible) gradient of head-forming potential while the other is an unstable (diffusible)
gradient with potential to inhibit head formation. Both gradients are high in hypostome and become lower toward basal disc. Head is produced, if not already present, when the head-forming gradient becomes sufficiently higher than the inhibiting gradient in a given tissue. Foot is produced when the relationship between the two gradients is reversed, although additional factor(s) may be also involved for this process (Hicklin & Wolpert, 1973).

Thus, in lateral grafting experiment with normal hydra (strain 105), head induction occurs when donor tissues with high head-forming gradient level (upper gastric) are grafted to the site of low inhibitor gradient level (lower gastric), but the same tissues regress when grafted to the site of a comparable inhibitor gradient level (upper gastric). The same tissue, however, does induce head formation in the same site when it is derived from a regenerating animal, indicating an increase of the head-forming potential in the distal ends of regenerating animals.

When compared to the results with strain 105, lateral grafting experiment with reg-16 hydra produced three important differences: (1) upper gastric tissues transplanted to lower gastric region regressed instead of inducing head formation; (2) the same tissue, upon grafting to the upper gastric region, sometimes induced foot formation instead of regressing; (3) increase of head-forming potential was not observed in the distal tissues of regenerating animals.

The simplest way to explain these observations in the light of the gradient theories is to assume that the head-forming gradient of reg-16 is labile in the wounded tissues. Then, the upper gastric tissues removed from donor animals would behave as if they were taken from lower gastric region and, upon grafting, produce results as (1) and (2) above. At the distal ends of regenerating animals, the head-forming gradient level would drop, instead of remaining at the same level and later becoming higher, thereby failing to induce head determination at this site.

Nerve cells of reg-16

Nerve cells are thought to play vital roles in hydra morphogenesis and also in maintaining axial polarity (Bode et al. 1973). Also, low-molecular-weight substances having properties expected for morphogens have been isolated from nerve cells (Schaller & Gierer, 1973; Schaller, 1973; Berking, 1977). We therefore thought that regeneration deficiency of reg-16 might be associated with the nerve cells of this strain. To examine this possibility, we introduced nerve cells of reg-16 into nerve-free hydra strain named nf-1 (Sugiyama & Fujisawa, 1977b). This strain, like the colchicine-derived strain produced by Campbell (1976), contains no interstitial cells, nerve cells or nematocytes. When a tissue of a normal strain was temporarily grafted onto nf-1, migration of interstitial cells (and thus, through differentiation, of nematocytes and nerve cells) took place from the normal tissues into nf-1. No migration of epithelial cells occurred during the same time. These observations were made by using donor tissues labelled with
tritiated thymidine and following the cell migration by radioautography (Sugiyama & Fujisawa, 1977b). After separation of the graft, the interstitial cells introduced into nf-1 multiplied there, resulting in the formation of a chimaera strain containing epithelial cells of nf-1 origin and interstitial cells and their derivatives of another origin.

Using the same procedure, several new chimaera strains were produced between nf-1 and various mutant strains previously described (Sugiyama & Fujisawa, 1977a). When mutants were used that had characters known to be associated with interstitial cells or their derivative cell types (nematocyte deficiency, nerve cell deficiency) such characters were transferred to chimaeras, establishing that chimaeras were indeed produced as the result of interstitial cell introduction from donors to nf-1 (to be reported later elsewhere).

When reg-16 was used as the interstitial cell donor, its regeneration deficiency was not transferred to the chimaera strain, suggesting that this character was not associated with the nerve cells. Marcum & Campbell (1977) and Sugiyama & Fujisawa (1977b) recently reported on the morphogenetic capabilities of nerve-free hydra and suggested the possibility that nerve cells play very limited roles in hydra morphogenesis. This suggestion appears to be supported by the present finding that the regeneration deficiency of reg-16 was not located in the nerve cells.

Genetic background

Reg-16 was obtained through sexual inbreeding of wild strains originally collected from a pond in Ugowada, Akita, Japan (Fig. 1). Presumably, the genes responsible for the regeneration deficiency of this strain were carried in the original parents in recessive and heterozygous forms, and they became homozygous in reg-16 through inbreeding. Plural numbers of genes are probably involved since strains showing different levels of regeneration deficiency have appeared among the progenies of the inbreeding (Fig. 1). However, precise genetic analysis has not been done since high mortality of sexually produced progenies due to inbreeding depression makes such an analysis extremely difficult (Sugiyama & Fujisawa, 1977a).

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REFERENCES


Hydra regeneration mutant


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