

Head formation at the basal end and mirror-image pattern duplication in *Hydra vulgaris*

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ABSTRACT Head and foot in *Hydra* are organizing centers and considered to be sources of long-range inhibitory morphogens that prevent head and foot formation elsewhere. In a previous study the apparent long-range head inhibition was shown to coincide with long-range foot promotion exerted by the head. Here it is shown that: (1) ring-shaped pieces of the body column taken from a near-foot position form feet – frequently circular – if inserted into the midgastric region; this ectopic foot formation is strongly dependent on assistance by the head. (2) Bisection causes a transient increase in positional value at the wounded basal end of the upper body column. This transient development in the head direction in turn promotes ectopic foot formation by transplants and thus has an effect as though the source of a foot-inhibiting morphogen were removed. The existence of long-range foot inhibition is open to question. (3) If a ring with low positional value is present in the midgastric region, the increase in positional value at the basal end is stable and results in mirror-image head formation instead of foot regeneration in up to 100% of cases. Even before the ring forms a foot it acts like a ligature and subdivides the body column into two developmental compartments. (4) The basal head in turn organizes a mirror-image duplication of the body pattern. In pattern regulation, *Hydra* follows rules of intercalation known from other organisms.

KEY WORDS: *pattern formation, positional value, intercalation, regeneration, Hydra vulgaris*

Introduction

Hydra is a well established model organism for investigating morphogenetic pattern formation. Three features made the freshwater polyp a popular system for experimental studies at the organismal level: (1) the body architecture is relatively simple, exhibiting only one axis of asymmetry ("polarity") with the apical head (hypostome and tentacles) and the glandular foot as terminal reference structures (Fig. 1). (2) *Hydra*'s body is not a static structure but a dynamic steady-state assembly of an ever-changing cell population. In a process of perpetual self-renewal new cells are constantly produced from stem cells to replace aged or lost cells. While the new cells differentiate and migrate they respond to ever-available positional information. (3) The high capacity for regeneration makes it possible to initiate large-scale reorganization at any time.

Classical studies analyzed the properties of the regulatory system by grafting together segments of the body column of various lengths and positional origins or by inserting small lateral grafts into the body wall (e.g. King, 1901, 1903; Browne, 1909; Mutz, 1930; Tardent, 1954; MacWilliams *et al.*, 1970; Wilby and Webster, 1970a,b; Hicklin and Wolpert, 1973; Hicklin *et al.*, 1973; MacWilliams and Kafatos, 1974; Wolpert *et al.*, 1974; MacWilliams, 1983a,b).

This study resumes this type of experimental approach because transplantations allow both short-range and long-range interactions between the parts of the body to be detected. Only some general features of this regulatory system can be reviewed here.

The hypostome of *Hydra* is an organizing center capable of exerting inducing influence on neighboring tissue, comparable to the amphibian upper blastopore lip (Browne, 1909; discussed in Lenhoff, 1991, and Müller, 1996). In addition, the hypostome exerts apical dominance by inhibiting competitive head formation elsewhere along the body column. This apparent inhibition has been attributed to a hypothetical long-range head-inhibiting morphogen (Webster, 1971; Shostak, 1972; Wolpert *et al.*, 1972, 1974; Berking, 1977, 1979; MacWilliams, 1982, 1983b; Meinhardt, 1993).

The foot has been considered to be a second organizing center with reciprocal activities and the source of a long-range foot-inhibiting morphogen (MacWilliams and Kafatos, 1968, 1974; MacWilliams *et al.*, 1970; Shostak, 1972; Hicklin and Wolpert, 1973; Berking, 1977, 1979; MacWilliams, 1982; Meinhardt, 1993).

Later it was shown that the apparent long-range head inhibition coincides with long-range foot promotion exerted by the head

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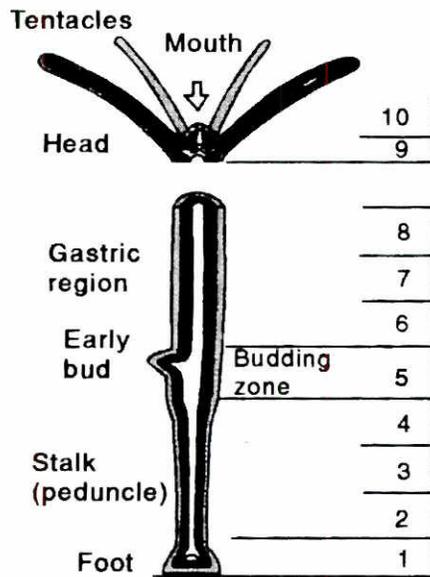


Fig. 1. Body regions of a *Hydra* and notation used in this study.

(Müller, 1990, 1995b). This observation has led to an alternative interpretation of the experimental observations: the apparent head inhibition is explained as a consequence of competition for limited cellular resources and hormonal factors: the existing head binds and removes hormonal head-promoting factors (Müller, 1995a,b; Sherratt *et al.*, 1995). By doing so, it prevents competitive head formation elsewhere and simultaneously promotes foot formation.

In more formal terms, transplants form ectopic heads if their positional value is substantially higher than that of their new surroundings; they form a foot if it is substantially lower. Inferences from such observations about the existence of morphogens are indirect and speculative.

On the other hand, long-range influences do exist in *Hydra* whether they are mediated by morphogens or other mechanisms such as depletion of precursor cells or factors. This study was undertaken to reexamine long-range influences with emphasis on the hypothetical long-range foot inhibition. The results were surprising, for in certain experiments the basal end formed a head instead of a foot, although the strain of *Hydra* used is known to rigidly maintain its original polarity.

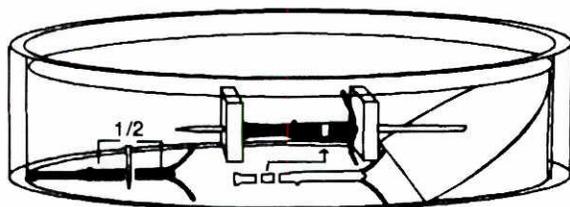


Fig. 2. Axial grafting procedure. The dish was designed by Stefan Berking. Dishes of this type allow up to 100 transplantations to be performed per day and person.

Results

Ring-shaped transplants frequently form circular feet, leading to the separation of the body column as in the case of budding

The primary aim of the present study was to examine whether the head has a supporting influence on the frequency of ectopic foot formation by transplants as the competition-for-resources model suggests. In preparation of the planned experiments various grafting procedures were tested. The procedure should fulfil two criteria: (1) it should produce consistent control values and (2) no other ectopic structures besides feet should be evoked. Eventually the following type of axial grafting was chosen.

Ring-shaped transplants taken from a low positional level of the body column (peduncle, stalk), henceforth called low-p rings, form feet if inserted into the mid-gastric region of a host. Ring-shaped transplants may form a patch-like foot as do lateral transplants. But unlike lateral grafts, ring-shaped transplants sometimes form more than one foot and frequently a ring-shaped



Fig. 3. Feet formed by ring-shaped transplants. Feet were made visible with a peroxidase stain (Hoffmeister and Schaller, 1985). All stained tissue represents "foot" as far as peroxidase activity defines foot tissue. The original vital stain bleached in the course of the peroxidase reaction. Of the 4 specimens shown, 3 have a ring-shaped foot, one has a patch-shaped foot. Ring-shaped feet lead to the eventual physical division of the body column.

foot as do buds (Fig. 3). Feet appear in the transplant close to its upper or lower border, or anywhere within the transplant. (Quantitative data on the relative frequencies of the various types of feet and their precise location were not collected). Like the circular foot in buds, the circular foot of the transplant eventually contracts and causes a transverse physical separation of the body column. However, this splitting did not occur until one to four weeks after transplantation. When it occurs, the column splits into two separate but different specimens: a normal animal with head and foot and an abnormal specimen with feet at both ends. Usually, these pieces with bipolar feet did not regenerate a head unless the middle of the piece was injured (no quantitative data collected).

In the following no distinction is made between specimens with ring-shaped feet and those with patch- or spot-shaped feet. In

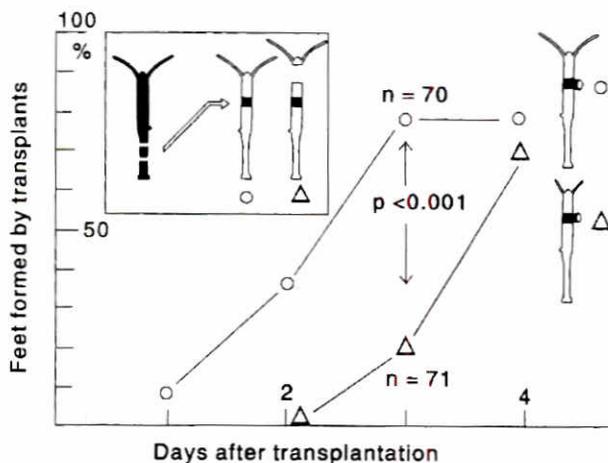


Fig. 4. Promotion by the host's head of ectopic foot formation by transplants.

independently performed experiments the frequency of ectopic foot formation by low-p-rings displayed some variance (for example, 80% in Fig. 4 and 68% in Fig. 5), but the differences among independent but otherwise identical experimental series did not exceed the level of significance at $p = 0.5$.

The head of the host strongly supports ectopic foot formation by transplants

When the position-dependent formation of feet by transplants and the apparent inhibition by the existing host foot were investigated (MacWilliams and Kafatos, 1968, 1974; MacWilliams *et al.*, 1970; Shostak 1972, 1973; Berking, 1979), the long-range assistance of foot formation by heads and buds (Müller, 1990, 1995b) was not known. Therefore in the present study the frequency and speed of foot formation by transplants were reexamined in the presence and absence of a host head. Low-p-rings taken from the stalk were intercalated into the middle of the gastric region. The head of the host was either left or removed 1-2 h following the grafting procedure. The results show: the head has a strong promoting influence on foot formation in the grafts (Fig. 4). In the decapitated animals ectopic foot formation began only after the head was regenerated.

Positional values upstream and downstream from the transplant are of significance

The following experiment was designed to examine whether or not assistance by high positional values of ectopic foot formation in transplants is a polarized event. The low-p ring was confronted with high positional value at only its apical side or at both its apical and basal side. When the *Hydra* is arbitrarily divided into 10 regions of different positional values (Fig. 1), the constructs had the composition 10...7/3/4...1 versus 10...7/3/7...1. The results (Fig. 5) clearly show that low-p rings consisting of a middle part of the stalk (value=3) made feet faster and more frequently when high positional values were present both downstream and upstream from the transplant than when only the upstream values were high.

A traditional interpretation (for example, MacWilliams and Kafatos, 1974) would ascribe these differences to longer and shorter distances between the transplant /3/ and the foot ...1, the

main source of the supposed foot inhibitor. The effect of foot removal was reexamined in the following experiments.

Removal of the host foot supports ectopic foot formation – but with a surprising end result

Foot formation in the transplants

Removal of the existing foot of the host promoted ectopic foot formation by lateral grafts in *Hydra viridis* (MacWilliams and Kafatos, 1974). Several considerations prompted a reexamination of this effect in the context of the present study. (1) Different species of *Hydra* and even different strains within a species can behave quite differently. (2) In the present study, the axial grafting procedure is used, in former studies (MacWilliams and Kafatos, 1974) the lateral grafting method was employed. In transplantations designed to test the apparent head inhibition the lateral and axial grafting procedures gave rather different results (Shimizu and Sawada, 1987; and my own results with *Hydra vulgaris*; data not shown). (3) A possible influence of the head (or a bud) on foot formation by transplants was previously not taken into account.

In a first study the frequency of foot formation by low-p rings was compared in animals whose own foot was removed or left. In most of these grafts a decline of long-range foot inhibition upon foot removal was not evident (only data of one experiment that apparently shows the expected decline of inhibition will be presented below). Frequently, feet formed in the transplants in the close vicinity of the regenerating host's feet (Fig. 6).

Lack of apparent mutual inhibition might be attributed to unfavorable phase-relationships in the kinetics of foot differentiation at the two neighboring locations. A synchronous start of foot

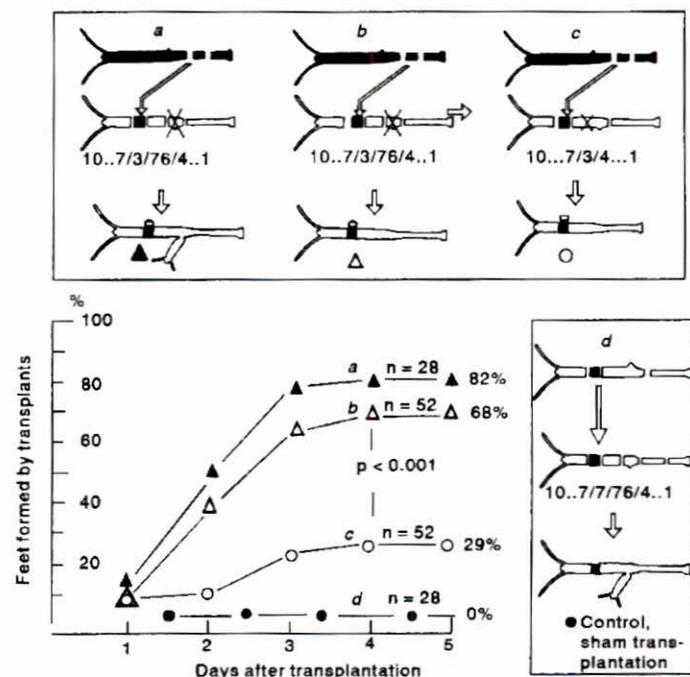


Fig. 5. Influence of positional values downstream from the implanted ring on ectopic foot formation by the ring.

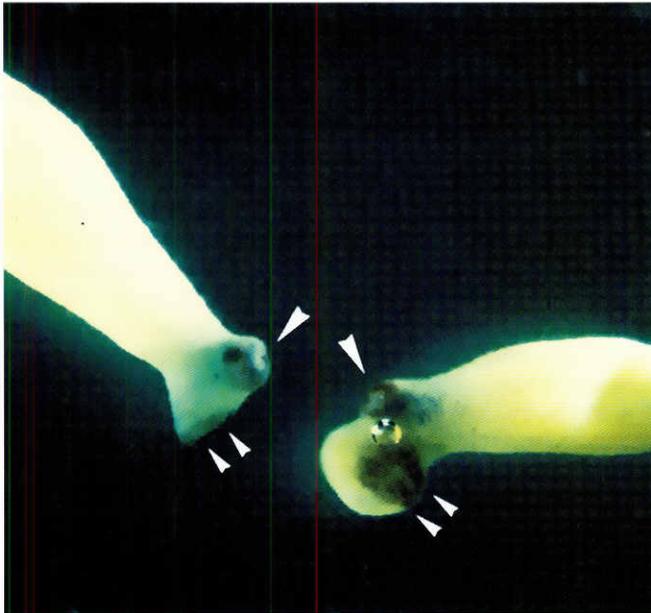


Fig. 6. Feet formed close to each other. The photos show two examples seen in the experiment shown in Figure 7 and were performed to examine whether the neighborhood of an ectopic foot prevents foot regeneration at the basal end or, conversely, a basal foot prevents foot formation by transplants. The foot-bearing part of the host was removed close to the transplant and 1 h after the low-p ring had been inserted. Even very close proximity often did not prevent competitive foot formation. One arrow: foot formed by the transplant. Two arrows: foot formed by the host.

formation might lead to a mutual inhibition of equal strength (Bode and Bode, 1984). To disrupt a possible synchronism, the experiment was modified in several ways. One such modification was the following: to increase speed and frequency of ectopic foot formation, the transplants were confronted with high positional value on both sides and the following combinations prepared: 10...7/32/764321 (with foot) and 10...7/32/7643 (without foot).

In this situation the data seemingly demonstrated a significant decline of long-range foot inhibition following foot removal. The low-p ring formed a foot more frequently and faster when the foot of the host was removed than in its presence. After 4 days 95% of the low-p rings inserted into the gastric region of foot-deprived hosts had formed an ectopic foot, while in the group of foot-bearing hosts only 68% of the transplants had formed an ectopic foot (Fig. 7).

Head formation at the basal end

The decline of apparent foot inhibition upon removal of the host's foot was associated with a surprising event. By day 4, in most cases the basal end of the host's body column was still footless. This is unusual. Seemingly, the ectopic foot of the transplant prevented normal foot regeneration. But some days later, many of the foot ends had formed – a head! (Fig. 8).

In an additional study, the conditions of head formation at the basal end were analyzed in more detail. Not surprisingly, the frequency of head formation at the lower end of the host's body column is a function of the level where the host is cut, being higher

at near-head and lower at near-foot cut levels. Up to 100% of the animals formed a head instead of regenerating a foot when the lower cut was in the gastric region (halfway between the transplant and the budding zone). Lower values were scored when the lower cut was in the stalk (below the removed budding zone, Fig. 9). If the lower cut was close to the budding zone, not a head but a detaching bud appeared.

When the frequencies and kinetics of ectopic foot formation by transplants and head formation at the basal end of the hosts are compared, a clear positive correlation is evident: head formation at the basal end occurred exclusively in specimens in which the transplants had formed a foot or were about to form a foot. If the t_{50} 's are compared, visible foot formation in transplants preceded visible tentacle formation at the basal end by one day (Fig. 10). This correlation suggested a causal relationship.

The apparent transient drop of 'foot inhibition' observed after foot removal coincides with a temporary increase in 'head activation potential' at the lower cut

Head formation by the basal end presupposes a local increase in positional value (also called the "head activation potential": MacWilliams 1982, 1983b). This increase in positional value in turn should influence foot formation in the transplants previously inserted into the midgastric region. As stated above, high positional value not only upstream but also downstream from a transplant promotes foot formation within the transplant.

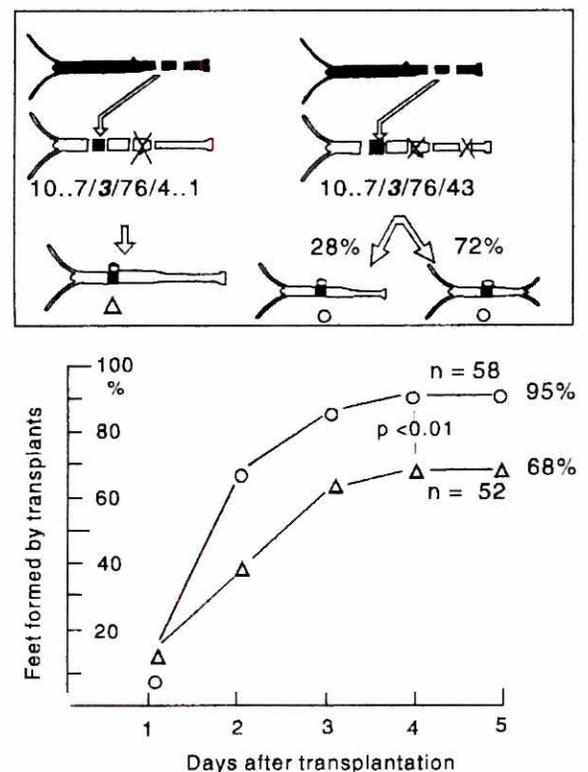


Fig. 7. Apparent promotion of ectopic foot formation in the transplants by the removal of the host's own foot. The removed foot is frequently replaced by a head instead of a foot (Figs. 7 and 8).

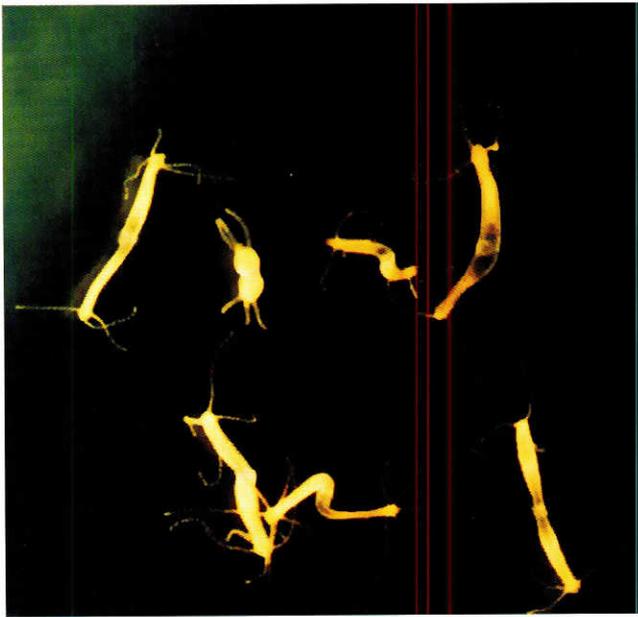


Fig. 8. Mirror-image heads in *Hydra vulgaris*. In spite of the presence of the original head a second head formed at the basal cut if a ring-shaped transplant of low positional value had previously been inserted into the gastric region. The interval between grafting the ring and removal of the host's lower body part was 1-2 h.

When in previous studies removal of the host's foot was shown to favor foot formation in transplants, this effect was ascribed to the disappearance of a supposed long-range foot inhibitor emanating from the existing foot (MacWilliams and Kafatos, 1968, 1974; MacWilliams *et al.*, 1970).

Therefore, the promoting effect of foot removal could be attributed either (a) to the disappearance of a foot-inhibiting substance after removal of its source, or (b) to an increase in positional value at the lower cut of the host due to wounding; this increase in turn would indirectly promote foot formation in the transplant.

I reexamined the effect of foot removal by methods that allow a decrease in foot inhibition to be distinguished from an increase in positional value.

Animals were bisected in the middle of the gastric region. From the upper, head-bearing fragments ring-shaped pieces comprising the lower end were taken as probes and inserted into the stalk region of host animals (Fig. 11) or grafted onto the lower end of hosts whose foot had been removed (Fig. 12). Previous pilot studies had shown that significant percentages of ectopic head formation could be expected only if the head of the host as well as developing buds are absent. Therefore, the hosts were reduced to the stalk region. The grafts were carried out 2 h after the donor animals were bisected and, for a control, immediately after the cut was made. If removal of the foot resulted in a drop of foot inhibition, the two-hour probes should show an increased probability of forming feet, since MacWilliams and Kafatos (1974) had shown the apparent decline of inhibition to occur within 0.5 to 3 h after foot removal. At least a decreased probability of forming heads was expected. If bisection resulted in an increase in positional value, the probes should display an increased capacity to form head structures and/or a decrease in the tendency to form feet.

In both experiments, 2 h after bisection the basal end of the body column did not display an increased capacity for foot formation but an increased capacity for head formation (Figs. 11, 12). The probability of head formation by the probes was improved in the two-hour probes compared to the 0-hour probes. Although the increase in positional value at the basal end was only slight (but significant) even a long-range competition effect was observed: concomitant with the increased capacity for head formation in the probes, the capacity for head formation at the apical end of the host was reduced.

The higher incidence of head formation by the two-hour probes might be attributed to an additional injury effect. Prior to grafting the probes the freshly closed wound at their basal end had to be prised open again. On the other hand, if the foot region were source of a diffusible long-range foot inhibitor, wounding at this position should not promote head formation but support foot formation.

Moving cells did not cross a ring that was about to form a foot

Epithelial layers in *Hydra* are not static tissues but move from the gastric region in the apical and basal directions, and onto an emerging bud (for example Campbell, 1967; Shostak, 1967; Shostak and Kankel, 1967). In the present context it was of interest to see that stained cells did not cross a low-p-ring located in the midgastric region of the host. Even before the ring visibly displayed a foot, while the gastric cavity was still entirely continuous allowing free exchange of food, the ring was an effective barrier to cells moving in the epithelial layers. Normally, epithelial cells move from the midgastric region upward to enter the tentacles and downward to enter a bud or the stalk region. As vitally stained donors or hosts were used, the movements could easily

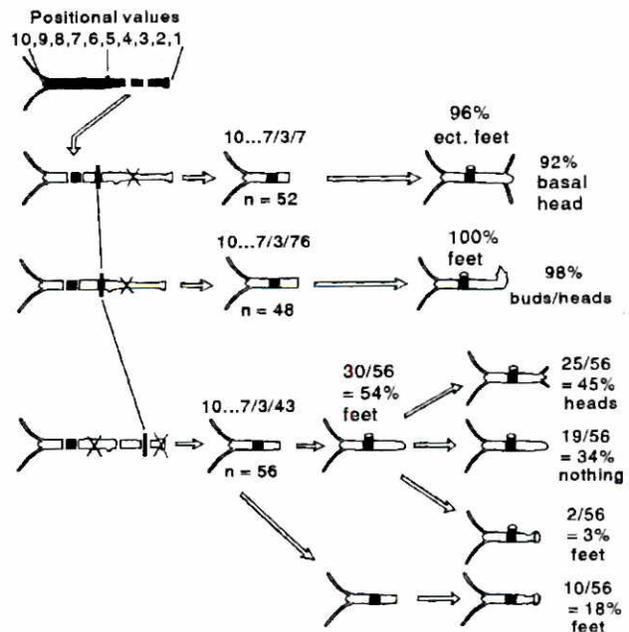


Fig. 9. Overview of the possible development at the basal end downstream from a foot-bearing transplant. The data are from actual experiments.

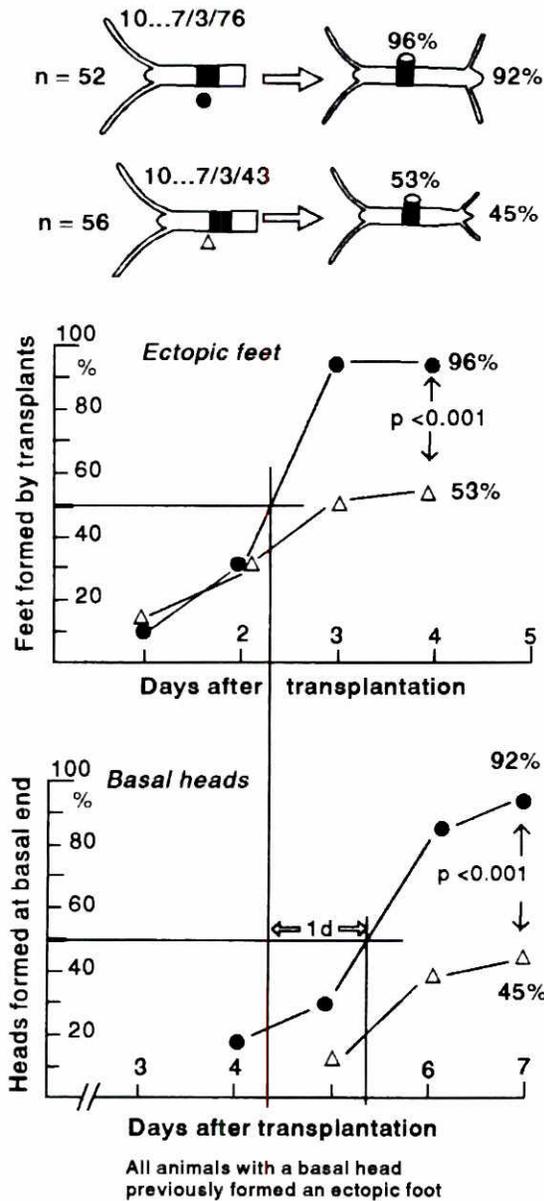


Fig. 10. Correlation between the development of heads at the basal end and the previous development of feet in the transplants.

be followed (see Fig. 13). If the transplant was stained, blue spots indicating endodermal cells with internalized blue pigment granules began to disperse 3-5 days after grafting and moved into the tentacles of the host's head or into buds as described in the literature (Kankel, 1967; Shostak, 1967; Shostak and Kankel, 1967). By contrast, if the host was stained blue and the pathway in the gastric region interrupted by an unstained low-p-ring, stained host cells from the upper gastric region did not cross this region even when the host was about to develop a bud or a second head below the transplant. Thus emigration of cells from a foot-forming transplant occurred but not immigration and transit. The circular transplant formed a barrier even when it formed merely a small lateral foot and long before the ectopic feet split and separated the body column physically.

Ectopic heads frequently form at graft/host junctions

When in the previous sections ectopic head formation was ascribed to the transplant, this attribution was not entirely correct. Ectopic heads were formed at the graft/host junction and comprised tissue contributed by both partners (Fig. 13). Ectopic feet were also formed preferentially near the boundaries but within the transplant. The interpretation of this phenomenon has a trivial and a non-trivial component. The trivial component is that in the transplants the highest and lowest positional values are at the transplant/host boundaries. The non-trivial aspect is that head (or bud) formation does occur and that both partners contribute to the emerging structure. This observation is reminiscent of induction and intercalation phenomena in other developmental systems.

Heads at the basal end document polarity reversal and give rise to mirror-image body duplications

In the experiment shown in Figure 12, the probes were always grafted onto the lower end of a host with their polarity oriented in the direction of the host's polarity. Nevertheless, heads were formed with their polarity oriented opposite to that of the host's body column. Eventually, a mirror-image duplication of the body resulted. In a few cases (2 out of 81) a second whorl of tentacles was formed at the donor/host boundary. Basal head formation always resulted in a mirror-image duplication of the axial body pattern (Figs. 8, 9, 10 and 12).

Hydra follows rules of intercalation observed in other animals

Pattern duplication that starts with ectopic head formation implies intercalation. In the course of this study intercalation of missing structures was often observed. For example, in biheaded animals the entire body column was eventually transformed into two mirror-image animals sharing a common foot for some time (Figs. 8, 9, 10, 12), until the foot split to give rise to two separate individuals. Such a mirror-image duplication of the body pattern first follows the "rule of distal transformation" (Müller, 1986, and references therein): the sequence 10,9...6/10 is transformed into 10,9...6...9,10. But subsequently the rule of distal transformation is invalidated and the sequence is completed by the insertion of lower p-values:

10,9...6...9,10 → 10,9...1...9,10 (photographs in Müller, 1989). Decrease of positional values in the middle of such biheaded animals is caused by the foot-promoting action of the two opposite heads. Eventually, the common foot ...1... is split up and two complete individuals 10...1 and 1...10 separate from each other.

Furthermore, the V-shaped conjoined twins shown in Figures 11 and 13 eventually displayed a complete, mirror-image duplicated sequence of axial regions, including a budding zone, provided the animals were fed. When an ectopic head was present along the body column (as shown in Fig. 13) and the animal fed daily, the ectopic head gave rise to a second body axis. The animal acquires the form of a Y (Fig. 14A,B,C). The second axis in the conjoined twin displays a duplication of the axial pattern present in the primary axis between the head and the bifurcation. Later, the point of bifurcation is displaced downward and the Y is transformed into a V. Finally, the foot splits up and the V-forms give rise to two complete individuals. A ring-shaped foot facilitates the splitting process.

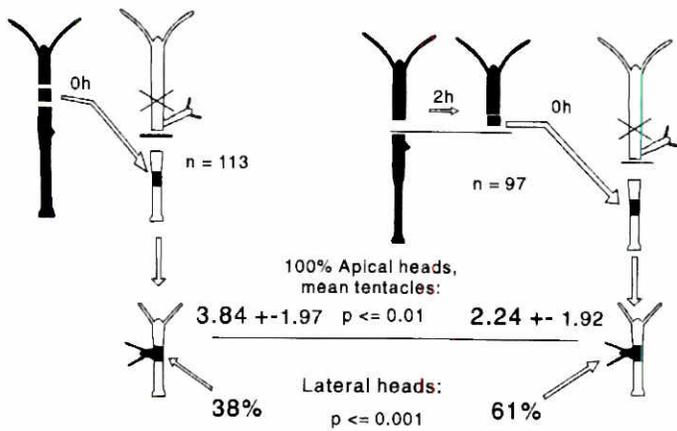


Fig. 11. Increase in positional value at the basal cut as measured by the capacity to form a head in the axial grafting procedure. In order to obtain ectopic heads in sufficient quantities the competing head of the host as well as the budding zone were removed.

By contrast, lateral ectopic feet usually do not give rise to a long secondary axis. Feet are rapidly displaced downward and arrive in the peduncle before the secondary axis has the opportunity to elongate. One of the very few second basal axes induced by a foot-forming transplant is shown in Figure 14B. However, if feet (with some stalk tissue) are directly coupled to the lips of the mouth secondary body axes emerge frequently and give rise to conjoined twins with duplicated lower bodies (not shown).

To further verify that *Hydra* obeys the rules of intercalation, a classic experiment, first conducted with insect legs (Bohn, 1971) was transposed to *Hydra vulgaris* in a way that allowed the animals to be fed. A complete or almost complete animal is coupled at its basal end with the lip of another animal (Fig. 15). Since healing together presupposes contact of the endodermal layers, the foot end of the first and the mouth field of the second polyp were injured to expose the endoderm. An additional head was grafted onto the basal end of the polyp whose mouth was attached to the neighbor and therefore closed and unable to ingest food. The second, grafted head allowed the polyp to be fed.

Twenty eight of such tandem grafts were prepared. After some days, between the head and foot of the adjoining polyps a tube-like structure emerged. However, since the induced structure was initially thin and the risk of breakage high, of 28 tandem grafts only 3 'survived' for more than 2 weeks and intercalated a complete body. The induced second body was derived from the foot end of the one partner. It elongated by growth and eventually displayed a complete set of positional values, however with inverted polarity: 10...5...1/10...5...10 → 10...5...1...5...10...5...10. The development of such specimens (Fig. 15) up to the separation of individuals takes 2-4 weeks.

A variant of the method easier to perform (and suitable for student courses) is shown in Figure 16. Heads and feet are removed and the remaining columns joined together. Under these conditions, usually a common head is formed at the junctions, where high and low positional values are in contact. Of 28 grafts 25 remained attached for 2 weeks. Then the polyps began to separate by splitting the common feet.

Discussion

Head formation at the basal end

This study gives the first report of head formation at the basal end of *Hydra vulgaris*. The strain used is known to rigidly maintain polarity in regeneration. Even repeated treatment with diacylglycerol, a procedure evoking ectopic head formation and bipolar head regeneration in *Hydra magnipapillata*, strain wt105, (Müller, 1989, 1990, 1995b), causes multiplication of the apical head in *Hydra vulgaris* rather than increase in positional value in lower body regions (Müller, 1995a).

It should be emphasized that the basal heads as shown in Figure 8 did not arise from grafts (as, for example, in Hicklin *et al.*, 1973) but from the host's own lower body end, and that the polarity of the host's body column was in no part physically changed. Throughout this study the polarity of all parts of a construct were assembled in the same, original direction. Transplants were always inserted with their polarity in the direction of the host's polarity. In addition, the length of the transplants was kept small and constant, and the design of the experiment was such that in hundreds of grafts besides ectopic feet no other ectopic structures were evoked (except deliberately in the experiment of Figure 11 and in the studies on intercalation).

Data presented here cannot be directly compared with data of classical transplantation studies. In those former studies small pieces of donor tissue were laterally inserted into the body wall of hosts (Browne, 1909; Webster and Wolpert, 1966; Hicklin and Wolpert, 1973; MacWilliams, 1983a,b), or segments of the body column of different lengths and positional values were confronted with each other, sometimes with opposite polarity, and the occurrence of ectopic head or foot structures at the junctions was observed (Wilby and Webster, 1970a,b; MacWilliams *et al.*, 1970; Wolpert *et al.*, 1972, 1974; Hicklin *et al.*, 1973; MacWilliams and Kafatos, 1974). Moreover, different species of *Hydra* were used. On the other hand, several findings in the literature are consistent with data presented here and disclose general features of the pattern-forming system. Thus a supportive influence of the head on foot formation can be inferred from data collected in various contexts with *Hydra littoralis* (Hicklin *et al.*, 1973; Table 4 versus Table 1) and *Hydra vulgaris* (MacWilliams and Kafatos, 1974). Eventually, assistance of the head in foot formation was explicitly stated in studies using *Hydra magnipapillata* (Ando *et al.*, 1989; Müller, 1990, 1995b). The present study gives the additional information that this supportive influence is not polarized and is effective not only at the basal end of the body column.

It was these transplantation studies cited above that prompted the development of several theoretical models of pattern formation and regulation. At present, pattern regulation in *Hydra* is explained by two different speculative models both of which are formulated mathematically to allow computer simulations.

(1) The Gierer-Meinhardt reaction-diffusion model (Meinhardt, 1993). This model produces standing waves of four morphogens: head activator, head inhibitor, foot activator, and foot inhibitor. The waves extend along the entire length of the body. In the framework of this model foot formation, for example, is triggered at any position when the local concentration of the foot inhibitor drops below a critical level. An overcritical drop enables the autocatalytic production of the foot activator. However, the existence of long-range morphogens is not well compatible with several findings reported in previous studies (Müller, 1990,

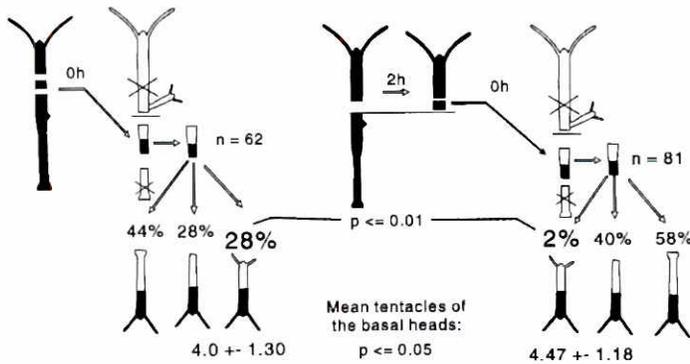


Fig. 12. Increase in positional value at the basal cut measured by the speed and quality of head formation in the transplanted probes. Note that heads are formed at the lower end although the polarity of the probes was originally identical with that of the host. Apparently, polarity reversal was quick and presumably caused by the longer opening of the basal wound. Note furthermore that the head at the basal end reduces the quality of the head at the apical end as measured by the number of tentacles formed.

1995a,b) and in the present study. For example, feet can appear close to each other (Fig. 6). Promotion of foot formation by the head is not included in this model.

(2) The receptor-based model of competition for hormonal factors, basically developed by the present author (Müller, 1990, 1995b, 1996; Sherratt *et al.*, 1995). In this model, the head end is the winner and the foot end is the loser in the competition for head-promoting factors. The formation of a foot by a transplant reflects its inferior ability to compete for the locally available factors. Its inferior ability in turn reflects its paucity of receptors for such factors. Promotion of foot formation by body regions with high positional value is an integral feature of this model.

The present study was not designed to verify or disprove former computer simulations and therefore will not discuss alternative interpretations based on these two models. Only a brief intuitive interpretation in terms of the competition model will be given. The primary aim of the following discussion is to examine which long-range interactions can be inferred from the results.

Does head formation at the basal end reflect a preexisting 'head activation' potential?

Head formation at the basal end may reflect a high local 'head activation potential' (MacWilliams, 1982, 1983b) present at the cut level even before the animals were bisected, caused by the proximity of a bud. Budding has been equated to secondary head formation (Meinhardt, 1993) and shares features with head regeneration from basal body levels (Technau and Holstein, 1995). Although the budding zone was removed, 'head activation potential' might have been high in the adjacent regions above and below the budding zone. The competence for budding extends beyond the narrow budding zone in the direction of the head and foot (Ando *et al.*, 1989; Müller, 1995b).

On the other hand, at the cut levels chosen the animals normally regenerate a foot whether or not a bud is present. Head formation began visibly only one or several days after the transplants had formed feet, and was morphologically no different from head regeneration at the apical end if the host was cut in the gastric region some distance above the budding zone.

A prerequisite for head formation at the basal end was the previous insertion into the gastric region of a ring-shaped piece of body column with low positional value, here called the low-p-ring. This ring formed a foot, often shaped like the circular foot normally found at the base of a mature bud. Presumably circular feet promote ectopic head formation at the basal end more effectively than patch-shaped lateral feet. (As this effect was unexpected, the type of feet formed by each single transplant was not noted).

Is help of foot formation by the head supplemented by a reciprocal help of head formation by the foot?

A rule formulated by the present author states that in *Hydra* additional heads evoke the development of additional feet but feet do not evoke the development of additional heads (Müller, 1990, 1995b, 1996). Is this rule no longer valid? Several arguments make a direct assistance of head formation by feet unlikely. *Hydra vulgaris* can easily be caused to form ring-shaped or multiple patch- and spot-shaped lateral feet, for example by prolonged lithium treatment (Hassel and Berking, 1989, 1990; Hassel *et al.*, 1993) or simply by inducing exhaustive budding (Müller, 1995b). Even a multitude of such feet does not evoke ectopic heads or cause head formation instead of foot regeneration at the basal end. Two conditions are essential for basal head formation to occur:

- (1) The animal must be cut. Injury is known to promote head formation (MacWilliams, 1983b; Kobatake and Sugiyama, 1989; Shimizu and Sugiyama, 1993).

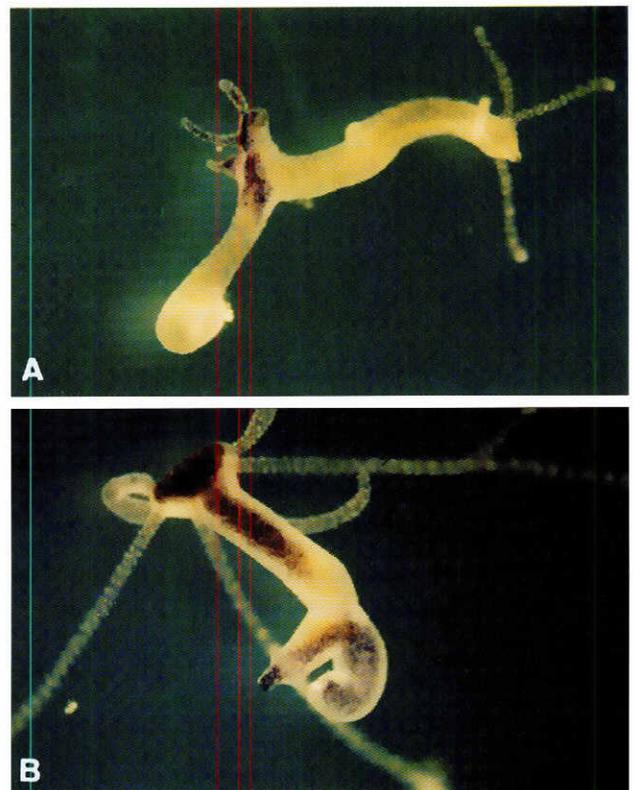


Fig. 13. Formation of a head (A) or a bud (B) at the transplant/host junction. Both, stained and unstained tissue moves into an emerging ectopic head or bud. (A) shows a head being formed by a transplanted foot end (experiment in Fig. 11).

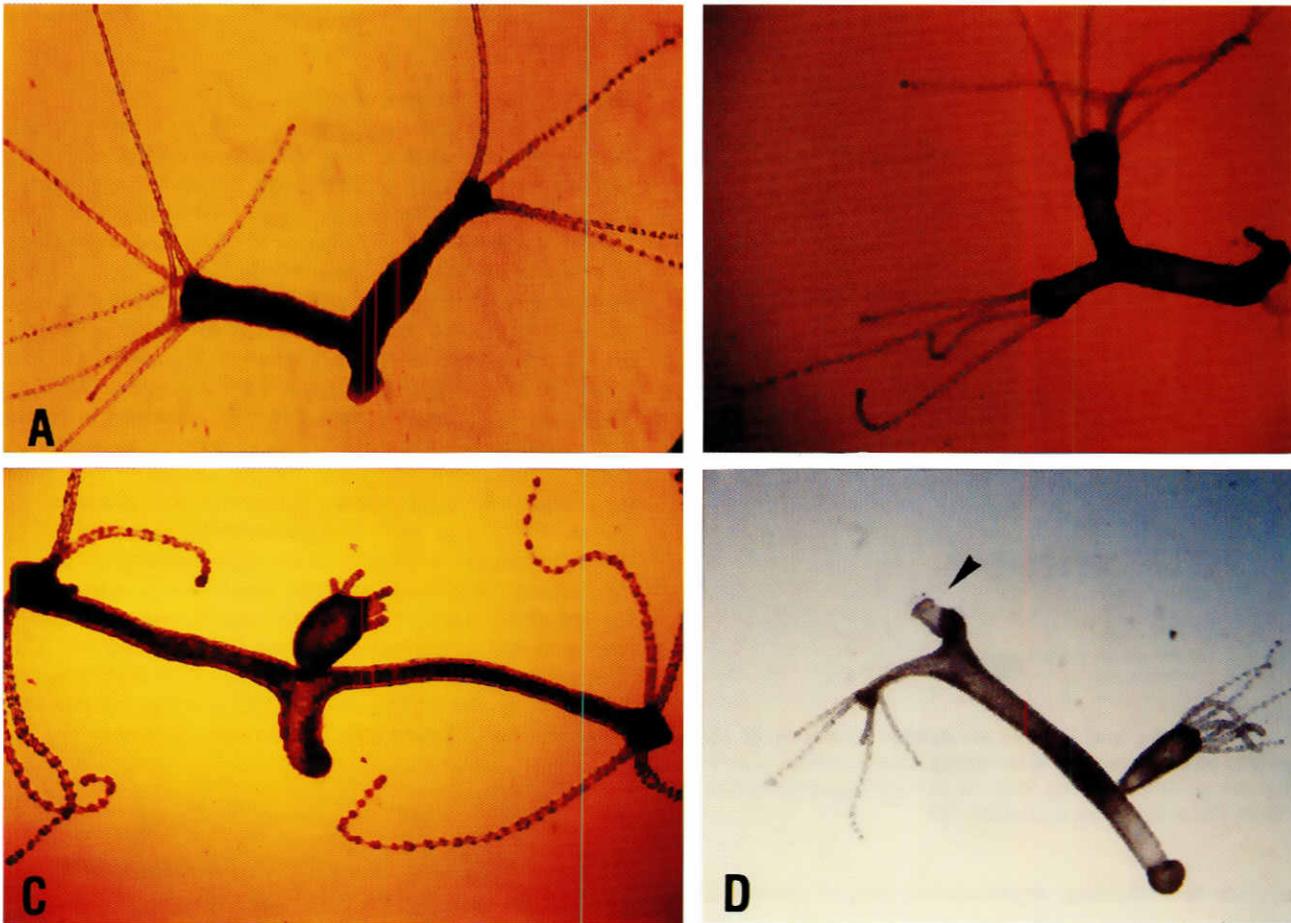


Fig. 14. Y-shaped forms documenting the validity of the intercalation rules in *Hydra*. (A,B,C) Ectopic head formation was induced by a lateral graft as shown in Figure 11. The second axis emerged from a transplant that was inserted into the body wall of the primary body axis. Between the point of insertion and the head a second axis intercalated in the course of 2-3 weeks. (B) Difference between a true second body axis and a bud, which formed at the bifurcation of the two body axes. (D) One of the very few basal axes observed in the course of the present study. The arrow points to the foot that formed by a transplant and gave rise to the second body axis.

(2) The implant must have a ring-shaped structure. The facilitating effect of the low-p ring appears to be indirect in that it causes a physiological splitting of the body column into two separate developmental compartments. A low-p ring acts like a ligation in the insect egg (Sander, 1975) and interrupts long-range interactions (although the gastric cavity remains open). Normally, positional value – in *Hydra* also called the "head activation potential" (MacWilliams, 1982, 1983b) – begins to increase at the lower cut but the increase is soon stopped and converted into a decrease by the action of the competing existing head.

How does basal head formation fit into the competition model?

Not only the supportive influence of the head on ectopic foot formation by transplants but also the observed basal head formation is readily explained in terms of the competition-for-resources model of pattern control (Müller, 1995b): the low-p ring barrier protects the lower body half from the competitive access of the existing head to the cellular resources and head-promoting factors produced in the lower half of the gastric column. The observation that moving cells do not cross a low-p ring supports this

interpretation. Protected from competition, the lower body part can form a head itself (Fig. 17).

New assessment of the apparent foot inhibition gradient

In previous concepts of pattern control in *Hydra* regenerative foot formation has been attributed to the decay of a foot-inhibiting morphogen whose main source is the existing foot. This concept gave a simple explanation why removal of the lower body part causes regenerative foot formation: without the suppressing activity of the supposed morphogen cells were thought to differentiate feet autonomously and automatically due to an inherent 'foot activation' (Cohen and MacWilliams, 1975). In extensive studies it was shown that removal of the host's foot promotes ectopic foot formation by lateral transplants (MacWilliams *et al.*, 1970; MacWilliams and Kafatos, 1974). The apparent foot inhibition declined within one hour after the foot of the host was cut off, and returned within 1-4 h, presumably – so the authors concluded – because the host was about to regenerate the lost foot and to restore the source of inhibition (MacWilliams and Kafatos, 1974).

In the present study no convincing, unambiguous evidence for long-range foot inhibition was found. Not only do feet which

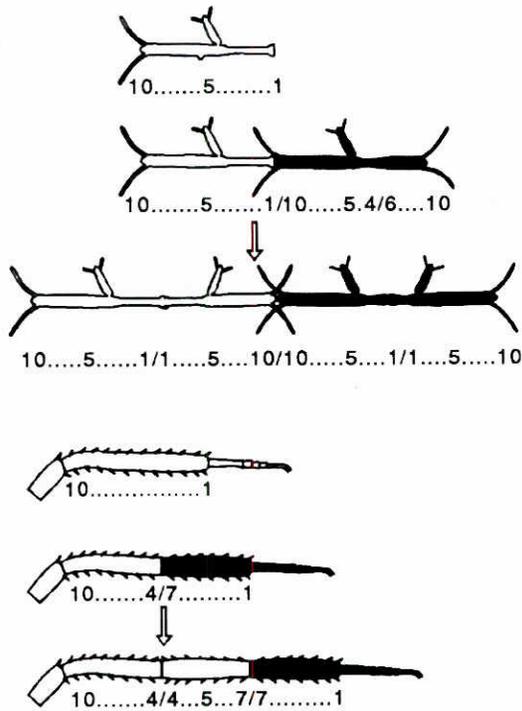


Fig. 15. Intercalation and pattern duplication in chains of *Hydra*, compared to intercalation in the insect leg. To enable healing together, adjoining feet and mouth fields were injured and pressed together for some hours. Food was taken up at both ends.

appear close to each other argue against long-range inhibition. The apparent decrease in foot inhibition in the midgastric region where the transplant was located was correlated with an increased capacity of the host's basal end to form heads rather than regenerating feet. Thus, the apparently diminished 'foot inhibition' may actually be due to promotion of foot formation in the transplant by the increase of positional value at the host's basal end, and not to the decay of a foot inhibitor. Transiently, the terminal tissues at both the apical and the basal end of the host compete with the transplants for head-promoting factors. In fact, this study showed that (a) ectopic foot formation by transplants depends on assistance by heads, and (b) high positional values both above and below a transplant cooperatively promote ectopic foot formation.

Upon cutting, positional value increases not only at the apical but also at the basal end but normally only transiently

Normally, the increase in positional value at the injured lower body end is temporary because it is subsequently suppressed by the existing head (supported by buds). The low-p ring prevents the correcting influence of the apical head.

This observation is of significance for the interpretation of local gene activities, for example by *in situ* hybridizations. Enhanced gene expression at the basal cut observed in the first 4 h after cutting does not necessarily reflect incipient foot formation or mere wound effects but can well reflect activities associated with increase in positional value – that is, with development in the direction of head formation. Thus, a decline in *Cnox-2* expression, which is associated with head formation, has been observed also

to occur temporarily at the basal end where later the foot was formed (Shenk *et al.*, 1993).

Observations on intercalation

Phenomena of intercalation in *Hydra* were actually observed by several investigators and are indicated in several published figures (Mutz, 1930; Tardent, 1954, 1960, 1972; Lenhoff *et al.*, 1969; Berking, 1979; Müller, 1989, 1990) but have not been explicitly pointed out and coherently described. *Hydra* observes the rules of intercalation followed by other systems, provided the animals are fed and the continuous loss of cells in the terminal body regions is more than merely compensated by cell proliferation in the middle of the body column. The rule of intercalation states that missing body regions are interposed when tissues of disparate positional values are directly apposed to each other. For example, the sequence 10...6/10 is completed to 10...6...10. Such a regulation has been clearly shown in the hydrozoan *Hydractinia echinata* (Müller, 1982) but also in *Hydra oligactis* (Mutz, 1930) and *Hydra magnipapillata*, strain wt105 (Müller, 1989). However, in contrast to the legs of insects or vertebrates, *Hydra* displays an extremely high flexibility in its response and has an extended ability to regulate and to correct patterns. For example, due to the foot-promoting activity of the two heads the sequence 10...6...10 is subsequently supplemented to 10...6...1...6...10 (Müller, 1989, 1990, and Fig. 8 in this study). In doing so, the animals also demonstrate that they are ready to

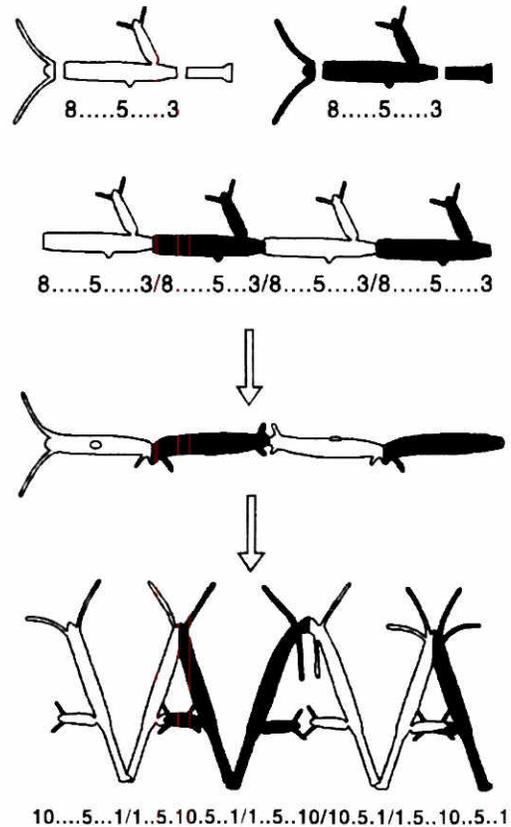


Fig. 16. Intercalation and pattern duplication in chains of *Hydra* pieces. To facilitate grafting, the original heads and feet were removed and the remaining body columns strung onto a needle (similar to Fig. 2).

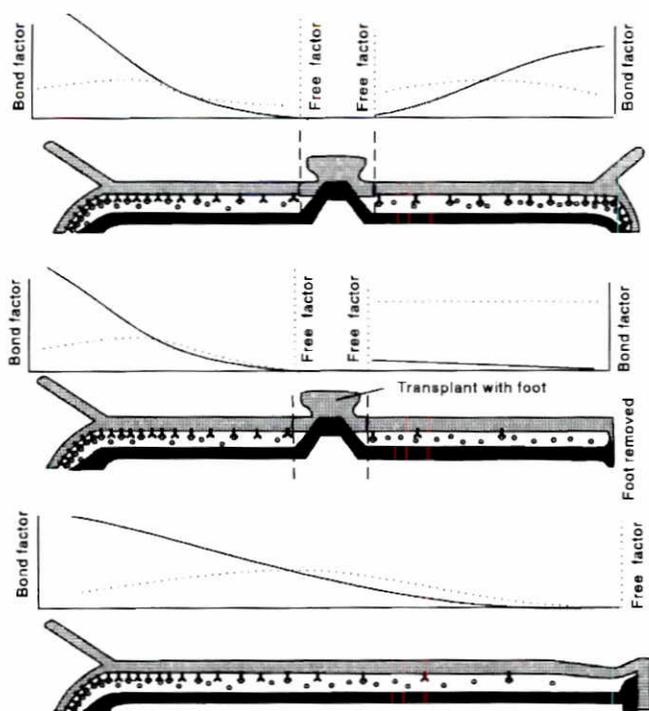


Fig. 17. Interpretation of head formation at the basal end in terms of the competition model. Head-promoting factors are produced in the gastric region and distributed in the interstitial spaces where they are picked up by epithelial cells with receptors and removed through internalization and enzymatic degradation. (For clarity, in the graph only the ectodermal epithelium is provided with receptors). The density of the receptors forms a gradient along the length of the body. In the undisturbed *Hydra* (bottom) the limited production of factor and its permanent removal have entered a steady state. In this steady state the amount of bound factor reflects and determines positional value. A transplant with low positional value has only a few receptors, loses in the competition for the locally available factor, and forms a foot. When such a foot-forming transplant subdivides the body into two compartments, the concentration of free factor increases in the lower body part because the cells of the lower body region have only few receptors to remove the continuously produced factors and this region is shielded from competition by the head cells (middle). As a consequence, the cells develop more receptors because the regulation of the receptor density includes a positive feedback: ample provision with factor causes the cells to synthesize more receptors (principle of induction of receptors by ligands) until the concentration of free factor falls below a critical level. Normally more apically located body regions have a "head start" because they possess more receptors from the outset, and win the competition. However, if the gradient in receptor density is shallow an injury effect can give the basal end an advantage (top).

change polarity, in contrast to, for example, the legs of insects and vertebrates (reviewed in Bryant *et al.*, 1977). The conjoined twins thus formed will then separate and give rise to two complete individuals. Y-shaped forms result from animals that have been induced by any means (oblique cuts, grafted pieces, diacylglycerol treatment) to form a second head. The second body axis demonstrates that values missing between a second head and the point of bifurcation are readily intercalated (Fig. 14A,B,C). By contrast, feet usually evoke the development of a second axis only if directly grafted onto the mouth region (Mutz, 1930). The same has been found in *Hydractinia* (Müller, 1982).

Therefore, while the head of *Hydra* is an organizing centre comparable to the upper blastopore lip in amphibians (reviewed in Müller, 1996), the foot is not a center of similar inductive capacity, and is probably not a source of long-range signals (this study). The system of pattern regulation is not symmetrically organized as assumed in current models based on long-range morphogens (Hicklin and Wolpert, 1973; Wolpert *et al.*, 1974; Meinhardt, 1993).

Conclusion

Pattern control in multicellular organisms obeys some common rules and principles such as rules of intercalation. This suggests common molecular mechanisms underlying the establishment and regulation of continuously graded positional values. However, these molecular mechanisms are largely enigmatic at present.

By contrast, the principles governing the basic decision head versus foot in *Hydra* are now emerging in broad outline: high positional value that endows the tissue with competence for head formation is associated with a high ability to attract precursor cells and to absorb head-promoting factors. The losers in this competition are caused to form the foot. These principles are further analyzed in the accompanying paper.

Materials and Methods

Animal culture and experimental manipulation

For this study clone Zurich of *Hydra vulgaris*, once collected by Pierre Tardent and now grown in many laboratories, was used. A population of animals was vitally stained blue by feeding *Artemia salina* nauplii that had previously been cultured in a suspension of 5 mg Evans blue in 100 ml of seawater. The particulate stain is endocytosed and stored by the endodermal epithelial cells.

This paper uses a notation for the regions of the body axis somewhat different from the traditional notation once introduced by Wolpert (Wolpert *et al.*, 1972). In the present study only numbers and not a combination of numbers and letters are used, and the region with the highest positional value is designated with the highest number (Fig. 1).

To induce ectopic head or foot formation the classic transplantation method was employed, adopting the axial grafting version (Tardent, 1972; Hicklin *et al.*, 1973; Shimizu and Sawada, 1987). Ring-shaped pieces comprising about one-tenth to two-tenths of the body column were excised from donors and inserted into the body column of host animals as shown in the figures. The pieces were joined together by stringing them one after another onto a stainless steel needle (Fig. 2). The diameter of the needle was slightly larger than the diameter of the gastric cavities of the donor and host animals. Thus the risk of distortions and mismatches between the host and donor tissues was minimized. The polarity of the transplants and host pieces was always kept in the same direction. After 1-1.5 h the polyps were removed from the needle.

Conventional lateral tissue transplantations (Webster and Wolpert, 1966; Wolpert *et al.*, 1974; MacWilliams, 1983a,b; Takano and Sugiyama, 1983; Müller, 1990) generally produce a higher yield of ectopic head formation. Nonetheless, this method was not used because the yield is critically dependent on conditions which are not easy to control, such as time of wound opening (Shimizu and Sugiyama, 1993) and perfection of the morphological integration of the implant into the body wall (Shimizu and Sawada, 1987).

To standardize the experimental design, rules observed in the laboratory of Tsutomu Sugiyama (Shimizu and Sugiyama, 1993, and references therein) were adopted. For example, polyps were selected that bore one just emerging bud, indicated by a small triangular protrusion of the body wall. Such a selection gives certainty that no further latent bud that could

influence the result is present. In addition, the site halfway between the tentacle whorl and the bud is a well-defined location for excising or intercalating grafts, because this point does not shift its relative position as the animal contracts or expands. If the bud was known or supposed to influence the result, the budding zone was removed immediately before the pieces were strung onto the needle. In a few experiments (Figs. 11, 12) starving animals whose last bud was about to detach were used.

In a given experiment, half of the transplants were taken from stained donors and inserted into unstained hosts, while in the other half the transplant was unstained and the host stained (Fig. 2). The values for the two series were generally almost identical and therefore averaged. Percentages of ectopic foot or head formation were statistically evaluated using the Fisher-Yates χ^2 test, mean tentacle numbers using the Mann-Whitney Test.

For photos, feet were made visible with the peroxidase stain (Hoffmeister and Schaller, 1985).

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