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Characterization of the head organizer in hydra

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SUMMARY

A central process in the maintenance of axial patterning in the adult hydra is the head activation gradient, i.e. the potential to form a secondary axis, which is maximal in the head and is graded down the body column. Earlier evidence suggested that this gradient was based on a single parameter. Using transplantation experiments, we provide evidence that the hypostome, the apical part of the head, has the characteristics of an organizer in that it has the capacity to induce host tissue to form most of the second axis. By contrast, tissue of the body column has a self-organizing capacity, but not an inductive capacity. That the inductive capacity is confined to the hypostome is supported by experiments involving a hypostome-contact graft. The hypostome, but not the body column, transmits a signal(s) leading to the formation of a second axis. In addition, variations of the transplantation grafts and hypostome-contact grafts provide evidence for several characteristics of the organizer. The inductive capacity of the head and the self-organizing capacity of the body column are based on different pathways. Head inhibition, yya signal produced in the head and transmitted to the body column to prevent head formation, represses the effect of the inducing signal by interfering with formation of the hypostome/organizer. These results indicate that the organizer characteristics of the hypostome of an adult hydra are similar to those of the organizer region of vertebrate embryos. They also indicate that the Gierer-Meinhardt model provides a reasonable framework for the mechanisms that underlie the organizer and its activities. In addition, the results suggest that a region of an embryo or adult with the characteristics of an organizer arose early in metazoan evolution.

Key words: Hydra, Organizer region, Head activation gradient

INTRODUCTION

A defining characteristic of an organizer region is its ability to induce the formation of a secondary axis when transplanted to another region of an embryo. Among vertebrates, this is true for the dorsal lip of frog embryos, Hensen's node in chick embryos, the embryonic shield of zebrafish embryos and the node region of the mouse embryo (Smith and Schoenwolf, 1998). In adult hydra, a similar phenomenon exists. The ability of tissue to form a secondary axis upon transplantation is graded down the single axis of the animal being maximal in the head (MacWilliams, 1983a). This gradient is commonly referred to as the head activation gradient. However, this capacity differs in hydra from the examined vertebrates in two ways. First, the capacity is found in an adult instead of an embryo. This most probably reflects the tissue dynamics of the animal, which require that the pattern forming processes be continuously active to maintain the form of the adult (Bode and Bode, 1984). Second, this capacity appears to be spread throughout most of the animal instead of being localized in a specific region.

There is some evidence to suggest that the properties of the head activation gradient are not the same throughout the animal (Yao, 1945; MacWilliams, 1983a). We show that the head activation gradient consists of two components. The hypostome region of the head has the inductive capacity of an organizer, while the ability of body column tissue to form a second axis is due to a self-differentiating or self-organizing property. In addition, we extend the understanding of the signals transmitted by the organizer to surrounding regions. The head is known to produce two long-range signals that are transmitted into the body column. One sets up the head activation gradient in the body column (Wilby and Webster, 1970a; Herlands and Bode, 1974; MacWilliams, 1983a), while the other inhibits head formation (MacWilliams, 1983b). We show that both are produced in the hypostome, and that the inhibitor of head formation is more precisely an inhibitor of hypostome/organizer formation.

MATERIALS AND METHODS

Hydra and culture conditions

One-day starved animals were used for all experiments. Experiments were carried out with the L2 strain of *Hydra vulgaris*, except for those involving LiCl treatment for which the Basel strain of *Hydra vulgaris* was used. Animals were fed three times a week and maintained as described previously (Martinez et al., 1997).

Tissue manipulations

Two kinds of transplantation experiments were carried out. In one, a modification of the normal lateral grafting procedure (Rubin and Bode, 1982) was used because the pieces of tissue were transplanted were often smaller than usual. A 0.05-0.10 mm diameter glass needle was passed through a piece of tissue excised from a specific region of a donor animal. The other end of the needle was passed through a small wound in the middle of the body column of a host animal perpendicular to the body axis, and out through the other side. Pieces of parafilm were threaded onto either end of the glass needle and brought snugly against the tissues to hold the transplant in contact with the body column at the site of the small wound. Transplants were allowed to heal for 2-3 hours before removing the glass needle. Subsequently, they were assayed for second axis formation. The size of the transplanted tissue was measured in terms of the number of epithelial cells using the maceration technique as described by David (David, 1973).

The second grafting procedure, a 'hypostome-contact graft', was carried out as described by Mutz (Mutz, 1930). The upper one-quarter to one-third of a donor animal was isolated, and the same type of glass needle as described above was threaded into the open basal end of the donor animal through the gastric cavity and out through the apex of the hypostome. Then, the same end of the needle was passed through a small wound in the middle of the body column of a host animal, and the hypostome of the donor was brought into contact with the edges of the wound. It was held in place with pieces of parafilm as described above, and allowed to heal. To ensure contact with the injured edge of the host, the tip of the hypostome of the donor was also injured. Depending on the experiment, the host was normal or decapitated. Periodically, thereafter, the inducing tissue was removed and a second axis allowed to develop on the host body column. To ensure that the host and donor tissue were separated at the graft junction, the endoderm of the host, or the donor, was labeled with India Ink (Campbell, 1973), or with 1 mg/ml fluorescent dextran (FL-DX) by injecting the dye into the gastric cavity a day before grafting. To insure a strongly labeled hypostome in some experiments, animals labeled with Fl-DX were decapitated 4-6 hours later, allowed to regenerate heads and used as donors. A secondary axis was defined by the presence of a hypostome with at least two tentacles. To identify emerging and developing tentacles on developing second axes in hypostome-contact grafts, immunofluorescence was carried out using TS-19, a tentacle-specific antibody as described previously (Bode et al., 1988).

Treatment with 0.5 mM LiCl was carried out as described previously (Smith et al., 1999).

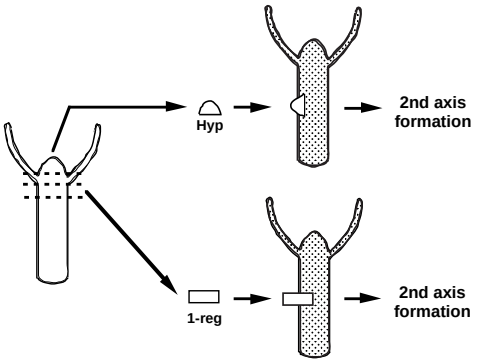
In situ hybridization

In situ hybridization analysis of wholemounts of animals was performed as described previously (Grens et al., 1996; Martinez et al., 1997). The antisense RNA probe for the *HyBra1* gene included the T-box domain (Technau and Bode, 1999). Samples were incubated at a probe concentration of 0.025 ng/ml for 36 hours.

RESULTS

The inductive capacity, or head organizer, is localized in the hypostome

There is some evidence that the second axis-forming capacities of the head and body column have somewhat different properties suggesting that they are based on different mechanisms (Yao, 1945; MacWilliams, 1983a). A critical difference for the formation of a second axis is that a small piece of hypostome tissue is sufficient, while a larger piece of body column tissue is necessary (Yao, 1945). One explanation is that the hypostome has an inductive capacity, while that of the body column is due to a self-organizing or self-differentiating capacity.



B.

Induction capacity of the three apical regions as measured by transplantation

Source of grafted tissue	Size of graft (Number of epithelial cells)	Number of grafts	Second axes formed N	Second axes formed %	Tentacles / 2nd axis source of tissue host	Tentacles / 2nd axis source of tissue donor
Hypostome	160 ± 23	19	19	100	4.2 ± 0.3	0
1-region	1930 ± 340	32	30	94	0	4.4 ± 0.3
1/4 1-region	480	24	3	12	1.0 ± 0	3.0 ± 0.2
1/4 tentacle zone	325	24	11	46	0.5 ± 0.2	1.4 ± 0.2

Fig. 1. Induction capacity of the three apical regions (hypostome, the tentacle zone and the 1-region) as measured by transplantation. (A) The procedure for the hypostome and 1-region, and (B) the results of the procedure. The number of epithelial cells per region is the average of three measurements. The value for the tentacle zone is 1300±190. Values for one-quarter regions are calculated from the measurements for the whole region. For the one-quarter tentacle zone, several axes derived from donor had a single apical tentacle instead of a complete head. All values are ±s.e.m.

To examine this possibility, either the whole hypostome or the 1-region of a donor animal, which is the apical eighth of the body column, was transplanted into the middle of the body column of an intact host whose endoderm was labeled with India Ink (Fig. 1A). Labeling of the host provided a means of determining whether the host or the donor tissue provided the tissue for the secondary axis. In both cases, secondary axes were formed in >90% of the transplantations (Fig. 1B). When a hypostome was transplanted, the body column, tentacles and part of the hypostome were labeled indicating they had been formed by tissue of the host (Fig. 2A). The remainder of the hypostome was derived from the transplant. In sharp contrast, when a 1-region was transplanted most of the tissue of the secondary axis was derived from the donor tissue (Fig. 2B). A comparison of the number of labeled tentacles in both types of grafts provides a quantitative measure of this difference (Fig. 1B). For hypostome grafts, all the tentacles of the second axis were labeled, indicating they were derived from the host. By contrast, none of the tentacles of 1-region graft was labeled, which shows they were all derived from the transplant

This difference in the source of tissue (host versus donor) for the formation of the second axis could be due to the size of the transplant, as the 1-region is much larger than the hypostome, as measured in terms of the numbers of epithelial

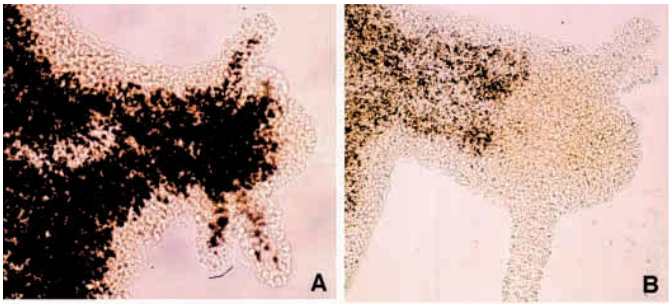


Fig. 2. Formation of second axes following transplantation. (A) Induction of a second axis by hypostomal tissue; (B) self-organization of a second axis by 1-region tissue. The host was labeled with India Ink.

cells (Fig. 1B). This possibility was examined by carrying out the same experiment using one-quarter of the 1-region, which is somewhat closer though still three times larger than the size of the hypostome. In this case, the fraction forming second axes was very low (Fig. 1B) indicating that there is a qualitative difference between the hypostome and the 1-region. The hypostome is capable of induction but the 1-region has little or no inductive capacity. In a similar experiment the tentacle zone, the region between the hypostome and the 1-region exhibited an intermediate capacity for axis formation (Fig. 1B). One quarter of the tentacle zone was used so that the size was comparable with that of the hypostome. As most (~75%) of the tentacles formed in both the one-quarter 1-region graft and the one-quarter tentacle zone graft were derived from the donor, axis formation resulted primarily from self-organization.

These results suggest that the head formation ability of the hypostome is based on an inductive capacity. By contrast, the head formation ability of the body column and tentacle zone is based on a self-organizing capacity.

The hypostome produces a signal that induces a second axis

Tissue that has an inductive capacity, or acts as an organizer, is assumed to be emitting a signal(s) to the surrounding tissue that affect its future development. Such signals have been identified in *Xenopus* (for reviews, see Harland and Gerhart, 1997; Smith and Schoenwolf, 1998). A transplantation experiment initially described by Mutz (Mutz, 1930), which we refer to as a hypostome-contact graft, provides a reasonably direct means for demonstrating that a signal transmitted from the donor hypostome to the host tissue induces the formation of a secondary axis. This axis consists of a hypostome, tentacles and a body column. As shown in Fig. 3A, the apical one-quarter to one-third of a donor animal is grafted through its hypostome to the middle of the body column of a decapitated host labeled with India ink. Thirty-six hours after graft formation, the host body column begins to evaginate at the donor hypostome contact site, and subsequently elongates into a cylindrical protrusion. By 72 hours, tentacles begin to emerge (Fig. 4A), which by 120 hours have elongated into normal tentacles (Fig. 4B). When the donor tissue is removed, a secondary axis develops (Fig. 4C), which is composed exclusively of host tissue.

Even a transient contact is sufficient to induce the formation of a secondary axis. Hypostome-contact grafts were prepared, and periodically thereafter the donor tissue was removed. As a

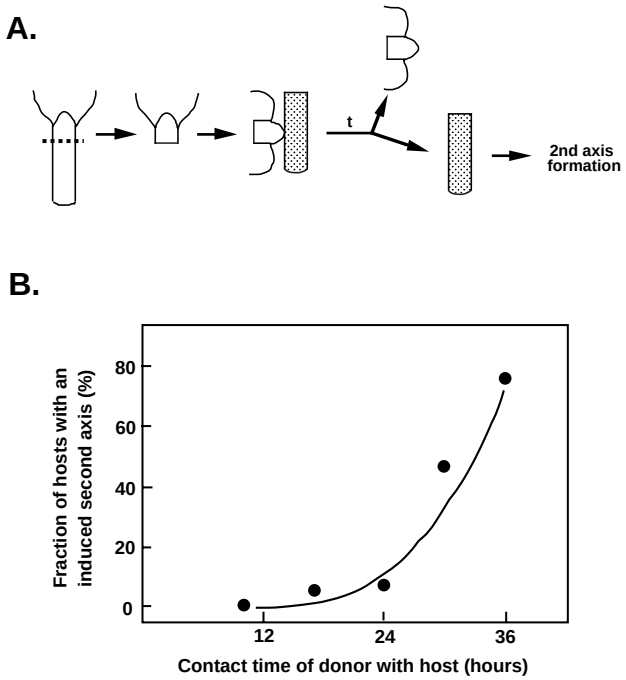


Fig. 3. Induction of a second axis using a hypostome-contact graft. (A) Graft procedure. (B) Increase in the fraction of second axes formed with increasing time of hypostome contact. The number of grafts/time point was 14-32.

single epithelial cell labeled with India Ink is clearly visible in the transparent tissue of hydra (Campbell, 1973), removal of all of the donor tissue was readily assessed. The ability of the tissue receiving the signal to form an axis increased with time, with about 75% forming a second axis after 36 hours of contact (Fig. 3B). As none of the donor tissues is involved in the formation of the secondary structures, as indicated by the absence of unlabeled tissue in the induced second axis, the secondary axis

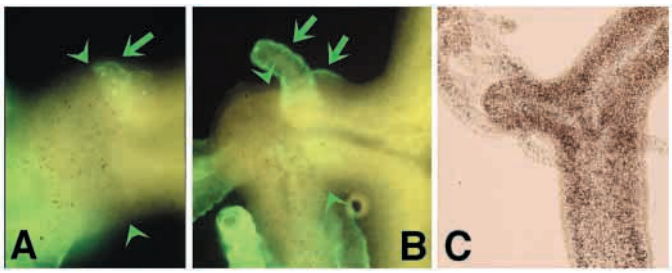
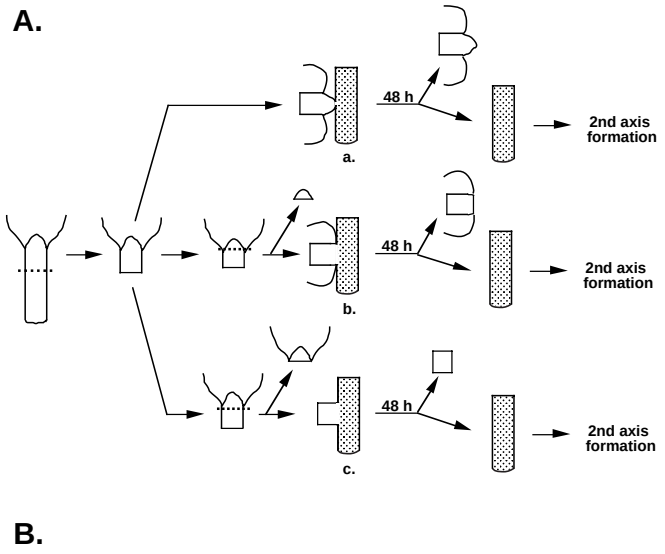


Fig. 4. Process of second axis formation in a hypostome-contact graft. (A) Seventy-two hour graft with an emerging second axis. (B) One hundred and twenty hour graft with tentacles forming at the apical end of the emerging second axis. In both A,B, the India Ink stained donor tissue (black dots) is on the left with the arrowheads indicating the border between donor and host. The arrows in A,B indicate emerging (A) or developing (B) tentacles. Samples in A,B were stained with the TS-19 antibody to identify the emerging and developing tentacles (Bode et al., 1988). (C) An induced second axis (head and body column facing left) after removal of the inducing donor tissue. The donor (on the left in A,B) was labeled with India Ink while the host was labeled in C. Arrowheads indicate the border between donor and host, and arrows indicate emerging or developing tentacles.



B.

Induction capacity of the three apical regions as measured with a contact graft

Apical end of donor	Number of grafts (N)	Second axes formed N	%
a. Hypostome	28	25	89
b. Tentacle zone	32	17	53
c. 1-region	33	2	6

Fig. 5. Induction capacity of three apical regions as measured with a contact graft. (A) Diagram and (B) results of the grafting procedure.

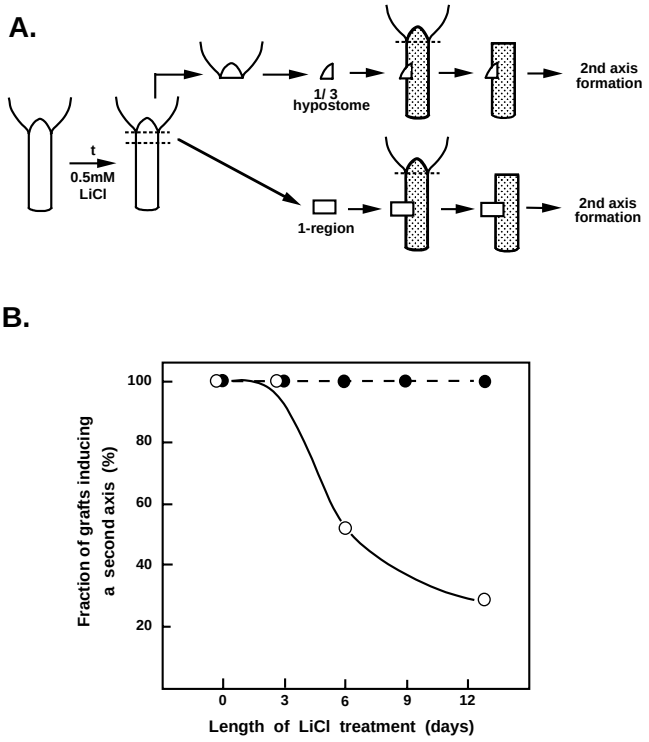


Fig. 6. Effect of LiCl treatment on the ability of the hypostome and 1-region to form a second axis upon transplantation. (A) Diagram and (B) results of the procedure. The number of grafts/time point was 15-32.

must have been initiated by the transmission of a signal(s) from the donor animal. Hence, this is a true induction.

To determine where in the apical part of the donor the source of the inducing signal was located, contact grafts were carried out using different regions as the apical end (Fig. 5A). Donors were grafted to the middle of the body column of host animals through their hypostome, or their tentacle zone, or their 1-region. Forty-eight hours later the donor tissue was removed and the host animals maintained for 3-4 days to see if they would develop a secondary axis. The ability to induce a secondary axis was maximal when the donor was grafted through the tip of a hypostome, lower in the tentacle zone, and minimal, when grafted through the 1-region (Fig. 5B). Hence, the inducing signal originates only in the head and primarily in the hypostome.

The inductive capacity of the hypostome is based on a different signaling pathway from the self-organizing capacity of the body column

The above experiments demonstrate that what has commonly been referred to as the head activation gradient (MacWilliams, 1983a) consists of two different capacities: an inductive capacity in the hypostome and the self-organizing capacity of the body column. Are they based on different signaling or metabolic pathways?

One way to answer this question is to examine the effects of reagents that alter the level of head activation. Prolonged treatment with 0.5 mM LiCl reduces the head activation gradient throughout the body column, expressed by a reduced capacity for head regeneration of body column tissue (Hassel and Berking, 1990). Based on these results, animals were treated with 0.5 mM LiCl and the capacity for second axis formation of either one-third of the hypostome or the entire 1-region was periodically measured by transplantation into a labeled untreated host that had been decapitated (Fig. 6A). The smaller piece of hypostome, instead of a complete hypostome, was used as it might provide a more sensitive measure of the effect of LiCl on the inductive capacity. The LiCl treatment affected the two regions differently (Fig. 6B). Even after 12 days of treatment, the capacity of a hypostome to induce a second axis remained unchanged and maximal. That most of the tentacles formed in the second axes were derived from the host indicated that these second axes were induced. By sharp contrast, the ability of the 1-region to form a second axis decreased substantially with increasing length of the LiCl treatment (Fig. 6B). Thus, the difference in effect of LiCl on the ability of the hypostome and 1-region to form a second axis provides evidence that the induction and self-organization properties are based on different pathways.

As treatment with 0.5 mM LiCl had no effect on the inductive capacity of the hypostome in transplantation experiments, one would expect the treatment not to affect the signaling capacity of the hypostome. To test this idea hypostome-contact grafts were carried out in which the donor had been treated with 0.5 mM LiCl for 12 days (Fig. 7A). After 72 hours of contact, the donor tissue was removed, and the hosts assayed. As expected, and as shown in Fig. 7B, the LiCl-treated donors were just as effective as control donors. In fact the LiCl-treated donors induced second axis formation more rapidly (Table 1), suggesting that LiCl treatment may increase the strength of the inducing signal.

Table 1. LiCl treatment of donor increases the rate of tentacle formation during the development of a second axis

Donor	Number of grafts	Number of tentacles formed on second axis	
		48 hours	72 hours
LiCl treated	34	1.3±0.2	3.5±0.2
Control	30	0.4±0.1	1.7±0.2

Head inhibition from the host represses the effect of the inducing signal transmitted from the donor hypostome

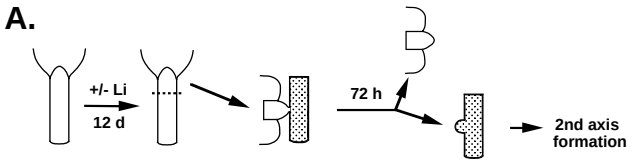
As part of the patterning processes controlling head formation in an adult hydra, the head produces an inhibitor that is transmitted to the body column and prevents head formation from occurring there (Wilby and Webster, 1970b; MacWilliams, 1983b). This inhibitor has a short half-life (2-3 hours) (MacWilliams, 1983b). Hence, when a head is removed, the level of inhibitor drops rapidly, thereby permitting the body column to initiate head regeneration. Using the hypostome-contact graft, the effect of host inhibition on the induction process was examined.

Hypostome-contact grafts were made using intact hosts. Thirty-three hours later, the inducing donor tissue was removed, and periodically thereafter the host head was removed from samples (Fig. 8A). Thirty-three hours was chosen because, as shown in Fig. 3B, 50-60% of the contacted hosts form second axes. Leaving the host head on continuously resulted in the formation of secondary axes in 31% of the cases, whereas if it was removed at the same time as the donor tissue, 80% formed second axes (Fig. 8B). Thus, the host head clearly had an inhibitory effect on some part of the process leading to the formation of a second axis. Furthermore, the decline in the fraction forming a second axis upon removal of the host head indicates that either the signal itself, or the early events in the inductive process, decay with a half-life of about 14 hours.

Head inhibition from the donor interferes with the formation of the hypostome in a developing second axis

To determine what part of the inductive process is affected by head inhibition, hypostome-contact grafts were again used. When the donor was removed after visible initiation of a second axis in a hypostome-contact graft, this axis (upon further development) invariably consisted of a hypostome, tentacle zone with tentacles and a body column. However, if the donor was not removed, it was unclear whether a hypostome had formed (Fig. 4B). Plausibly, the head inhibition transmitted from the donor blocked hypostome formation in the induced second axis.

This possibility was examined by making use of *HyBra1*, a hydra *Brachyury* homolog (Technau and Bode, 1999). The gene is expressed very early in the presumptive head during bud formation and head regeneration, even before the tissue has been committed to head formation. It continues to be expressed in the developing hypostome and subsequently in the adult hypostome. Hypostome-contact grafts were made using decapitated hosts, the donor tissue removed at 30 hours or 36 hours, and periodically thereafter samples were stained for *HyBra1* expression (Fig. 9A). These times were chosen as a hypostome contact-graft of 30 hours resulted in an induced axis



B.

Ability of hypostome - contacted host tissue to form a second axis

Donor	Host	Grafts (#)	Second axes formed (N)	(%)
control	control	37	33	89
Li-treated	control	58	50	86

Fig. 7. Effect of LiCl treatment on the induction of a second axis in a hypostome-contact graft. (A) Diagram and (B) results of the two grafting procedures.

forming in at least 50% of the hosts (Fig. 3). When host animals of the 30 hours hypostome-contact grafts were examined immediately after removal of the donor, no *HyBra1* expression was observed (Fig. 9B). However, 6 hours later, 50% of the samples expressed the gene (Fig. 9B) at a low level (Fig. 10A). With time, the level of expression rose (Fig. 10B-D) as is typical for this gene during hypostome formation (Technau and Bode, 1999). As expected for a 30 hour graft, the fraction forming a second axis as well as expressing *HyBra1* remained constant around 50% (Fig. 9B, 12 hour time point).

The delay in expression of *HyBra1* in the 30 hour hypostome contact-grafts can be interpreted in two ways. First, *HyBra1*

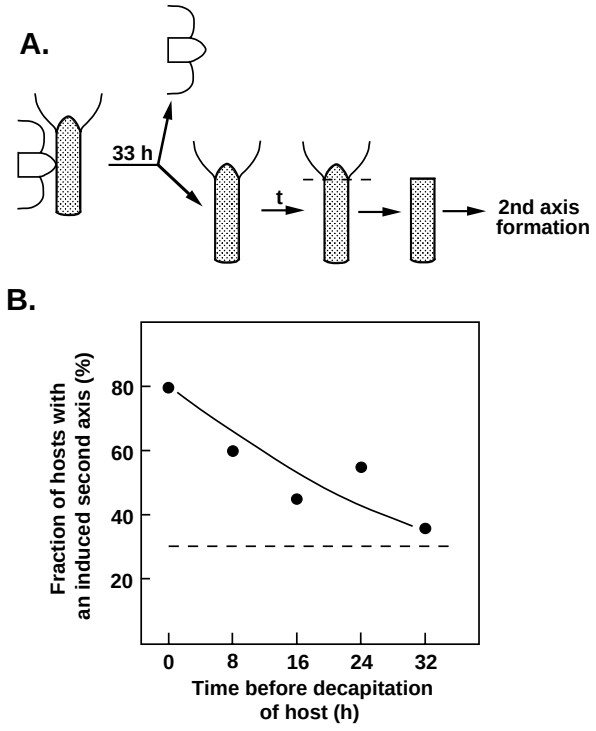
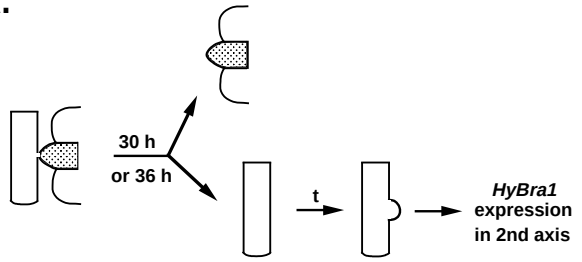


Fig. 8. Effect of head inhibition on the activity of the inducing signal. (A) Diagram and (B) results of the grafting procedure. The number of grafts/time point was 11-32.

A.



B. ***HyBra1*** **expression in induced 2nd axis**

Time of contact in hypostome-contact graft (h)	Time after graft removal (h)	Number of grafts	Grafts expressing *HyBra1* (N)	(%)
30	0	80	0	0
	6	70	33	48
	12	70	39	55
36	0	36	0	0
	6	35	12	34

Fig. 9. Effect of the presence of the donor hypostome on HyBra1 expression in the induced second axis. (A) Diagram and (B) results of the grafting procedure.

expression does not occur until around 36 hours after the initial contact of the donor hypostome with the host tissue. Second, head inhibition prevents *HyBra1* expression. In this case, the delay in expression after removal of the donor tissue reflects the rapid decay of the head inhibition ($t_{1/2}$=2-3 hours) (MacWilliams, 1983b) transmitted from the donor hypostome. The 36 hour hypostome contact-grafts clearly support the second interpretation. Again, there was no expression immediately after removal of the donor at 36 hours (Fig. 9B). Six hours later (42 hours after the graft was formed) the gene was expressed. The 6 hour delay in *HyBra1* expression in both the 30 hour and 36 hour grafts is consistent with the view that donor inhibition represses *HyBra1* expression. In turn, these results indicate that the donor hypostome specifically inhibits hypostome formation in the induced second axis, but does not affect formation of the tentacle zone – the lower part of the head.

Head inhibition interferes with the formation of the inductive capacity in a developing secondary axis

Because hypostome formation, when measured in terms of *HyBra1* expression, was blocked in the developing second axis, it was plausible that the development of the inductive capacity, which is located in the hypostome, was also blocked.

To measure the effect of head inhibition on organizer development more directly, a modification of the previous experiment was carried out (Fig. 11A). After removal of the donor animal, host tissue was isolated at different times, transplanted to a decapitated host and assayed for second axis formation. For 30 hour samples, tissue was isolated from the site where the donor was removed. For 42 hour samples, half of a developing protrusion was used. For 144 hour samples, tissue at the apical end of the secondary axis, including a developing tentacle, was used. The pieces were small to ensure that it was their inductive capacity that was being tested, not the ability to self organize. Here too there was a delay for the 30 hour time point. When the donor-contacted tissue was transplanted immediately after removal of the inducing tissue, it did not have the inductive capacity necessary to form a second axis (Fig. 11B). If the donor-contacted tissue was transplanted 12 hours after removal of the donor tissue, complete secondary axes were formed in 46% of the cases, which is again similar to the number (50%) that would have formed if the protrusion had been allowed to develop on the host (see Fig. 3).

That this delay was not simply a reflection of the time of development of the inductive capacity of the developing hypostome is shown by the latter samples. Were it simply such a delay, then one would expect a transplant of a 42 hours protrusion to form secondary axes in about half the samples. Instead, they formed in only a quarter of the samples, whereas when the protruding tissue was transplanted 24 hours after removing the donor, all induced second axes (Fig. 10B). Even in the case where the second axis developed for almost 6 days, the apical tip was not very effective in inducing a second axis immediately after removal of the donor tissue.

The reduced ability of the developing tissue to, in turn, induce another secondary axis, suggests that it was hindered by the presence of head inhibition from the original donor. Once head inhibition had decayed upon removal of the donor, then presumably the hypostome/organizer could complete development to the point where it could induce the host tissue. The cases where second axes did not form could reflect the limited half-life of the inducing signal (Fig. 8). Or, more likely, they could reflect the instability of the early head development processes sometimes referred to as unstable head activation (MacWilliams, 1983a).

DISCUSSION

A gradient of head formation capacity known as the head activation gradient controls head formation in hydra. A common view based on transplantation studies is that this gradient reflects the distribution along the body axis of a single property that is maximal in the head. However, some earlier results (Yao, 1945; MacWilliams, 1983a), as well as the results described here, indicate that this gradient is made up of two different components. One is an organizer region confined to the head, in particular the hypostome, while the

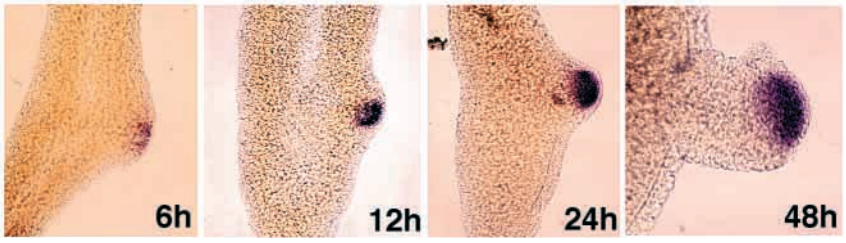
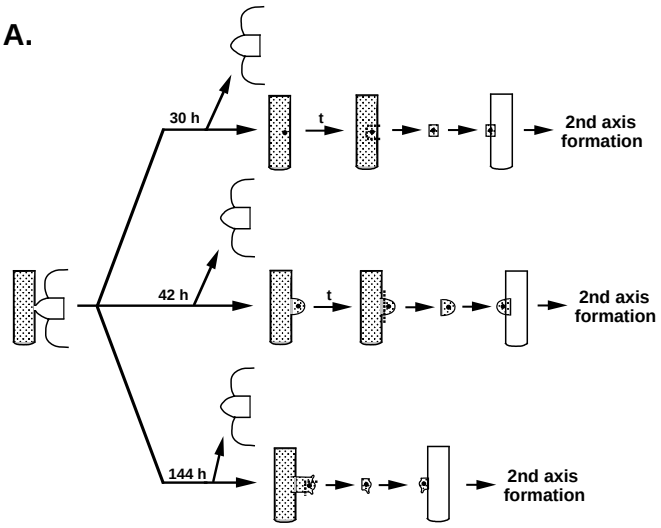


Fig. 10. Development of HyBra1 expression with time in host tissue after end of contact with a donor hypostome.



B.

Ability of contacted tissue to induce a second axis

Time of contact in hypostome-contact graft (h)	Time after graft removal (h)	Number of grafts	Grafts forming a 2nd axis (N)	(%)
30	0	19	0	0
	6	14	0	0
	12	13	6	46
	24	15	7	46
42	0	12	3	25
	24	10	10	100
144	0	16	5	30

Fig. 11. Effect of the presence of the donor hypostome on the ability of the apical end of the induced second axis to form another axis upon transplantation. (A) Diagram and (B) results of the grafting procedure.

second is a gradient of head formation capacity in the body column.

The hypostome acts as an organizer

As shown in several vertebrate species, the organizer region has the property of recruiting surrounding embryonic tissue to participate in the formation of one or both embryonic axes (Smith and Schoenwolf, 1998). The hypostome of hydra has similar characteristics. Transplantation of a hypostome (Browne, 1909; Yao, 1945) invariably leads to the formation of a second axis. To demonstrate that the formation of the second axis is due to induction of the host tissue, we have shown that an unlabeled transplanted hypostome induces tissue of a labeled host to form a second axis consisting of a labeled lower part of the head, or tentacle zone, and a labeled body column. This inductive capacity is confined to the head as a piece of the 1-region similar in size to the hypostome has a very low capacity to induce a second axis. Because the 1-region has the highest head activation capacity in the body column, it is very likely that the rest of the body column also lacks this inductive capacity. As a transplant of a piece of the tentacle zone has an intermediate capacity to induce a second axis, the inductive capacity is highest in the hypostome decreasing rapidly through the tentacle zone to a low level in the upper end of the body column.

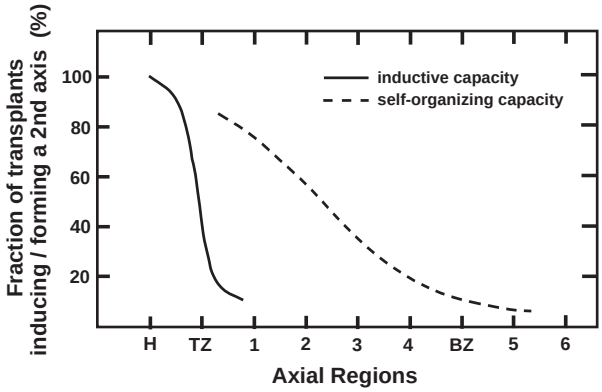


Fig. 12. Axial distribution of inductive capacity and the self-organizing capacity.

The hypostome-contact graft, which also results in the formation of a second axis, provided a more direct measure of the crucial characteristic of an organizer: the production and transmission of a signal(s) that carries out the inductive process. As all of the donor tissue is removed at the end of the procedure, the formation of a second axis derived from host tissue must be due to the transmission of an inductive signal from the donor. Contact grafts using different regions of the apical end of the adult clearly indicated that the inductive capacity was restricted to the head. It is highest in the hypostome, tapers off rapidly in the tentacle zone and is negligible in the upper end of the body column. (Fig. 12).

One final point concerns the capacity of the organizer. The second axes induced by the hypostome invariably consist of a complete head and most of the body column. However, these second axes never include a foot, implying that the organizer region in the hypostome is more precisely a head or anterior organizer. Transplantation experiments have shown that the basal one-eighth of the body column has an organizing capacity for foot formation (Hicklin and Wolpert, 1973).

The body column has a self-organizing capacity

If the hypostome has the characteristics of an organizer, what are the characteristics of head activation in the body column? They are clearly different from the inductive or organizing characteristics of the hypostome in several respects. The first is the size of the piece of the body column necessary for the formation of a second axis. A whole or a part hypostome will induce a second axis upon transplantation, but a similar-sized piece of the upper end of the body column almost never or rarely does (Yao, 1945) (Fig. 1). A piece approximating one-eighth of the body column, which is more than 10 times larger than a hypostome, is necessary for second axis formation. In fact, the differences are even greater, as a clump of ~10 epithelial cells derived from the hypostome (one-fifteenth of the hypostome) will induce a second axis (Technau et al., 2000).

The second difference concerns the source of tissue that forms the second axis. By labeling the host, it is clearly demonstrated that transplants of unlabeled body column tissue do not induce the host tissue to form the second axis. Instead, the transplant itself forms most of the second axis, with host tissue providing only part of the lower body column. This

suggests that it is the well-known regenerative or self-organizing property of the body column tissue that leads to the second axis (Bode and Bode, 1984). For example, an isolated one-eighth of the body column such as the 1-region will regenerate a head and a foot to form a complete hydra.

Another piece of evidence that separates the hypostome and body column components of the head activation gradient is based on the effects of LiCl. This treatment severely reduced the capacity of the tissue of the body column to form second axes upon transplantation; that is, the ability for self-organization had been reduced. However, LiCl treatment had no effect on the inductive capacity of the hypostome (Fig. 6). In fact, the treatment may have enhanced the inductive capacity when measured by the rate of tentacle formation in the developing second axes (Table 1).

Finally, MacWilliams (MacWilliams, 1983a) has shown that the stability of head activation in the two regions differs. He showed that head activation in a regenerating head, that is a developing organizer, has a half-life of 12 hours, while that of the body column was longer at 36 hours.

All of the evidence strongly suggests that the self-organizing property of the body column tissue has different properties than does the organizer activity of the hypostome. Thus, instead of a single head activation gradient that is maximal in the head, the axial distributions of the two properties are more accurately distributed as shown in Fig. 12. We suggest that only the property of self-organization in the body column be referred to as the 'head activation gradient', or as a head competence gradient (Technau et al., 2000).

The head organizer produces a signal which inhibits hypostome/organizer formation

That the adult head continuously produces a signal, head inhibition, that prevents head or second axis formation in the body column is well known (MacWilliams, 1983b). By studying the effect of head inhibition on the developing head using hypostome-contact grafts, more information has been obtained about organizer formation and the target of head inhibition.

Leaving the host head on for increasing lengths of time after the donor had been removed in hypostome-contact grafts reduced the number of secondary axes induced (Fig. 8). Hence, head inhibition produced by the host head interfered either with the inducing signal transmitted from the donor hypostome, or with the subsequent development processes initiated by the inducing signal. The ability of induced tissue to form a second axis declined with a half-life of about 14 hours (Fig. 8), which is comparable with the 12 hour half-life of the unstable head activation during head regeneration (MacWilliams, 1983a). As the rise in unstable head activation corresponds to the development of the organizer, it is likely that the head inhibitor blocks head formation/organizer development some stage after initiation by the inducing signal.

The hypostome-contact grafts have also provided a more precise indication of the role of head inhibition. As long as the donor hypostome is in contact with the host, a second axis consisting of a body column and a tentacle zone with tentacles will form (Fig. 4A,B), but no hypostome. Once the donor is removed, a hypostome will form at the distal end of the developing second axis (Fig. 4C), suggesting that hypostome formation might be the target of the inhibitor emanating from the donor. Two pieces of evidence support this view. (1) HyBra1, an early marker of hypostome formation (Technau and Bode, 1999), appears in the developing second axis 6 hours after the donor had been removed (Fig. 10). (2) The developing organizer in the developing hypostome was also tested in transplantation experiments to determine whether it could in turn induce another secondary axis. Regardless of the age of the developing head, a delay was still observed in its induction capacity in the first several hours after removal of the donor head (Fig. 11). The timing of the appearance of both the HyBra1 marker and the organizer activity in the developing hypostome after removal of the donor is consistent with the half-life, or decay rate of the head inhibitor transmitted from the donor hypostome.

Thus, the head inhibition is more accurately termed hypostome inhibition, as it prevents the full development of the hypostome/head organizer region. This view is reinforced by the finding that the hypostome inhibitor has no effect on the formation of the lower half of the head, the tentacle zone, in an induced second axis

Relationship of the head patterning components to the Gierer-Meinhardt model

The reaction-diffusion model originally proposed for hydra by Gierer and Meinhardt (Gierer and Meinhardt, 1972), and a subsequent modification of the model (Meinhardt, 1993) have provided a useful framework for explaining axial patterning in hydra. The central feature of the model involves an activator and an inhibitor. In a tissue that starts with a uniform distribution of activator and inhibitor, a random, or non-random, event will occur so that the activator concentration will rise autocatalytically in one location. When the activator concentration reaches a threshold value, that tissue becomes committed to the formation of a specific structure, such as the head of a hydra. The inhibitor, whose production depends on the activator, diffuses away from the activator peak, thereby preventing a second activator peak, i.e. a hydra head, from forming in the surrounding tissue. This mechanism shares features with the organizer in the hypostome.

The head organizer has characteristics of the activator

In vertebrates and other chordates, the organizer region is a transient structure that appears during early embryogenesis, where it is involved in setting up the overall organization of the embryo. Whether a similar situation exists in hydra embryos is not known. By contrast, in an adult hydra, the organizer region is a permanent structure that is continuously active. This is a consequence of the tissue dynamics of the animal. The epithelial cells of the body column are constantly in the mitotic cycle (Campbell, 1967a; David and Campbell, 1972), and the generated tissue is continuously displaced towards the extremities of the column, where it is eventually sloughed (Campbell, 1967b; Otto and Campbell, 1977). As the size and shape of the animal remain constant, the pattern forming processes must be continuously active to maintain the form of the animal, as well as the axial distribution of differentiated cells.

Tissue displaced up the body column flows through the tentacle zone, and out onto the tentacles, but not into the hypostome (Campbell, 1967b; Bode et al., 1988). A similar, but separate process occurs in the hypostome/organizer region. The epithelial cells of the lower part of the hypostome are continuously in the mitotic cycle (Dubel, 1989), and tissue of

the hypostome is constantly displaced towards its apical tip, where it is sloughed (Campbell, 1967b). Thus, the organizer must be constantly undergoing renewal as cells of the organizer move apically, are lost and are replaced with cells generated in the base of the hypostome. As a consequence, the organizer would be in a steady state. One way to maintain this steady state would be for the organizer in the apical part of the hypostome to produce a short-range signal that recruits neighboring cells in the basal part of the hypostome to become part of the organizer as they are displaced apically into the tip. In essence, this would be a positive-feedback loop. This idea is similar to that of the autocatalytic activator of the Gierer-Meinhardt model (Gierer and Meinhardt, 1972; Meinhardt, 1993)

Although the molecular basis of the organizer is unknown, an intriguing possibility is that the Wnt pathway is involved. *HyWnt*, a hydra *Wnt* homolog, is expressed at the very apical end of the hypostome, and only there, in an adult hydra (Hobmayer et al., 2000). It has also been shown that during bud formation, as well as in developing aggregates of hydra cells, the early expression of the gene is invariably associated with tissue that will form the hypostome. Furthermore, where examined, other members of the pathway are associated with head formation. Both *Hyb-cat* the hydra homolog of β-catenin, and *HyTcf,* the hydra *Tcf* homolog are strongly expressed in the developing head of a bud, while the latter is also expressed in the head of an adult (Hobmayer et al., 2000). Recent evidence indicates the Wnt pathway can act directly as a positive feedback loop as the *armadillo/tcf* complex binds to the *Wnt* promoter to stimulate *Wnt* production during wing disc development in *Drosophila* (Heslip et al., 1997). Finally, in our experiments, prolonged treatment with 0.5 mM LiCl increases the inductive activity of the hypostome, as expressed by a more rapid appearance of tentacles in the induced second axis (Table 1). LiCl is known to activate the Wnt pathway by inhibiting the activity of GSK-3 (Hedgepeth et al., 1997). Thus, it is plausible that, in hydra, the Wnt pathway is active in the formation and maintenance of the organizer in the hypostome.

The hypostome/organizer inhibitor has characteristics of the inhibitor

The characteristics of head inhibition described previously (MacWilliams, 1983b) have indicated a close correlation with the inhibition of the reaction-diffusion mechanism. Furthermore, Technau et al. (Technau et al., 2000) have recently demonstrated that the range of the activator is much shorter than that of the inhibitor, which is another crucial characteristic of the two components of the mechanism. Finally, one would expect the two components of the mechanism to be operating in the same location. Based on results described this is the case. The head inhibitor is more precisely an inhibitor of both hypostome and organizer formation. As the organizer is in the hypostome, the requirement for the same location is satisfied. Finally, the fact that the inhibitor blocks hypostome formation, but not formation of the lower part of the head, the tentacle zone plus tentacles, is consistent with Meinhardt's modification of the model (Meinhardt, 1993), which postulates separate mechanisms for hypostome and tentacle zone formation.

The organizer produces a signal that sets up the head activation gradient

One remaining issue is the relationship between the organizer in the hypostome, and the self-organizing capacity of the body column. The self-organizing capacity is not uniform along the body column. Instead, the head formation capacity, or head activation, is graded down the body column. This gradient plays a role in the context of the tissue dynamics of the adult. As tissue is displaced in an apical direction in the upper half of the body column, the level of head activation rises, and eventually passes a threshold value that leads to the conversion of body column tissue to that of the lower part of head, the tentacle zone. In addition, in the lower half, where the tissue is displaced in a basal direction, the level of hypostome inhibition drops below a threshold level permitting the initiation of organizer formation, and hence formation of a new bud.

Grafting experiments have demonstrated that the head produces a signal and transmits it to the body column, which sets up the head activation gradient (Wilby and Webster, 1970a; Herlands and Bode, 1974; MacWilliams, 1983a). The contact grafts indicate that this signal is produced in the hypostome (Fig. 5). When a hypostome-contact graft is left intact for more than 72 hours, a second axis is invariably induced that includes a body column with tentacles emerging from the distal end, but no hypostome (Fig. 4). The formation of a body column and a tentacle zone with emerging tentacles indicates the presence of the head activation gradient. Thus, the head activation gradient part of this self-organizing capacity of the body column is controlled by the head, or more specifically the head organizer.

In the Gierer-Meinhardt model, the signal that sets up the head activation gradient is assumed to be the diffusing activator. An equally plausible view would be that a short-range signal (possibly *HyWnt)* acts as the activator that sets up and maintains the organizer, while the organizer produces a second long-range signal that sets up the head activation gradient.

Summary

In the context of the tissue dynamics of hydra, the organizing capacity of the hypostome can be explained quite well in terms of the model described by Gierer and Meinhardt (Gierer and Meinhardt, 1972). The positive feedback loop of the organizer and the characteristics of the hypostome inhibitor fit quite well with the activator and inhibitor of the reaction-diffusion mechanism. While the signaling properties of an organizer are consistent with the signaling required to set up the head activation gradient.

Evolutionary considerations

The head organizer in the hypostome of hydra has characteristics similar to those described for organizers in chordates, ranging from amphioxus to mammals (Harland and Gerhart, 1997). The most important is the ability to induce a second axis. As with other organizers, the hydra hypostome self-organizes to form the hypostome of the second axis, and induces surrounding tissue to form the rest, tentacle zone and body column. Furthermore, in chick embryos, it has been demonstrated that the organizer region can regenerate (Yuan and Schoenwolf, 1998). In hydra, bisection of the body column followed by regeneration of a head with a normal hypostome indicates that the organizer can also regenerate in hydra. In addition, where examined in vertebrates, a set of genes has been found that is expressed in the organizer region. Among these genes are *goosecoid* (Cho et al., 1991;

Blum et al., 1992), *Brachyury* (Herrmann, 1991; Smith et al., 1991; Schulte-Merker et al., 1992) and *HNF3* (Ang and Rossant, 1994). Homologs of each of these genes are expressed in the hypostome of hydra (Broun et al., 1999; Technau and Bode, 1999; Martinez et al., 1997). In addition, as mentioned above, genes of the Wnt pathway are expressed in the hypostome as well as during the initiation of bud formation when a new organizer forms (Hobmayer et al., 2000), which is consistent with genes of the latter part of this pathway being involved in the formation of a vertebrate organizer (Harland and Gerhart, 1997).

Thus, the similarities between the head organizer in the hypostome and organizer regions in chordates suggest that this approach of using a small confined area of the embryo to set up the overall pattern of an early embryo arose early in metazoan evolution.

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