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HyAlx, an *aristaless*-related gene, is involved in tentacle formation in hydra

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SUMMARY

Developmental gradients are known to play important roles in axial patterning in hydra. Current efforts are directed toward elucidating the molecular basis of these gradients. We report the isolation and characterization of *HyAlx*, an *aristaless*-related gene in hydra. The expression patterns of the gene in adult hydra, as well as during bud formation, head regeneration and the formation of ectopic head structures along the body column, indicate the gene plays a role in the specification of tissue for tentacle formation. The use of RNAi provides more direct evidence for this conclusion. The different patterns of *HyAlx* expression during head regeneration and bud formation also provide support for a recent version of a reaction-diffusion model for axial patterning in hydra.

Key words: Hydra, *aristaless*, Reaction-diffusion model, Tentacle formation

INTRODUCTION

Developmental gradients play a central role in some processes of pattern formation. A prominent example is the graded distribution of the bicoid protein, which functions as a morphogen in establishing the anteroposterior axis of the *Drosophila* embryo (for a review, see St Johnston and Nüsslein-Volhard, 1992). Hydra, a Cnidarian, is another example where developmental gradients play a pivotal role in axial patterning.

The body plan of a hydra is very simple, consisting of a single apicobasal axis with radial symmetry. The structures along the axis are the head, body column and foot. Because of the tissue dynamics in an adult hydra, the axial patterning processes must be continuously active to maintain the pattern and morphology of the animal. An extensive body of transplantation and regeneration experiments (for a review, see Bode and Bode, 1984) has indicated that a pair of developmental gradients governs head formation. One, termed the head activation gradient, stimulates head formation, while the other, the heat inhibition gradient, prevents head formation. The behavior of these gradients can be explained in terms of a reaction-diffusion model (e.g. Gierer and Meinhardt, 1972; Meinhardt, 1982).

More recently, with the identification and characterization of molecular markers for the head, it has become clear that the patterning of the head cannot be considered in terms of the patterning of a single entity (Bode et al., 1988; Technau and Holstein, 1995; Mitgutsch et al., 1999). Instead, head patterning needs to be considered in terms of its two parts: the hypostome and the tentacle zone. The hypostome, a dome-shaped region where the mouth is located, is the upper half, while the lower half, the tentacle zone, is the region from which the tentacles emerge.

We have identified and characterized an *aristaless*-related gene in hydra, termed *HyAlx*, which is expressed exclusively in the head or a developing head. The expression patterns of *HyAlx* were examined during the formation of a head in several developmental contexts. The results along with an RNAi analysis indicate that *HyAlx* is directly involved in the specification of tissue for tentacle formation. To take into account the separate patterning of the two parts of the head, Meinhardt (1993) constructed a more refined version of the reaction-diffusion model for axial patterning in hydra. The data obtained here with *HyAlx* are remarkably consistent with the process and dynamics of tentacle formation as predicated by the model.

MATERIALS AND METHODS

Hydra culture

The Zurich L2 strain of *Hydra vulgaris* (Grens et al., 1996) was used for most experiments, while the Basel strain of *H. vulgaris* and the 105 strain of *H. magnipapillata* (Grens et al., 1996) were used where indicated. All three strains were cultured as described previously (Smith et al., 1999).

Isolation and characterization of a hydra *aristaless*-related gene

A fragment of an *aristaless*-related gene was isolated from hydra using the touchdown PCR method (Don et al., 1991). The degenerate oligonucleotide primers used corresponded to the first and third helices of paired-like homeoboxes were: forward, AA(A/G)(A/C)GI(A/C)(A/T)(A/C/T)(A/C)GIAC(A/T)GCNTT; reverse #1,

TT(C/T)TG(A/G)AACCANA(C/T)(C/T)TTNAC; and reverse #2 (for nested PCR), GTNC(G/T)(A/G)TT(C/T)TG(A/G)AACCA. The template used was first strand cDNA transcribed from total RNA isolated from adult hydra heads. The PCR reaction was carried out using Taq polymerase (Fisher) and standard reaction conditions. The reaction cycle was 94°C, 1 minute; annealing temperature, 2 minutes; 72°C, 1 minute. Successive annealing temperatures were every degree from 58°C to 46°C, with 3 cycles performed at each temperature. Resulting PCR products were TA-cloned using the pGEM-T cloning vector (Promega) and sequenced using the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (Amersham).

To obtain full-length clones, a hydra lambda ZAPII cDNA library (Sarras et al., 1994) was screened under moderate stringency, based on standard protocols (Sambrook et al., 1989). The probe used was a ^{32}P-labeled 150 bp fragment of *HyAlx*, the hydra *aristaless*-related gene obtained with PCR. Filters were washed with 0.5× SSC, 0.1% SDS at 45°C, and inserts were sequenced as above.

Southern analysis was carried out using standard procedures (Sambrook et al., 1989). A genomic Southern blot was probed with a ^{32}P-labeled full-length cDNA of *HyAlx* and washed at high stringency in 0.2× SSC, 0.1% SDS at 60°C.

RNAi

HyAlx dsRNA or firefly *luciferase* dsRNA was introduced into stage 2-3 developing buds with a novel localized electroporation procedure [LEP] as follows. *HyAlx* dsRNA or *luciferase* dsRNA was synthesized as described by Fire et al. (1998). Adults of the Zurich L2 strain of *H. vulgaris* were chilled to 4°C for 30 minutes, and then exposed to 3 mM heptanol in 4°C hydra medium for 10 minutes to immobilize them. An immobilized hydra was placed on a piece of plastic mesh mounted at an angle to the floor of the petri dish containing the heptanol solution. The angle chosen placed the animal axis perpendicular to the micropipette bearing the dsRNA to be delivered. Using a micromanipulator, the micropipette was placed firmly against the adult body column thereby enclosing the entire developing bud and a small ring of body column tissue surrounding the bud. The dsRNA at a concentration of 1 μg/μl in DEPC water was introduced into the enclosed tissue as follows. One electrode was threaded through the micropipette into the dsRNA solution at the micropipette tip, while the second electrode was inserted into the hydra medium surrounding the animal. Using conditions of 100 V and 250 ufd delivered by a BioRad Gene Pulser with a Capacitance Extender, dsRNA was then introduced into the bud tissue. After electroporation, the animal was immediately transferred to 4°C hydra medium for 30 minutes, and thereafter, incubated at as usual at 18°C. Neither bud nor the adult suffered any visible damage.

To determine the effect of *HyAlx* dsRNA, the level of *HyAlx* RNA was measured using RT-PCR as described previously (Technau and Bode, 1999). After introduction of *HyAlx* dsRNA into stage 2-3 buds, periodically samples of ten animals were collected, total RNA isolated from each sample, and RT-PCR carried out using primers for *HyAlx* RNA (forward, 5′ TTCGGAAATTCCCCTAGTAA3′; reverse, 5′ATGATCAGTATGGTCTGAAA3′) and the hydra EF-1α gene (Technau and Bode, 1999) for 30 cycles.

Phylogenetic analysis

The evolutionary relationships among *aristaless*-related genes and other paired-like genes were examined using phylogenetic analysis. Phylogenetic trees were constructed by parsimony analysis using PAUP 3.1.1 (Swofford, 1993) using the 60 amino acids of the homeodomain with *Drosophila NK-2* (*vnd* – FlyBase) as the outgroup (Jimenez et al., 1995). A heuristic search was performed, with tree bisection and reconstruction branch swapping (100 replicates, random addition).

In situ hybridization

HyAlx sense and anti-sense digoxigenin-labeled RNA probes were made using an RNA in vitro transcription labeling kit (Boehringer Mannheim). Two probes were generated: one with and the other without the homeobox. In situ hybridization was carried out on hydra whole mounts as described previously (Smith et al., 1999), using a final probe concentration of 0.1 ng/μl for hybridization. Both probes gave the same results.

Tissue manipulations

For head and foot regeneration experiments, adult polyps of the Zurich L2 strain of *H. vulgaris* were bisected directly beneath the tentacles, halfway down the body column, or just above the foot. For bud regeneration experiments, animals with stage 4 buds were selected. The apical half of the bud was removed, and the basal half (attached to the parent) was allowed to regenerate. All regenerates were incubated in HM at 18°C and samples were periodically taken for in situ hybridization.

Treatment with diacylglycerol (DAG) was carried out on adults of strain 105 of *H. magnipapillata* as previously described (Smith et al., 1999).

RESULTS

Isolation of *HyAlx*, a hydra *aristaless*-related gene

Using degenerate oligonucleotide primers, a fragment of an *aristaless*-related homeobox was isolated from hydra head first strand cDNA via a touchdown PCR method (Don et al., 1991). This fragment was then used to screen a hydra cDNA library (Sarras et al., 1994). One of the clones isolated, named *HyAlx*, contained a complete open reading frame of 810 bp encoding a predicted protein of 270 amino acids. Southern analysis indicated the existence of only one of these genes in hydra (data not shown).

The HyAlx protein has two salient features (Fig. 1). One, the homeodomain belongs to the paired-like class of homeodomains as it has a glutamine at position 50 of the recognition helix. Among the genes of this class, the HyAlx homeodomain is closely related to the aristaless family of homeodomains, which can be loosely grouped into three subfamilies (Fig. 1A). HyAlx appears to be most similar to the first group of proteins, which includes the *Drosophila* aristaless (AL) and mouse Arx proteins. HyAlx shows less similarity to the Cart1 group of proteins, and even less with the third group, the CVC class of proteins (Chx10/Vsx1/Ceh-10). Phylogenetic analysis supported the identification of *HyAlx* as an *aristaless*-related gene as it clearly grouped more closely with the aristaless-related proteins than with other members of the paired-like class (Fig. 2).

The HyAlx protein also has an eh1/GEH domain located near the N-terminus, which is involved in repression of gene function (Mailhos et al., 1998). This heptapeptide has been identified in engrailed and other paired-like proteins. Some aristaless-related proteins also contain the domain (Fig. 1B), although it is not found in the Cart1 proteins or some of the first group of proteins. The HyAlx eh1/GEH domain is highly conserved as it contains 7 of the 8 amino acids of the consensus sequence of this domain (Fig. 1B).

There are other conserved regions among aristaless-related proteins. The CVC proteins, for example, have a conserved motif, the CVC domain, which is located adjacent to the homeodomain (Svendsen and McGhee, 1995). Others contain a paired-tail (Mathers et al., 1997), which is found in several

paired-like proteins. HyAlx does not contain either of these domains.

HyAlx is associated with tentacle formation

Because hydra are amenable to a number of manipulations, the role of a gene can be explored by examining its expression pattern in several developmental contexts. Initially, the expression pattern of *HyAlx* was examined in the adult, during bud formation and head regeneration, to gain an indication of its role.

Adult animal

The possible role of *HyAlx* in hydra patterning can be explored in adult hydra, because of the unusual tissue dynamics of the animal. Epithelial cells of the body column divide continuously (Campbell, 1967a; David and Campbell, 1972), resulting in a constant displacement of tissue towards the extremities and onto developing buds (Campbell, 1967b). As cells change their axial location, they undergo changes in cell division and differentiation (Campbell, 1967a; Dübel et al., 1987). Since the morphology and the distribution of dividing and differentiated cell types remain constant within this dynamic context, the patterning and differentiation processes of the animal must be continuously active. Thus, genes that affect patterning may be investigated in the adult.

Using in situ hybridization on whole mounts, *HyAlx* expression was detectable only in the tentacle zone (Fig. 3A), the lower part of the head from which the tentacles emerge (Fig. 3C). More specifically, *HyAlx* expression is confined to a band of ectodermal epithelial cells that is 3-4 cells wide and bridges the tentacle zone/tentacle border (Fig. 3B and C). As part of the normal tissue movements, tissue from the tentacle zone is displaced onto the tentacles, and eventually sloughed at the tentacle tips (Campbell, 1967b). Thus, these cells transiently express *HyAlx* as the cells traverse the tentacle-tentacle zone border, suggesting the gene plays a role in the steady state patterning of the tentacle in the adult.

Bud formation

To determine if *HyAlx* was also involved in the de novo patterning of tentacles, its expression was examined during bud formation, the form of asexual reproduction present in hydra. Budding begins with the formation of a circular placode in the ectoderm of the budding zone, which is two-thirds of the way down the body column. The placode and surrounding tissue evaginate and subsequently extend to form a cylindrical protrusion perpendicular to the parent axis. Thereafter, head and foot develop at the apical and basal ends of the protrusion, respectively, and eventually the young polyp detaches from the parent (Otto and Campbell, 1977).

Buds at different stages of development were examined for

A

HyAlx	Hv	LRRNRTTFTT	YQLHQLERSF	DKTQYPDVFT	RENLALKLDL	SEARVQVWF**Q**	NRRAKWRKRE	% identity
Al	Dm	Q--Y-----S	F--EE--KA-	SR-H------	--E--M-IG-	T---I-----	--------Q-	70
Spalx	Sp	Q--Y-----S	---EE---A-	C--H------	--E--MRV--	T---------	----------	78
Arx	Mm	Q--Y-----S	---EE---A-	Q--H------	--E--MR---	T---------	----------	80
Cart-1	Xl	K--H-----S	L--EE--KV-	Q--H----YV	--Q---RTE-	T---------	----------	72
Alx3	Mm	K-------S-	F--EE--KV-	Q--H----YA	--Q---RT--	T---------	----------	75
Alx4	Mm	K--------S	---EE--KV-	Q--H----YA	--Q--MRT--	T---------	----------	75
Ceh-10	Ce	K--H--I--Q	--IDE--KA-	QDSH---IYA	--V--G-TE-	Q-D-I-----	--------T-	60
Vsx1	Dr	K--H--V--S	H--EE--KA-	NEAH----YA	--M--M-TE-	P-D-I-----	----------	63
Chx10	Mm	K--H--I--S	---EE--KA-	NEAH----YA	--M--M-TE-	P-D-I-----	----------	65

B

Consensus		FSIDNIL
HyAlx	Hv	----M--
Arx	Mm	YC--S--
Ceh-10	Ce	-A-HE--
Vsx1	Dr	-A-TDL-
Chx10	Mm	-G-QE--
en	Dm	---S---
Dgsc	Dm	-T--S--
Xgsc	Xl	-------
NK2	Dm	-H-SD--

Fig. 1. Comparison of the homeodomain and eh1/GEH domain of *HyAlx* with other proteins. Horizontal lines indicate residues identical to HyAlx. (A) Alignment of the homeodomains of *HyAlx* and other aristaless-related proteins. The conserved glutamine at position 50 is underlined and in bold. (B) Alignment of the *HyAlx* and other eh1/GEH domains. The organisms represented are *Hydra vulgaris* (Hv), *Drosophila melanogaster* (Dm), *Strongylocentrotus purpuratus* (Sp), *Mus musculus* (Mm), *Xenopus laevis* (Xl), *Caenorhabditis elegans* (Ce) and *Danio rerio* (Dr). GenBank Accession Numbers are HyAlx (AF295531); Al (L08401); Spalx (D85080); Arx (NM_007441); Cart-1 (L14018); Alx3 (NM_007441); Alx4 (NM_007442); Ceh-10 (AAA93063); Vsx1 (AF025348); Chx10 (NM_007701); En (M10017); Dgsc (U52968); Xgsc (M81481); and Nk-2 (X87141).

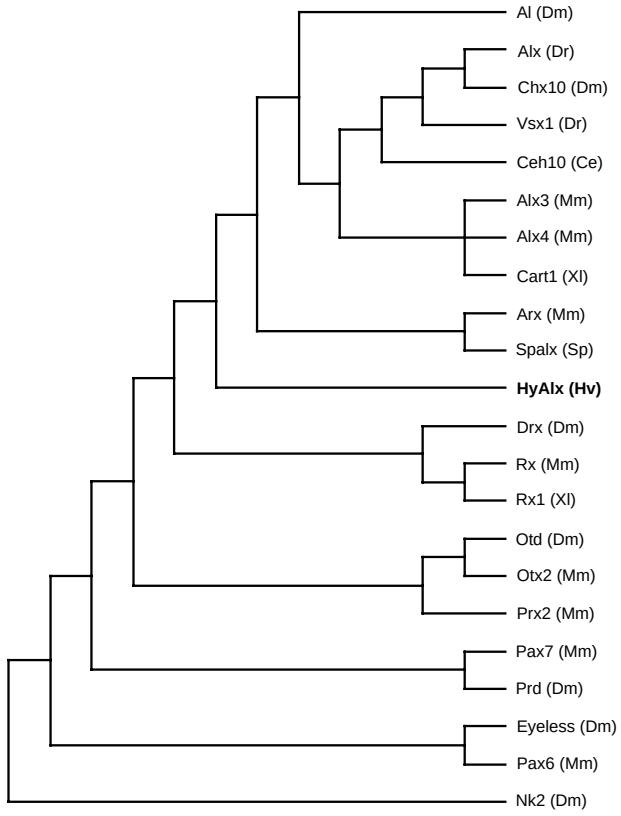

Fig. 2. Maximum parsimony tree illustrating relationships among *aristaless*-related genes and other members of the paired class. References for organism names and *aristaless*-related genes are as listed in Fig. 1. Additional accession numbers are Alx (U62898); Eyeless (X79493); Otd (X58983); Otx2 (P80206); Pax7 (AAA64491); Pax6 (P32117); Prd (P06601); Prx2 (CAA37055); Drx (AJ223300); Rx (AF001906); and Rx1 (AAB62325).

HyAlx expression. The gene first appeared as a faint band below the apical tip of the bud at stage 4 (Fig. 4A) when the bud has begun to elongate into a cylindrical protrusion. Since the bud tip corresponds to the future hypostome (Otto and Campbell, 1977), the *HyAlx* band appeared to mark the presumptive tentacle zone. By stage 5, the band became resolved into spots of a high level of expression (Fig. 4B), which are the sites of future tentacle evagination. The diameter of each spot appeared to correspond to the diameter of the emerging tentacle (data not shown). During this stage, the centers of some spots began to clear, resulting in rings of *HyAlx* expression (Fig. 4B). By stage 6 all of the spots had changed into rings (Fig. 4C), and tentacles began to evaginate from the centers of the rings. As the tentacles continued to elongate, the perimeters of the original spots remained as the final rings of expression at the tentacle zone/tentacle border (Fig. 4D), thereby establishing the adult pattern of *HyAlx* expression.

The timing of the appearance of tentacle buds provides further support for the correlation between *HyAlx* expression and the initiation of tentacle formation. The first two tentacles of these young buds form on the side of the bud facing the foot of the parent (Otto and Campbell, 1977). Similarly, the first two *HyAlx* spots appeared on this side of the bud (data not shown).

Head regeneration

When decapitated, a hydra regenerates a head, thereby providing another context for the examination of the role of *HyAlx* during tentacle formation. *HyAlx* was expressed in two separate phases during head regeneration.

During the first phase, *HyAlx* was transiently expressed as a cap extending across the entire regenerating tip. Two hours after decapitation, expression was visible as a faint cap, which gradually increased in intensity to reach a maximum by 6 hours (similar to Fig. 5A). Thereafter, expression faded disappearing by 12-16 hours of regeneration.

The second phase began with the reappearance of *HyAlx* about 20 hours after decapitation. Again, the gene was expressed in a cap covering the regenerating tip (Fig. 5A). Unlike the first phase, however, *HyAlx* was expressed very intensely as soon as it appeared as there was no gradual increase in intensity. By 24 hours the stain in the cap was diminishing and spots appeared in a ring at the lower edge of the cap (Fig. 5B). Then, by 28-32 hours, the cap stain had disappeared completely and only spots remained (Fig. 5C). which were located in the presumptive tentacle zone of the regenerate. They resembled the spots observed during budding. Thereafter, the spots evolved into rings just prior to tentacle formation (Fig. 5C), and tentacles subsequently evaginated from within these rings. By 48 hours of regeneration, fully developed tentacles were present and *HyAlx* was expressed in rings at their bases as in an adult (Fig. 5D).

To determine if both phases of *HyAlx* expression were directly involved in head regeneration and/or tentacle formation, additional regeneration experiments were carried out. Bisection of the body column at any axial level results in

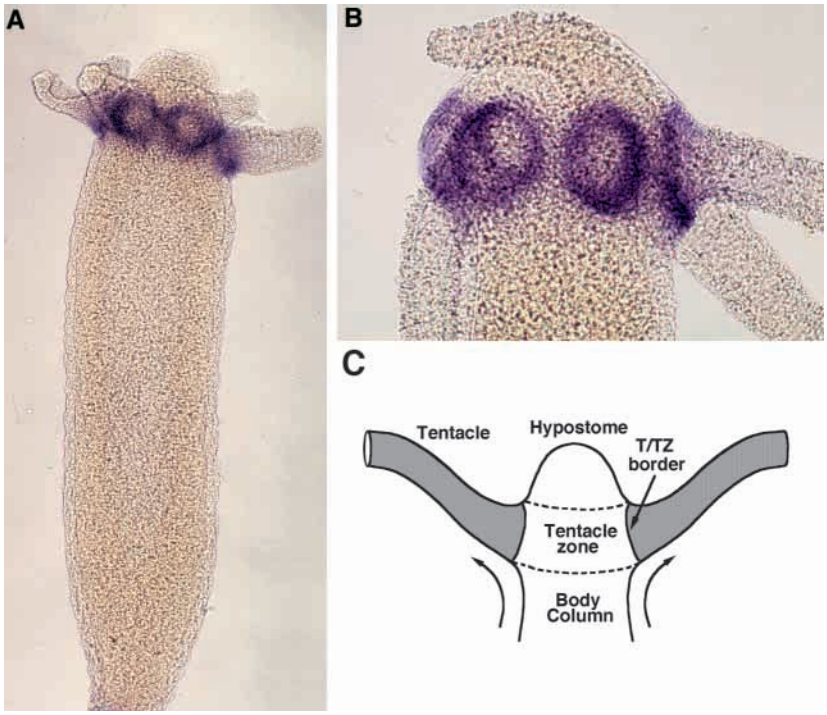

Fig. 3. Expression pattern of *HyAlx* in an adult hydra as detected by whole-mount in situ hybridization. (A) Whole animal; (B) high-magnification view of the head; (C) diagram of a hydra head. Arrows in C indicate direction of tissue displacement.

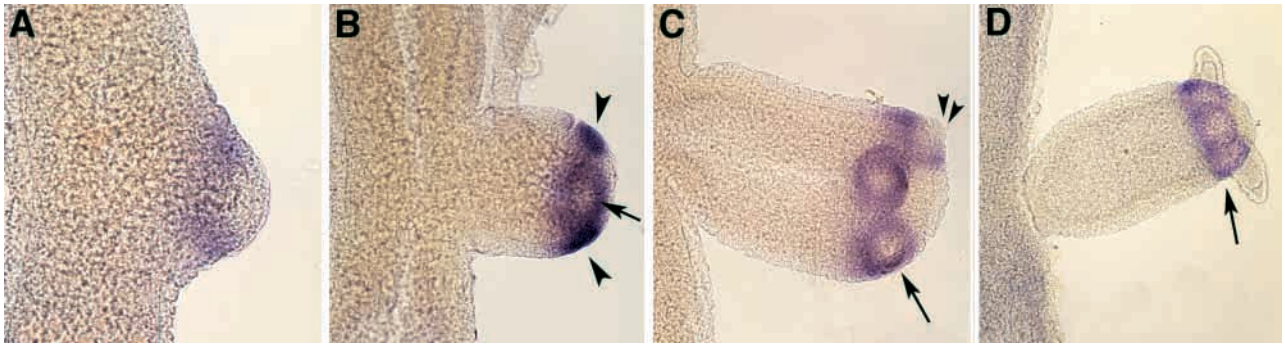

Fig. 4. Expression pattern of *HyAlx* during bud development. (A) Stage 4; (B) Stage 5; (C) Stage 6, with tentacle rudiment (double arrowheads); (D) Stage 9, shortly before detaching. Arrows indicate rings of expression and single arrowheads indicate *HyAlx* spots.

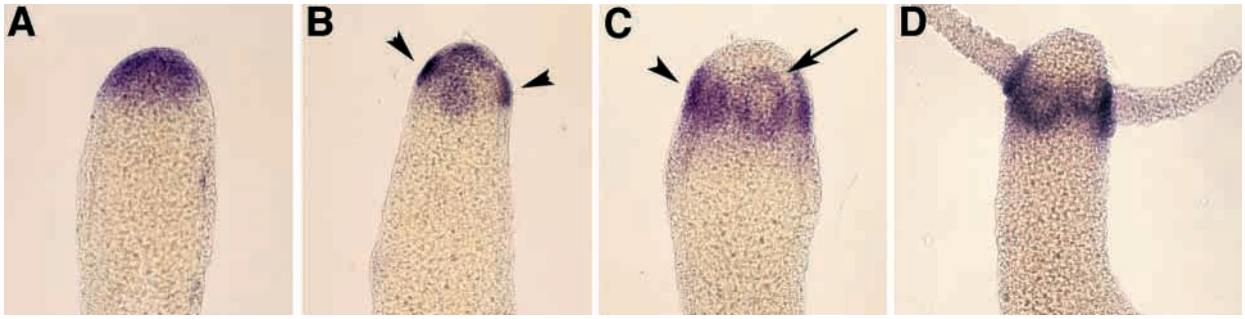

Fig. 5. Expression pattern of *HyAlx* during head regeneration. Time after decapitation: (A) 20 hours; (B) 24 hours; (C) 30 hours; and (D) 48 hours. Arrows indicate rings of expression and single arrowheads indicate spots.

the regeneration of a head at the apical end of the basal piece. However, the initiation of regeneration is increasingly delayed with increasing distance from the head (Webster and Wolpert, 1966). Thus, if a gene were closely related to the processes of head regeneration, one would expect that its expression would be delayed in mid-body column regenerates when compared with more apical regenerates.

In regenerates initiated by bisecting animals half way between head and foot, *HyAlx* was expressed in the same two phases as found in animals bisected directly below the head. The changes in the patterns for each of the two phases were the same. However, they differed in the timing of the initiation of the latter expression pattern. The first phase began 2 hours after bisection in animals bisected either directly beneath the head or in mid-body column. In contrast, the initiation of the pattern of the second phase was delayed by 8-12 hours in the mid-body column bisection compared with the beginning of this phase in decapitated animals. This change in the initiation of phase two is consistent with the slower rate of head regeneration after mid-body column bisection. Hence, phase two is correlated with head regeneration, whereas phase one is not.

That phase one expression of *HyAlx* was unrelated to head formation was supported by two other experiments. Following bisection of the body column, the basal end of the upper half always regenerates a foot. As a foot regenerated at the basal end in animals bisected in the lower part of the body column, the pattern and timecourse of *HyAlx* expression was identical to the first phase described above. Further, the second phase did not occur in these foot regenerates (data not shown). In a second experiment where both heads and feet were removed from animals, the apical and basal regenerating tips displayed the first phase of expression while the second phase appeared only in the head regenerating tips (data not shown). Therefore, the first phase of expression was not head- or foot-specific, and instead appeared to be related to a local injury effect that has been shown to occur at sites of bisection (MacWilliams, 1983b).

HyAlx is specifically associated with tentacle formation

The patterning of a hydra head during bud formation and head regeneration entails the formation of a hypostome, tentacle zone and tentacles. Though *HyAlx* appears to be associated with tentacle formation, its expression as a band during the early stages of budding and as a cap during head regeneration could suggest a more complex role in the patterning of the head. To determine if *HyAlx* was specifically associated with tentacle formation, animals were treated with DAG, which results in the formation of ectopic tentacles on the body column (Müller, 1990). Longer treatments with DAG will also induce the formation of ectopic heads.

Hydra were treated with DAG for 12 days, and samples examined periodically for expression of *HyAlx*. Ectopic tentacles, but no secondary heads, formed in these animals. The changes in *HyAlx* expression that occurred during the development of DAG-induced tentacles strongly resembled the expression pattern of this gene observed during the later stages of tentacle development in buds and regenerates. *HyAlx* was first expressed in spots along the body column (Fig. 6A), which subsequently hollowed out to form rings (Fig. 6A). Shortly thereafter, ectopic tentacles emerged from the rings (Fig. 6B). Developing and fully formed tentacles had the characteristic *HyAlx* pattern at the tentacle zone/tentacle border, as normally found in the adult head (Fig. 6B).

Thus, the *HyAlx* spot pattern is specifically associated with the formation of tentacles, and is not related to the formation of a whole head.

Direct evidence for the role of *HyAlx* in tentacle formation

To obtain more direct evidence that *HyAlx* plays a role in tentacle formation the RNAi procedure was employed.

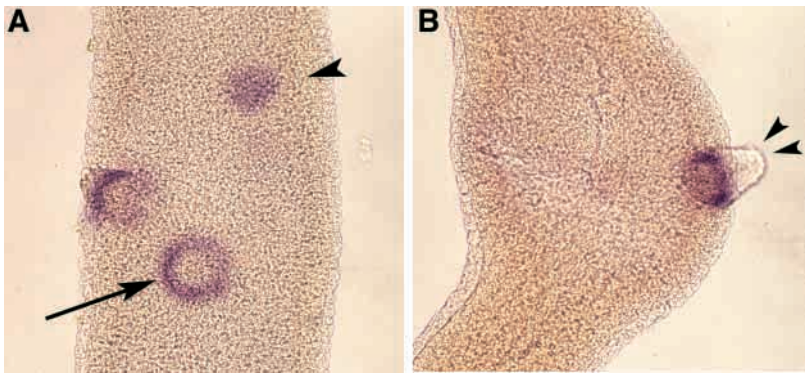

Fig. 6. Expression pattern of *HyAlx* following DAG treatment. Animals were treated for 4 days (A) and 8 days (B). An arrow indicates a *HyAlx* ring; a single arrowhead indicates a spot; and double arrowheads indicate an ectopic tentacle.

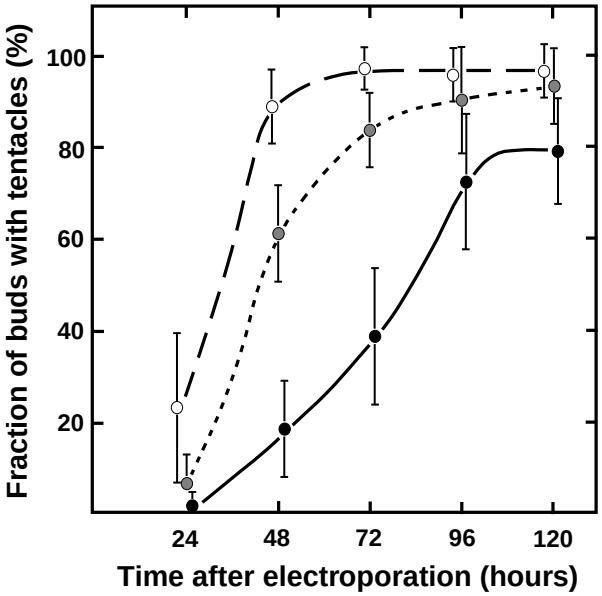

Fig. 7. Effect of *HyAlx* dsRNA (black circles) or *luciferase* dsRNA (grey circles) on tentacle formation in developing buds compared with untreated controls (open circles). Each data point is the average value ±s.e.m. for three to five experiments. Number of animals used for a particular condition in each experiment was 10-20.

Lohmann et al. (1999) have shown that the method is applicable to hydra. By introducing dsRNA of the *ks1* gene, a gene expressed in the head, by electroporation that they could delay head regeneration. We used a modified electroporation procedure in which the dsRNA was introduced not into the entire animal, but locally only into a developing bud (see Materials and Methods).

dsRNA derived from the *HyAlx* or firefly *luciferase* genes was introduced into a young bud that was just beginning to protrude (stage 2-3, Otto and Campbell, 1977). Normally tentacle buds appear in a ring just below the apical tip of the growing bud 24-48 hours later, and over the next few days develop into full-grown tentacles (see control in Fig. 7). Buds treated with *luciferase* dsRNA showed a slight delay, while those exposed to *HyAlx* dsRNA showed a significant delay (Fig. 7). The initial appearance of tentacle buds was often delayed by 48 hours compared with controls. This delay was accompanied by a transient reduction in the level of Alx RNA for about 24 hours after the introduction of *HyAlx* dsRNA (Fig. 8). Because the delay in tentacle formation occurred specifically with the *HyAlx* dsRNA, the data indicate that expression of this gene is required for this process.

The head formation capacity gradient is set up early during bud formation

Bud formation involves the formation of a new axis and, hence, a new gradient of head formation capacity. However, it is not known when this gradient is established. Removal of the apical half of a bud during or after stage 3-4 results in the formation of a new head at the distal end of the remaining basal half (Sanyal, 1966). The formation of this new head could occur due to head regeneration, which would reflect the presence of the gradient of head formation capacity. However, if this gradient is not yet established, the new head could be formed as a result of the initiation of head formation as normally occurs during budding. Since the early expression of *HyAlx* differs during head regeneration (a cap) and budding (a band), an analysis of the expression of this gene could distinguish between these two possibilities.

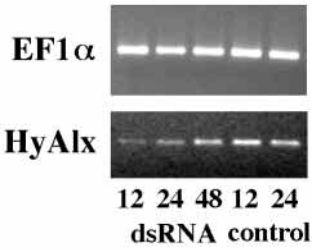

Fig. 8. Transient reduction of the level of *HyAlx* RNA in developing buds after the introduction of *HyAlx* dsRNA as measured by RT-PCR. Levels of *HyAlx* and *EF1α* RNA were examined 12, 24 and 48 hours after introduction of dsRNA. Controls were examined at 12 and 24 hours.

The apical halves of stage 4 buds were removed, and the basal halves attached to the parents allowed to regenerate. After 24 hours of regeneration, all buds displayed intense *HyAlx* expression in a cap covering the regenerating tip (Fig. 9A). Later, as the apical tip cleared, intense spots appeared at the base of the caps, which subsequently hollowed out to form rings as tentacles began to evaginate (Fig. 9B). Finally, by 64-72 hours, the normal *HyAlx* pattern was established (data not shown), and the fully patterned buds detached from the parent polyps. Thus, the *HyAlx* expression pattern was similar to the pattern observed during head regeneration, rather than that seen in normal bud development. This suggests that the gradient of head formation capacity has been established by stage 4 of bud formation. The experiment could not be carried out at earlier stages because the bisection of earlier stage buds is difficult and not reproducible.

DISCUSSION

aristaless-related genes appeared early in metazoan evolution

The hydra gene *HyAlx* has been identified as an *aristaless*-related gene, based on sequence comparisons and phylogenetic analysis. The HyAlx homeodomain is up to 80% identical with other genes belonging to this family, and appears to be more closely related to the first subgroup of genes (Fig. 1A). In addition to the homeodomain, the HyAlx protein contains an eh1/GEH domain at its N terminus, which is found in some, but not all, *aristaless*-related genes. The difficulty in precisely assigning *HyAlx* to a specific subgroup of these genes may be a reflection of the evolutionary distance separating them. However, the identification of *HyAlx* as well as two other *aristaless*-related genes in hydra (Gauchat et al., 1998) supports the idea that this family of genes arose early in metazoan evolution.

HyAlx is involved in the specification of tissue to form tentacles

The results described above demonstrate that *HyAlx* is tightly associated with the specification of tissue to form tentacles. This occurs during de novo tentacle formation as well as during the maintenance of the tentacles in adults.

Role in the de novo formation of tentacles

The de novo formation of tentacles occurs during budding,

head regeneration and as a result of DAG treatment. Although the initial stages of *HyAlx* expression vary in the three developmental contexts, the latter stages are the same. Immediately prior to tentacle formation, a necklace of spots of intense *HyAlx* expression appear. These spots transform into rings as *HyAlx* expression vanishes from the center of each spot, and subsequently, tentacles emerge from the centers of the rings. In addition, the *HyAlx* pattern during the formation of DAG-induced tentacles demonstrates that this phase of *HyAlx* expression is not related to a general head-patterning process, but is specifically related to the formation of tentacles.

Additional observations illustrate that *HyAlx* is a very precise marker for the tissue which is going to form a tentacle. First, there appears to be a correlation between *HyAlx* spot size and tentacle size in both budding and regeneration: large diameter spots give rise to large diameter tentacles, while smaller spots give rise to tentacles with smaller diameters (data not shown). Second, the appearance of *HyAlx* spots is tightly coupled with the timing of the initial evagination of tentacles. The spots appear sequentially in the same order as the order of the emergence of individual tentacles during budding. The first two spots appear on the basal side of the bud, which is also where the first two tentacles arise on these buds.

Thus, the spot and ring pattern of *HyAlx* expression is consistently associated with the emergence of tentacles indicating the gene is involved in the specification of tissue to form tentacles. The RNAi experiments provide more direct evidence for this conclusion. After introducing *HyAlx* dsRNA into developing buds at a stage before *HyAlx* expression had begun, the appearance of tentacles was delayed significantly compared with controls. This delay was specifically due to the *HyAlx* dsRNA since a control dsRNA, namely *luciferase* dsRNA had very little effect on the appearance of tentacles.

The fact that the dsRNA treatment caused a delay, but did not eliminate tentacle formation reflects the continuously regulative nature of hydra tissue. Since the patterning processes are continuously active, while the presence of dsRNA is transient, interference with a gene affecting tentacle formation would also be expected to be transient, but not permanent.

Role in the maintenance of the tentacles in the adult

Once tentacles are formed, *HyAlx* continues to play a role in tentacle patterning in the adult. In the adult, as cells are displaced from the tentacle zone onto the tentacles (see Fig. 1C), they undergo changes in their cellular properties. While in the tentacle zone, the epithelial cells are continually proliferating, but as they cross the tentacle zone/tentacle border and enter a tentacle, they become permanently arrested in the G2-phase of the cell cycle (Dübel et al., 1987). At the same time, the ectodermal epithelial cells undergo terminal differentiation to form tentacle-specific battery cells (Campbell, 1967a; Dübel et al., 1987).

The border between tentacle zone and tentacle is sharp and very precise, so that a cell on the tentacle zone side of the border exhibits dramatically different properties from its immediate neighbor on the tentacle side. This abrupt transition is reflected in the expression of several molecular markers. *CnOtx*, an *Otx* gene (Smith et al., 1999) and *Cnox3*, a *Hox* gene (M. A. Shenk and H. R. B., unpublished) are expressed in the ectodermal epithelial cells of the tentacle zone. The expression of both of these genes stops suddenly at the border, so that neither *Cnox3* nor *CnOtx* is expressed in the tentacle. Conversely, as cells cross the border, several genes not expressed in the tentacle zone are expressed at a high level as soon as these ectodermal cells enter the tentacle. These include an insulin receptor homolog, *HTK* (Steele et al., 1996), an annexin gene (Schlaepfer et al., 1992) TS19, which is a cell-surface antigen (Bode et al., 1988); and a hydra metalloproteinase, HMP1 (Yan et al., 1995).

HyAlx is expressed in rings of ectodermal cells that are approximately 3-4 cells wide, bridging this border. As ectodermal cells are displaced through the tentacle zone, they abruptly begin to express *HyAlx*, then cross the border, and only a couple of cell diameters past the border, they stop expressing *HyAlx*. Its expression at this border suggests that *HyAlx* might be involved in initiating some of the changes which take place in the tentacle zone cells as they prepare to cross the border. For example, *HyAlx* could have a role in driving cells from a proliferative to a differentiated state. The gene could also, or instead, be involved in changes in cell shape, as the ectodermal cells switch from columnar body column cells to the flat battery cells of the tentacle (Campbell, 1967a; Dübel et al., 1987).

In sum, *HyAlx* is very tightly associated with the patterning of tentacles. The gene appears to be involved in the specification of patches of cells in a developing head to form tentacles, as well as in the specification of tentacle zone tissue to become tentacle tissue in the context of continuous tissue movement in the adult.

HyAlx expression patterns are consistent with a reaction-diffusion model

Tissue-level analysis of the axial patterning processes in hydra have shown that head patterning is governed by three elements: head activation, head inhibition and a gradient of head formation capacity (MacWilliams, 1983a,b). The behavior of these three components has been most effectively explained with a reaction-diffusion model (Gierer and Meinhardt, 1972; Meinhardt, 1982). (1) Head activation is an autocatalytic process localized to a small region, a head activation center, which is responsible for head formation. In an adult hydra, this center is located in the head. (2) The head activation gradient, or source density gradient is the graded distribution of the head formation capacity along the body column. It is set up by a signal from the head activation center. (3) Head inhibition, whose production is directly coupled to the level of head activation, diffuses away from the head activation center, and prevents head formation in the body column.

In a more recent version of this model (Meinhardt, 1993), the head activation/head inhibition mechanism has been split into two components, hypostome activation/hypostome inhibition and tentacle activation/tentacle inhibition corresponding to the two regions of the head. As described in the following, the details of the dynamics of the *HyAlx* expression patterns during de novo head formation are very similar to those of tentacle activation.

Head regeneration versus bud formation

There is an important difference in *HyAlx* expression between the initial stages of head formation during head regeneration and bud formation. During head regeneration, *HyAlx* is initially expressed over the entire presumptive head region, and only

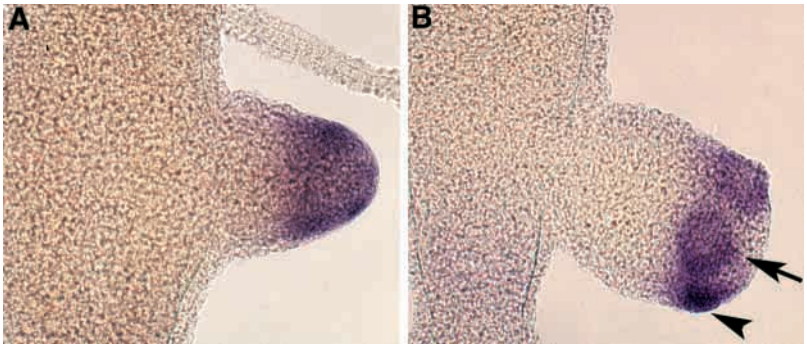

Fig. 9. Changes in *HyAlx* expression during regeneration of a bud head. (A) 24 hours and (B) 64 hours after removal of the apical half of the bud. A *HyAlx* ring is indicated by an arrow and a *HyAlx* spot is indicated by an arrowhead.

later is expression restricted to the presumptive tentacle zone (Fig. 10B). In contrast, during bud formation, the gene is not expressed at the tip in the presumptive hypostome, but only in the region below where tentacles will form (Fig. 10A). This difference is readily explained by the model in terms of differences in the order of the rise of hypostome activation and tentacle activation.

There are four relevant elements of the model. (1) Hypostome activation (HyA) rises when the level of head formation capacity, or source density (Sd), is greater than the level of head inhibition (HyI), that is when Sd>HyI. (2) In contrast, tentacle activation rises only after two conditions are met. The level of source density must be higher than the level of tentacle inhibition (TI), and it must also be greater than a threshold level of the source density, Sd_T, that is Sd>Sd_T. (3) Hypostome activation inhibits, or suppresses tentacle activation. (4) Tentacle inhibition is less stable than hypostome inhibition, and therefore decays more rapidly.

In the model, tentacle activation precedes hypostome activation during head regeneration following bisection just below the head (see Fig. 11B). Since tentacle inhibition is less stable than hypostome inhibition, a condition is rapidly reached whereby Sd>TI. Since Sd>Sd_T at the upper end of the body column, where the bisection takes place, the two conditions for a rise in TA have been met and TA rises in the regenerating tip (second red panel, Fig. 11B). This corresponds to the appearance of *HyAlx* all over the cap during the second phase. Hypostome inhibition falls more slowly, but eventually Sd>HyI in the tip and hypostome activation begins to rise (purple panels, Fig. 11B). Since HyA inhibits TA, the level of TA will begin to fall in the tip, and be confined to a ring below the tip (3rd and 4th red panels, Fig. 11B). This corresponds to the loss of *HyAlx* at the tip, and the change from a cap of *HyAlx* expression to a ring of expression in the presumptive tentacle zone (Fig. 10B).

The model works slightly differently during bud formation. Hypostome inhibition is graded down the body column eventually falling below the local level of hypostome formation capacity, or source density, that is Sd>HyI. This occurs in the region known as the budding zone. Thus, hypostome activation rises, and reaches a level where hypostome formation is initiated (purple panels, Fig. 11A). At the same time bud evagination begins either as part of the same, or a related process. The developing hypostome is confined to the apical tip of the evaginating bud (Fig. 10A). Since the level of Sd is much lower in the budding zone, Sd<Sd_T (compare first green panels in Fig. 11A and 11B), tentacle activation cannot occur. In fact, tentacle activation is delayed until the hypostome activation process has proceeded to such an extent that a signal from the developing hypostome is transmitted into the emerging bud body column, thereby generating a source density gradient in the developing bud (purple and green panels, Fig. 11A). At some point, the rising Sd will surpass Sd_T and tentacle activation will begin. Since hypostome activation has reached a maximum in the tip of the bud, TA cannot occur in the tip and the rise is confined to the region below the tip, or a ring below the tip (compare middle red panels in Figs 11A and 11B). This behavior of TA

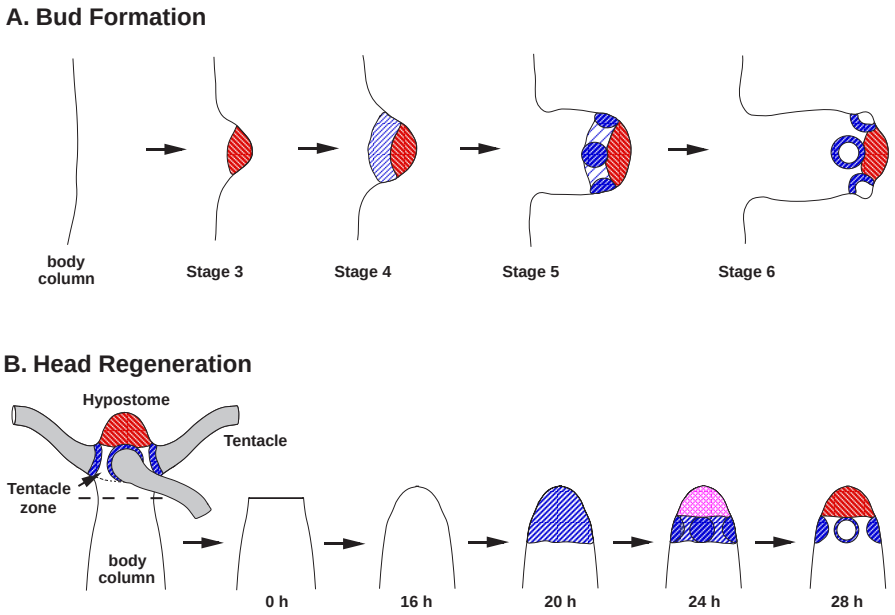

Fig. 10. Development of hypostome and *HyAlx* expression during (A) bud formation and (B) head regeneration. Red-colored regions indicate hypostome activation, while blue-colored regions represent *HyAlx* expression. The pink region indicates rising hypostome activation

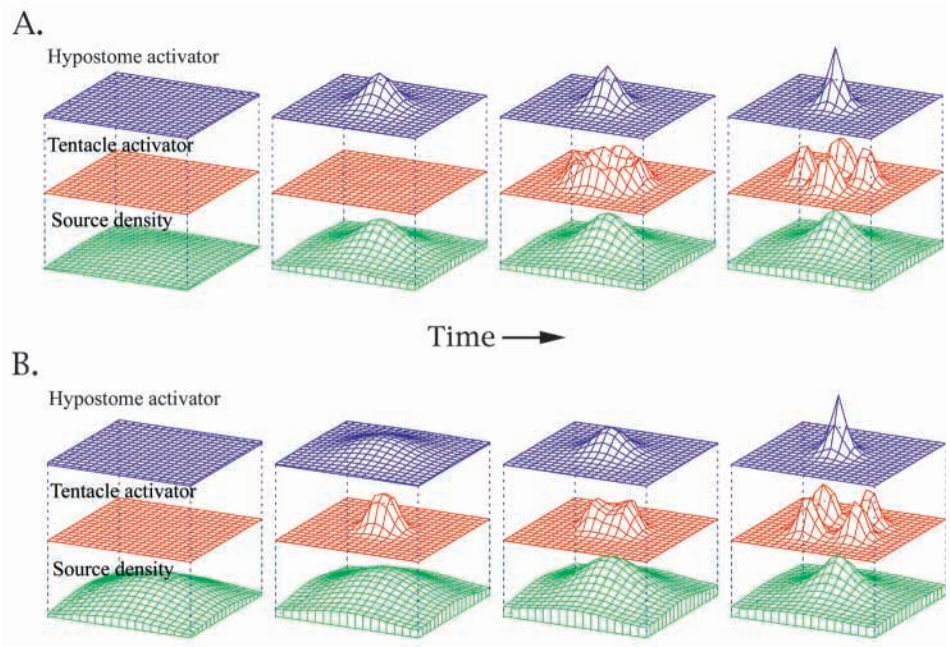

Fig. 11. Changes in hypostome activation, tentacle activation and source density as seen in a two-dimensional simulation. (A) Changes that occur in a region of low source density as in the budding zone. Hypostome activation reaches a peak in the center causing a rise in source density. Subsequently tentacle activation rises, but is confined to a ring surrounding the center since the peak of hypostome activation is already present. (B) Changes that occur after bisection in a region of high source density as occurs in the upper body column. Tentacle activation reaches a high value before hypostome activation in the center. Subsequently, as hypostome activation rises. tentacle activation is displaced from the center to a ring surrounding the center, and the ring breaks up into peaks.

is consistent with the expression of *HyAlx* during bud formation (Fig. 10A). The gene is first expressed as a ring below the apical tip of the bud, and does not appear until bud formation is well under way.

The bud regeneration results are also consistent with this interpretation. As just mentioned, the band of *HyAlx* expression by stage 4 implies that $Sd>Sd_T$ in the apical half of the bud. Thus, removal of the apical half of the bud at this stage is similar to decapitation of an adult. With $Sd>Sd_T$ and tentacle inhibition falling rapidly, tentacle activation rises all over the apical tip and, as observed (Fig. 9), *HyAlx* is expressed all over the apical tip. Thus, by stage 4, the source density gradient has been set up to permit head regeneration to occur in a bud.

The emergence of tentacle buds

The later stages of *HyAlx* expression are also consistent with the model. During bud formation, the ring of a low level of *HyAlx* expression breaks up into a necklace of spots which express the gene intensely (Fig. 10A). As shown in Fig. 11A (last two red panels) this reflects the shift from a ring of varying levels of tentacle activation to one of several uniform peaks of quite high levels of tentacle activation. In contrast, during head regeneration, the uniform high level of expression of *HyAlx* over the cap changes as the level in the center, which corresponds to this cap, drops and spots begin to emerge in a ring surrounding the center (Fig. 10B). The same changes are observed in the model (last two red panels, Fig. 11B).

Conclusion

Thus, the model quite faithfully reflects the changes in *HyAlx* expression in these two developmental contexts where de novo head formation occurs. It explains the differences between the early stages of *HyAlx* expression during head regeneration (cap) and bud formation (band). These differences in the sequence of hypostome and tentacle zone formation during head regeneration and bud formation were also observed by Technau and Holstein (1995) using markers specific for each of the two regions. Furthermore, the model describes the later changes in *HyAlx* expression from the uniform cap or band into a necklace of spots. These results support the view that this reaction-diffusion model provides a reasonable explanation for the specification of tentacle tissue and, plausibly, more generally for axial patterning in hydra. In turn, *HyAlx* should be part of the tentacle activation process.

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