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Patterning Systems in *Hydra vulgaris*

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The Cnidarian, hydra, is an appealing model system for studying the basic processes underlying pattern formation. Classical studies have elucidated much basic information regarding the role of development gradients, and theoretical models have been quite successful at describing experimental results. However, most experiments and computer simulations have dealt with isolated patterning events such as the dynamics of head regeneration. More global events such as interactions among the head, bud, and foot patterning systems have not been extensively addressed. The characterization of monoclonal antibodies with position-specific labeling patterns and the recent cloning and characterization of genes expressed in position-specific manners now provide the tools for investigating global interactions between patterning systems. In particular, changes in the axial positional value gradient may be monitored in response to experimental perturbation. Rather than studying isolated patterning events, this approach allows us to study patterning over the entire animal. The studies reported here focus on interactions between the foot and the head patterning systems in *Hydra vulgaris* following induction of a foot in close proximity to a head, axial grafting of a foot closer to the head, or doubling the amount of basal tissue by lateral grafting of an additional peduncle-foot onto host animals. Resulting positional value changes as monitored by antigen (TS19) and gene (*ks1* and *CnNK-2*) expression were assessed in the foot, head, and intervening tissue. The results of the experiments indicate that positional values changed rapidly, in a matter of hours, and that there were reciprocal interactions between the foot and the head patterning systems. Theoretical interpretations of the results in the form of computer simulations based on the reaction-diffusion model are presented and predict many, but not all, of the experimental observations. Since the lateral grafting experiment cannot, at present, be simulated, it is discussed in light of what has been learned from the axial grafting experiments and their simulations. © 1999 Academic Press

Key Words: **hydra; head regeneration; foot regeneration; pattern formation; positional value; proportion regulation; reaction diffusion model.**

INTRODUCTION

The Cnidarian hydra arose early in metazoan evolution. Its simple morphology and lifestyle make it amenable to a wide variety of experimental manipulations, and it has been used as a model system to study biological processes since the 1700s (Lenhoff and Lenhoff, 1986). Numerous genes involved in axis formation and specification of body regions in vertebrates have been conserved during metazoan evolution and are expressed in Cnidarians (Schummer *et al.*, 1992; Shenk *et al.*, 1993a,b; Grens *et al.*, 1995; Martinez *et al.*, 1997). Thus, hydra is an appealing model system for the study of basic processes underlying the establishment of pattern. A rich literature documenting the existence of developmental gradients of head and foot formation and inhibition in hydra exists (Javois, 1992; Müller, 1996, for review). It has provided the basis for the formulation of theoretical models of pattern formation describing many experimental results (Wolpert *et al.*, 1972; Gierer and Meinhardt, 1972; MacWilliams, 1982; Meinhardt, 1993; Müller, 1995; Sherratt *et al.*, 1995). Until recently, most work on hydra pattern formation has focused on head, foot, or bud formation as isolated patterning events, although the influence of the head on budding has long been known. Recent studies also have documented interactions between the head and the foot patterning systems, indicating that the head can accelerate foot formation (Ando *et al.*, 1989; Javois and Frazier, 1991; Müller, 1995). However, basal influences on more apical patterning events have not been thoroughly

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studied or characterized. Recent work in this laboratory has documented interactions between the foot and the bud patterning systems (Schiliro *et al.*, in press) and the present study was undertaken to examine interactions between the foot and the head patterning systems.

The combination of monoclonal antibodies with position-specific labeling patterns (Bode *et al.*, 1988) and the recent cloning and characterization of two genes that are expressed in position-specific manners (Weinziger *et al.*, 1994; Grens *et al.*, 1996) now provide us with the means to monitor positional value changes along the hydra head to foot axis. Rather than studying isolated patterning events, this approach allows us to study patterning over the entire animal. More specifically, these markers can be used to investigate what influence the foot patterning system has on the apical end of the axis. Three approaches were used. In the first, a foot was induced to form in close proximity to a head by axially grafting a donor head to an isolated host upper body column and head. In these constructs, a new foot forms in the formerly upper body column tissue near the graft junction. In the second approach, the head and foot were placed closer together by excising the budding zone and lower body column. In the third experiment, the amount of basal tissue was doubled by laterally grafting an additional peduncle-foot onto host animals. In all cases, positional value changes as monitored by antigen and gene expression were assessed in the foot, head, and intervening tissue. The results of these studies indicate that positional values can change very rapidly (in a matter of hours) and the head and foot patterning systems reciprocally influence one another. Theoretical models of pattern formation may be used to simulate the axial grafting experiments, and the ability of the models to predict the observed changes in positional value can be assessed. Such simulations based on the reaction-diffusion model (Meinhardt, 1998) are presented and discussed.

MATERIALS AND METHODS

Animals and culture conditions. Asexual *Hydra vulgaris* (clone 203) originally obtained form Pierre Tardent, Zurich, Switzerland, were used for all experiments. For all experimental and control groups, 20–30 animals were used. Adult budding animals were divided into eight regions (H1234BPF) for means of description (Fig. 1a). The H represents the head that includes the tentacles and hypostome. The 1234 represents the gastric region divided into four equal segments from apical to basal. The B represents the budding zone; the P represents the peduncle, and the F represents the foot.

The animals were maintained at 18°C in hydra medium (HM) containing 1 mM $CaCl_2$, 1 mM $NaHCO_3$, 0.1 mM KCl, and 0.025 mM $MgCl_2$. The medium was changed daily. The animals were fed 3-day-old *Artemia nauplii* on Monday, Wednesday, and Friday. Animals used for experiments were starved for 24 h unless otherwise indicated. Animals were not fed during experiments unless otherwise stated. For some grafting experiments, vitally stained blue animals were used. The addition of 1 ml of 1% (w/v) Evans blue to 2-day-old *A. nauplii* 24 h prior to feeding stains the shrimp blue. The tissue of hydra fed three times with blue shrimp is dark blue and can easily be distinguished from unstained tissue.

Graft constructs. In general, graft constructs were created by axial grafting using 10-lb. Stren monofilament fish line (DuPont, Wilmington, DE) and PE-20 polyethylene tubing (Intramedic, Becton Dickinson, Parsippany, NJ). The tissues were allowed to heal for 30–60 min before the tubing and fish line were removed. Graft constructs were maintained without feeding.

The H12/H* animal was constructed by bisecting at the 2–3 border to produce an H12 tissue piece, which was strung on a fish line. A second animal was cut just below the tentacles to isolate a donor H*. This H* was placed on the fish line adjacent to the H12 with opposite polarity and allowed to heal (Fig. 1b). When it was necessary to distinguish between H and H*, the H* was taken from a vitally stained Evan's blue animal.

The H1/PF animal was constructed by bisecting below the budding zone and stringing the PF-region on a piece of fish line. The H1 tissue from the same animal was then removed and placed next to the PF on the fish line, maintaining the original polarity (Fig. 1c). The tissue pieces were held in place with PE tubing and allowed to heal. A sham graft for the H1/PF animal was created by bisecting an animal just below the budding zone. Both parts of the animal were strung on fish line with the original orientation to re-create the same animal. Sham grafted animals were maintained and compared to experimental animals.

The H1234B$(PF)^2$ animal was constructed by lateral grafting. An incision was made in the host animal at the B–P junction. The host animal was placed on the fish line and PF tissue removed from a donor animal was strung on the fish line at the incision with the same polarity as in the host, held in place with tubing, and allowed to heal (Fig. 1d). The resulting H1234B$(PF)^2$ was monitored for maintenance of the $(PF)^2$ morphology and only those that maintained the $(PF)^2$ morphology were analyzed further. A sham graft for the H1234B$(PF)^2$ was created by making a small incision at the B–P junction and stringing the animal on fish line inserted through the mouth and out the incision. The injured animal was compared to experimental animals.

Morphological evaluation of positional value. Apical positional value (PV) is associated with the ability to regenerate a head. Morphological evaluation of apical PV was determined by documenting head regeneration 72 h following head removal. Heads were removed from control and experimental animals at the same time. The presence of tentacles at 72 h indicated that the animal had the ability to regenerate a head. The absence of tentacles at 72 h indicated that the animal had lost the ability to regenerate a head within the normal time frame.

Basal PV is associated with ability to form a foot. Morphological evaluation of basal PV was determined by documenting the presence or absence of a sticky basal disc. If the animal stuck to the dish or to forceps when probed, a functional basal disc was present. In some cases, differentiation of the basal disc was confirmed by staining for the peroxidase-like enzyme activity associated with the basal disc using the method of Hoffmeister and Schaller (1985). Depending on the experiment, the morphology of basal PV was evaluated at different times.

Immunocytochemistry. Molecular evaluation of apical and basal PV was performed by whole-mount immunocytochemistry using the ectodermal epithelial cell-specific monoclonal antibody, TS19 (gift of H. R. Bode, Irvine, CA). Apical TS19 labeling was performed 32–37 h following decapitation just beneath the tentacles using the procedure of Bode *et al.* (1988) as modified below. All incubations and washes were for 10 min, and following the last

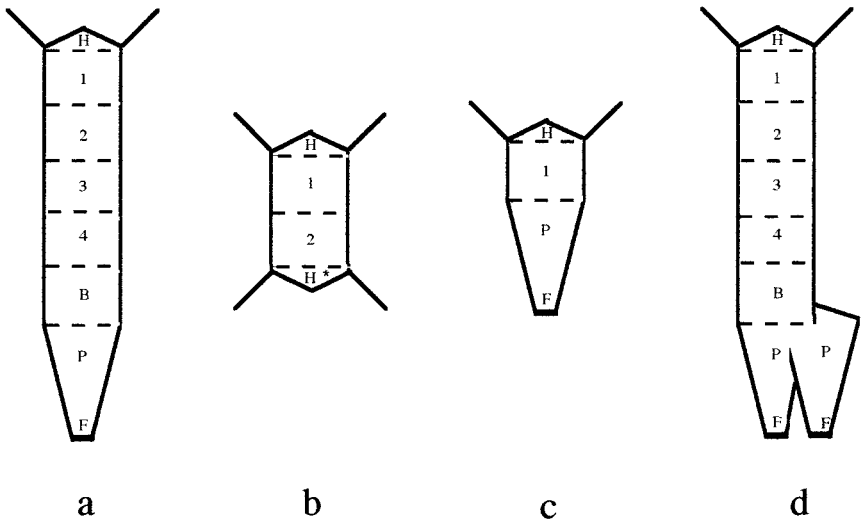


FIG. 1. Diagrams illustrating a steady-state hydra and graft constructs. Abbreviations for body regions are described in the text. (a) Normal nonbudding animal; (b) H12/H* axial graft; (c) H1/PF axial graft; (d) H1234B(PF)^2 lateral graft construct.

wash the animals were fixed in 70% EtOH. The animals were mounted between glass coverslips using fluorescence mounting medium (70% v/v glycerol, 30% v/v 0.1 M Tris, pH 9.0, 5% w/v *n*-propyl gallate). TS19 labeling was scored by viewing animals under fluorescence optics using an Olympus BHT compound microscope equipped with a BP490 excitation filer and a BH2-DM500 dichroic mirror with a 0- to 515-nm barrier filter. In the normal steady-state animal TS19 labels the tentacles (Fig. 2A) and a ring in the peduncle just above the basal disc (not shown).

Quantitation of apical TS19 label. There is a distinct progression of TS19 labeling during head regeneration from apical levels (Bode *et al.*, 1988; Technau and Holstein, 1995). TS19 initially labels a broad area of the apical ectoderm. As tentacle cells begin to differentiate, the TS19 label is localized into patches. These patches evaginate to form bumps, which then elongate into tentacles. Since head regeneration rate is correlated with apical PV, a quantitative method was devised to evaluate apical PV using this progression of TS19 label during head regeneration. After 32–37 h, regenerating animals were TS19 labeled, fixed, and evaluated as follows: an area of ectodermal label was given a value of 1; a tentacle patch was given a value of 2; a tentacle bump was given a value of 3; a tentacle of length equal to width was given a value of 4; a tentacle of length longer than width was given a value of 5; and, a tentacle of length much longer than width was given a value of 6. Each regenerating control and experimental animal was evaluated for complete tentacle morphology and given a total score (e.g., Fig. 2B). Scores were then averaged and compared using the Student *t* test to evaluate significance. To compare regeneration rates, the ratio of Experimental to Control average TS19 scores were compared over time. A ratio of 1 indicated equal regeneration rates, while ratios above 1 indicated that the experimental animals regenerated faster, and ratios below 1 indicated that the experimental animals regenerated slower.

Whole-mount in situ hybridization. Molecular evaluation of apical and basal PV also was performed using whole-mount *in situ* hybridization and the apical-specific probe *ks1* or the basal-specific probe *CnNK-2*. The *ks1* probe was transcribed from the pBS-*ks1* plasmid (gift of T. Bosch, Jena, Germany). The *CnNK-2* probe was transcribed from the pBS-*CnNK-2* plasmid (gift of H. R. Bode, Irvine, CA). Digoxigenin-labeled probes were prepared using Boehringer Mannheim's reagents and protocol (Boehringer Mannheim, 1996).

In situ hybridization was performed as described in Grens *et al.* (1996) to evaluate the expression patterns for experimental and control animals with the following modifications. When the control animals displayed normal labeling, the staining reaction was stopped in all wells at the same time by washing twice for 20 min in NTMT. The animals were placed into a solution of 50% PBS:50% glycerol + 0.3% sodium azide, analyzed, and stored at 4°C.

Photography. Animals were mounted between glass coverslips for photography. An Olympus BX60 microscope equipped for epiflourescence and differential contrast microscopy was used for photography. Black and white photos were taken using Tri-X Pan 35mm film (ISO 400) (Kodak, Rochester, NY), which was processed with DIAFINE (Acufine, Inc., Chicago, IL) 2-bath developer. Color photos were taken of animals using Ektachrome 64T professional 35mm film (Kodak). The film was commercially processed into slides and the slides were digitally reproduced into prints.

Computer simulations. Computer simulations of the axial graft constructs were performed using a program provided by Professor Hans Meinhardt, Tübingen, Germany, and a Gateway 2000 personal computer. This version of the reaction-diffusion model is similar to a recently published version (Meinhardt, 1998; see GT 12-6c) except that six activator–inhibitor systems, instead of four, were used to establish the source density gradient.

RESULTS

To examine the role of basal tissue in hydra pattern formation and potential interactions between the basal and the apical patterning systems, hydra body proportions were altered. The three manipulations performed are illustrated

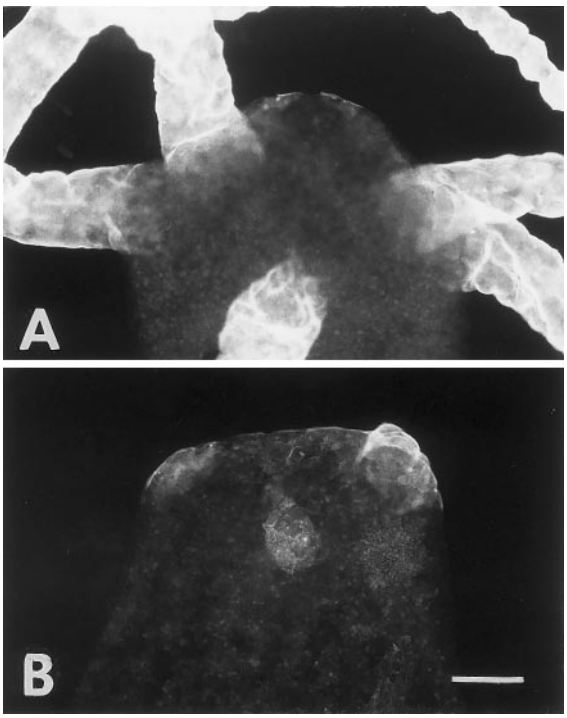


FIG. 2. Whole-mount immunocytochemistry illustrating apical TS19 labeling. (A) Steady-state control animal illustrating tentacle-specific ectodermal labeling; (B) 40-h regenerate; quantitation of TS19 label for this animal would be "7" (two patches and one tentacle bump; see Materials and Methods). Scale bar, 50 μm.

in Fig. 1. A foot was induced to form in close proximity to a head by grafting a donor head to the basal end of a host upper body column and head, creating a H12/H* animal (Fig. 1b). A foot was moved closer to the head by removing the 234B-region and grafting the H1 and PF back together (Fig. 1c). In addition, the amount of basal tissue was doubled by laterally grafting a second PF onto a host animal (Fig. 1d). The resulting changes in PVs over time for both apical and basal tissues were evaluated in the various constructs.

Positional values were defined using both morphological and molecular criteria. At the morphological level, high apical PVs were characterized by the ability to regenerate a head in 72 h. Furthermore, faster rates of head regeneration were correlated with higher apical PVs. Therefore, head regeneration rates were compared by quantifying the extent of tentacle ectodermal epithelial cell-specific monoclonal antibody TS19 labeling using whole-mount immunocytochemistry (Bode *et al.,* 1988) (Fig. 2). Additionally, apical PVs were evaluated with whole-mount *in situ* hybridization using the apical-specific probe, *ks1* (Weinziger *et al.,* 1994). In the steady-state animal, *ks1* expression was seen in the ectodermal epithelial cells at the base of the tentacles and the intertentacle zone (Fig. 4I).

Low basal PVs were characterized by the presence of a basal disc and the ability to stick to the substrate. At the molecular level, basal PVs were evaluated by whole-mount immunocytochemistry using TS19, which in addition to its apical labeling pattern also lightly labeled the peduncle ectodermal epithelial cells just above the basal disc. Whole-mount *in situ* hybridization with the basal-specific probe, *CnNK-2* (Grens *et al.,* 1996), also was used to evaluate basal PVs. In the steady-state animal, heavy *CnNK-2* expression was localized in the endodermal epithelial cells of the peduncle below the budding zone with some light expression up into the body column (Fig. 8A).

Evaluation of Apical Positional Values Following Alteration of Body Proportions

Induction of a foot in close proximity to the head resulted in a rapid loss of head regeneration ability and apical-most positional values. Previous work has shown that PV in the 1-region of H12/H* *H. oligactis* constructs decreased with time after graft construction (Javois and Bessette, 1996). This phenomenon was examined in greater detail using *H. vulgaris.* The host head, H, was removed from H12/H* animals at 3, 6, 12, and 24 h following graft construction, and head regeneration was documented 72 h later by observation under a dissecting microscope. Each time point was analyzed twice as summarized in Fig. 3. As early as 3 h and continuing through 24 h, the ability of the 1-region to regenerate a head was greatly reduced or lost.

Animals were fixed over a similar time course following graft construction, hybridized with *ks1,* and compared to control animals. Three hours following graft construction, both H and H* displayed a more intense *ks1* label when compared to control animals (cf. Figs. 4A and 4B with 4I). The majority of H displayed a heavy label, H* displayed roughly equal heavy and medium label, and control animals displayed medium or light label (Figs. 5A and 5B). However, in animals fixed 8 h following graft construction, H and H* expressed *ks1* similar to each other and much more like the expression seen in control animals (Figs. 4C, 4D, 5A, and 5B). By 24 h after graft construction only a few animals expressed *ks1* (Figs. 5A and 5B).

Moving the foot closer to the head by axial grafting resulted in a slower rate of head regeneration and loss of apical-most positional values. Basal tissue was moved closer to apical tissue by creating H1/PF animals, and morphologically the apical and basal tissues in the H1/PF graft construct remained unchanged from normal animals. The H1-region maintained the appearance of a body column with hypostome and tentacles while the PF-region maintained the less opaque appearance of a peduncle with a basal disc.

Heads were removed from the H1/PF animals 24 and 72 h following graft construction, and head regeneration was evaluated under a dissecting microscope and compared to regeneration in control animals. All of the experimental and control animals regenerated heads with two to four tentacles, and the average number of tentacles for H1/PF animals was only slightly less than that for controls.

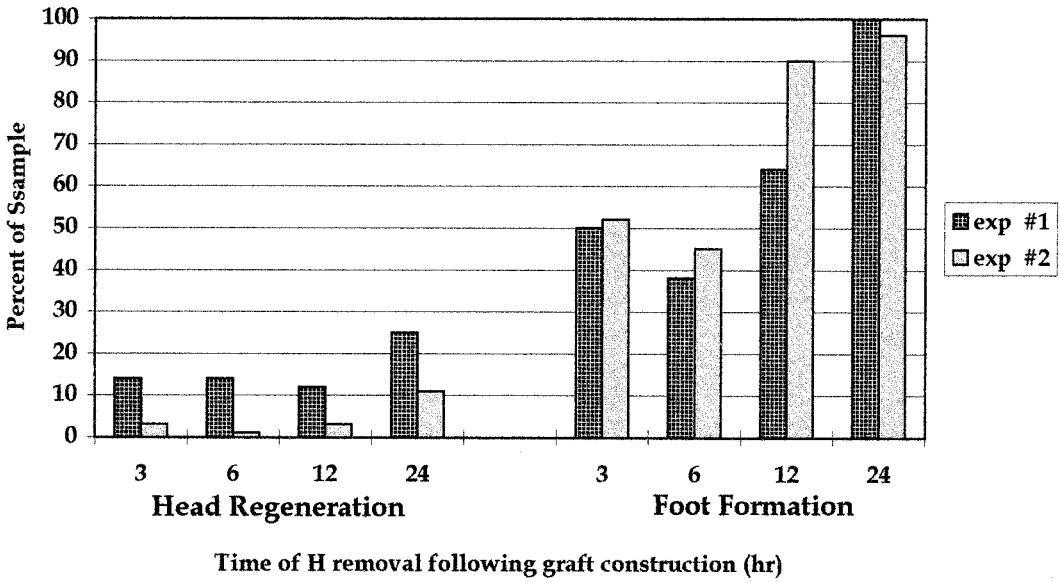


FIG. 3. Morphology 72 h following host head (H) removal from H12/H* construct. H was removed from the H12/H* constructs at various times following graft construction. Animals were analyzed for H regeneration and foot formation under a dissecting microscope. An animal was considered to be positive for head regeneration if one or more tentacles were present. An animal was considered to be positive for foot formation if it was sticking down or stuck to the forceps when probed. Each time point was examined twice and both results are illustrated. For each trial at each time point, $N = 24–30$.

However, tentacle length on many of the experimental animals appeared to be shorter than on control animals, suggesting that while the ability to regenerate a head was not lost in the H1/PF graft construct, regeneration might be slower. To evaluate this, the rate of head regeneration at earlier points during regeneration was examined using whole-mount immunocytochemistry and TS19. The TS19 labeling pattern during early head regeneration from apical levels passes through a very characteristic sequence that arbitrarily can be assigned quantitative scores as described under Materials and Methods. Heads were removed from H1/PF animals at 10, 19, 30, and 48 h following graft construction. Heads were removed from control animals at the same times. After 32–37 h of regenerating, all animals were labeled with TS19, evaluated, and given a TS19 score. The scores for each experimental and control group were then averaged. The H1/PF:Control average TS19 score was calculated and plotted such that a ratio of 1 indicated an equal rate of head regeneration and a ratio <1 indicated that regeneration was slower for experimental animals (Fig. 6A). At the earliest time point of 10 h, head regeneration was slower for the H1/PF animals. Throughout the time course the ratio of TS19 scores remained low, experimental head regeneration was slower, and the difference in rate was statistically significant (Student's t test; $P < 0.003$). As the morphology of these constructs was stable, later time points were not analyzed.

Following graft construction, H1/PF animals were fixed at early, intermediate, and late time points, hybridized with *ks1*, and compared to control animals. Five hours after graft construction, the *ks1* label was more intense and expressed in cells further out along the tentacles in the H1/PF animals compared to controls (Fig. 4E). A majority of the H1/PF animals had heavy label while the majority of the control animals had a medium label (Fig. 5C). However, 24 h following graft construction, the label in H1/PF animals was lighter than controls, and by 48 h light label was present on only a few experimental animals (Figs. 4F and 5C).

Doubling PF tissue initially slowed the head regeneration rate and lowered apical positional values, but then the head regeneration rate and apical positional values increased. Initial assessment of the ability of apical tissue in the H1234B(PF)^2 animals to regenerate was performed by decapitating and examining the extent of head regeneration 72 h later. All of the animals regenerated heads by 72 h. There was a slight increase in tentacle number compared to controls, but this increase did not appear to be significant. Head regeneration rates were evaluated using TS19 labeling on animals decapitated 8, 22, 67, and 94 h following graft construction and control animals decapitated at the same times. TS19 scores for each group were averaged and the Experimental:Control was plotted such that a ratio <1 indicated a slower rate of regeneration and a ratio >1 indicated a faster rate of regeneration for experimental animals (Fig. 6B). At 8 h, the rate of regeneration was slower for the H1234B(PF)^2 animals and the decreased rate was statistically significant (Student's t test; $P < 0.0004$). How-

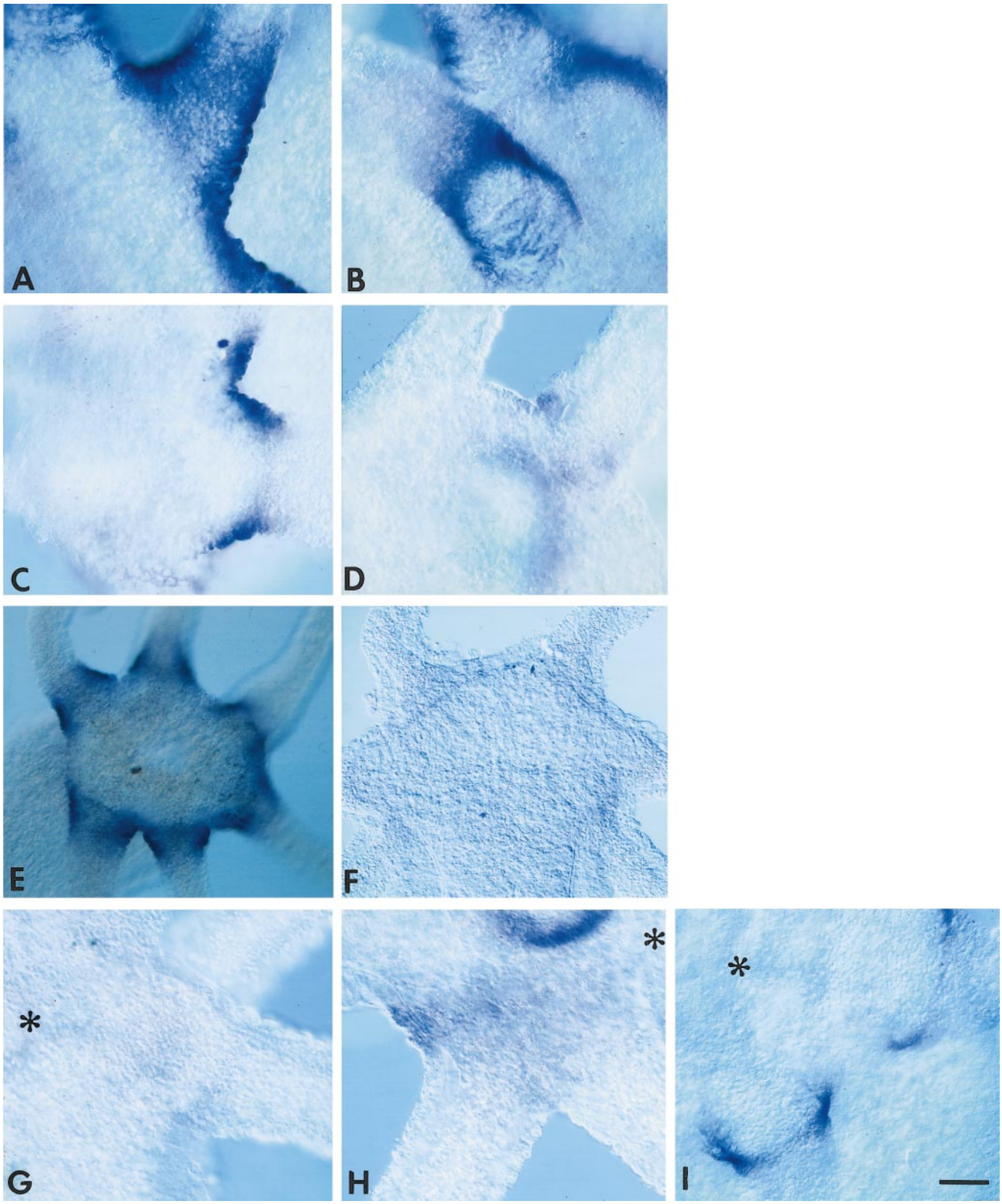


FIG. 4. Apical *ks1* labeling in graft constructs and control animal. *Ks1* labeling in H (A, C) and H* (B, D) of H12/H* constructs. 3 h (A, B) following graft construction labeling is more intense and extends further out on the tentacles compared to the control (I). After 8 h (C, D), labeling decreased (cf. C with A; D with B). (E) 5 h after construction of the H1/PF graft, *ks1* labeling shifted further out the tentacles. (F) 24 h after H1/PF graft construction *ks1* labeling is gone. (G) 8 h following construction of the $(PF)^2$ graft, *ks1* labeling was lost. (H) 67 h after grafting *ks1* labeling returned to the base of the tentacles. (I) Control adult hydra illustrating *ks1* labeling restricted to the base of the tentacles. Asterisks indicate the position of the hypostome relative to the tentacles in (G–I). Scale bar, 100 μm (A–D, G–I); 50 μm (E and F).

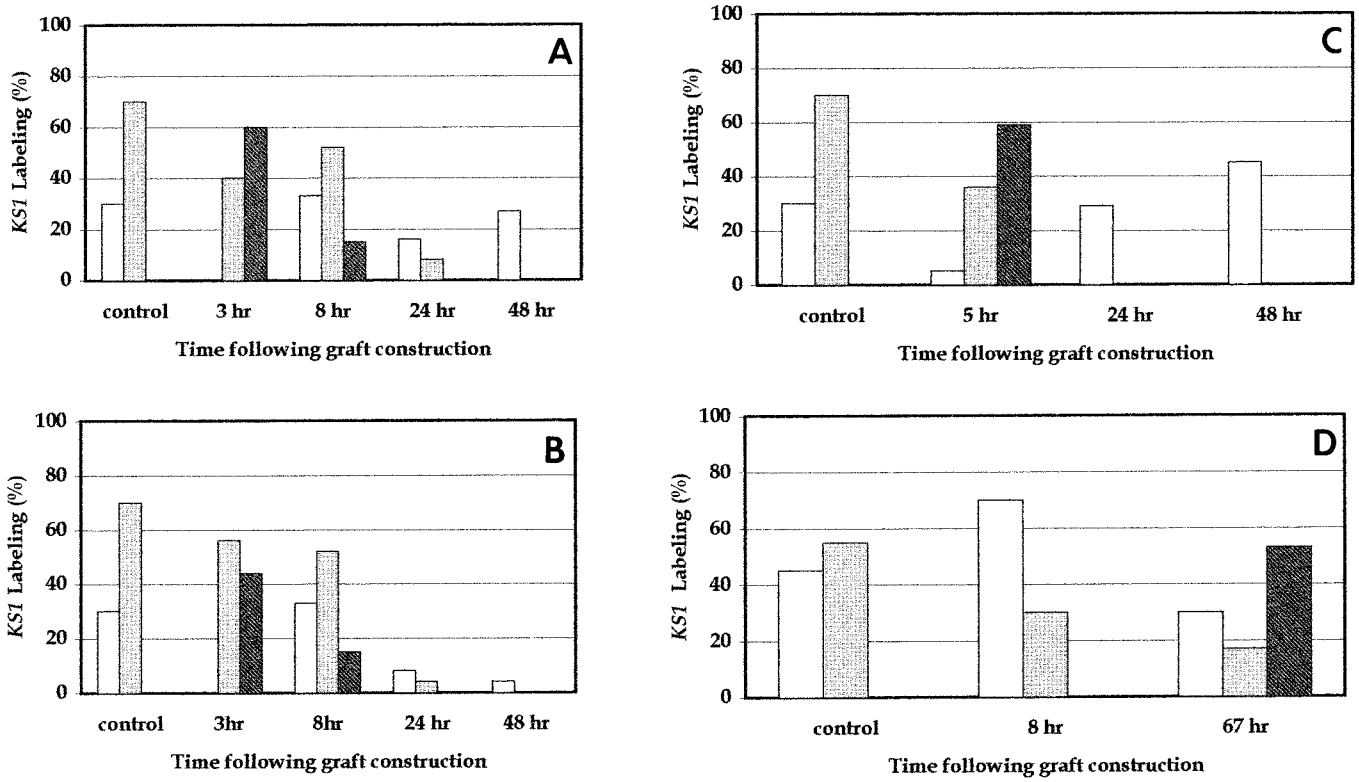


FIG. 5. Summary of *ks1* labeling in the graft constructs. Animals were examined under a dissecting scope and labeling was arbitrarily assigned to categories of light, medium, or heavy as indicated by shading of the bars. *Ks1* labeling of the H (A) or H* (B) in H12/H* constructs; for each time point, $N = 28–31$. (C) *Ks1* labeling in H1/PF constructs; for each time point, $N = 22$. (D) *Ks1* labeling in the $(PF)^2$ constructs; for each time point, $N = 26–31$.

ever, from 22 h and throughout the remainder of the time course the rate of head regeneration was faster for the $H1234B(PF)^2$ animals, peaking at 67 h, when the average TS19 score was more than twice the average control score. This difference was statistically significant (Student's *t* test; $P < 0.008$).

Since the most dramatic changes in head regeneration rates were observed at 8 and 67 h, animals were fixed at these two times following graft construction, hybridized with *ks1*, and compared to controls. Eight hours following graft construction, *ks1* expression was reduced (Fig. 4G). In comparison to the majority of control animals that displayed a medium-intensity label, the majority of graft constructs displayed only light *ks1* label (Fig. 5D). However, 67 h following graft construction, more than 50% of the constructs displayed a very intense heavy label (Figs. 4H and 5D).

Evaluation of Basal Positional Values Following Alteration of Body Proportions

Induction of tissue with basal positional values was dependent on the presence of the host head (H) and was complete by 48 h. Induction of tissue with basal positional values in the H12/H* animals was first evident as expression of *CnNK-2*. Animals were fixed starting at 6 h and periodically thereafter until 96 h after graft construction, hybridized with *CnNK-2*, and compared to control animals. The *CnNK-2* labeling seen in the H12/H* animals was determined to be negative, diffuse throughout the body column, or localized to the 2-region. Some animals had diffuse body column label and a heavier localized label. Because the trend was toward the localized label, these animals were included with those having localized label (Fig. 7A). As early as 6 h following graft construction, *CnNK-2* diffuse label was seen in 16% of the animals. By 12 h >50% had localized label, and by 24 h 100% of the animals were labeled with basal-specific *CnNK-2*. The overall trend is toward localized label, which was seen 96 h after graft construction (see Figs. 7A, and 8B).

Other observations further defined the events of foot induction. First, between 24 and 48 h, the animals took on a "V" shape with the 2-region forming the base of the V. Second, by 42 h following graft construction all animals were $TS19^+$ with the labeling encompassing a partial to complete circumferential band in the 2-region adjacent to the graft junction ($N = 25$). Third, it was observed that all

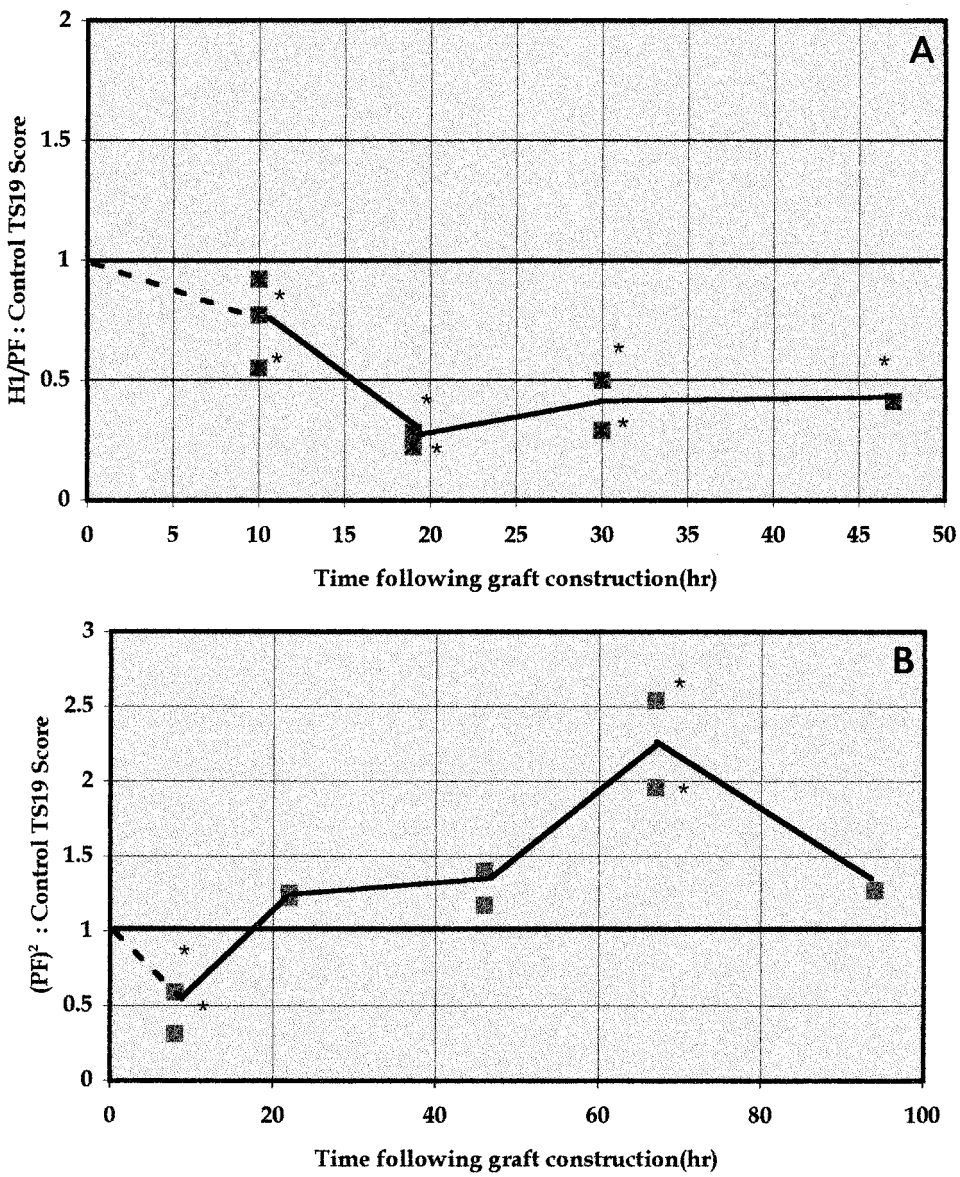


FIG. 6. Head regeneration in H1/PF (A) and $(PF)^2$ (B) constructs vs Controls. The ratios of Experimental:Control average TS19 scores are plotted over time. A ratio of 1 indicates an equal rate of regeneration, a ratio of <1 indicates a slower rate of regeneration, and a ratio >1 indicates a faster rate of regeneration in experimental animals compared to controls. For (A) each time point was repeated at least twice with the exception of 48 h, N = 21–27. For (B) each time point was repeated at least twice with the exception of 96 h, N = 22–32. Apical TS19 labeling was scored as described under Materials and Methods and illustrated in Fig. 2B. Asterisks indicate data points for which the difference between experimental and control average TS19 scores was significant (see text for details).

animals formed a foot in the 2-region and were sticking down by 48 h. Peroxidase labeling confirmed the presence of the foot in the 2-region by 48 h in all animals examined (N = 28).

This foot induction was dependent upon the presence of the host head. When the host head was removed at 3 or 6 h following graft construction, about half of the animals failed to form a foot by 72 h (Fig. 3), through some of these animals had peduncle-like TS19 labeling. For example, following host head removal after 3 h, 55% of the animals did not form feet; however, of these, 47% expressed peduncle-like TS19 labeling (N = 56). Only when the host head was left on for 24 h did 100% of the animals form a foot.

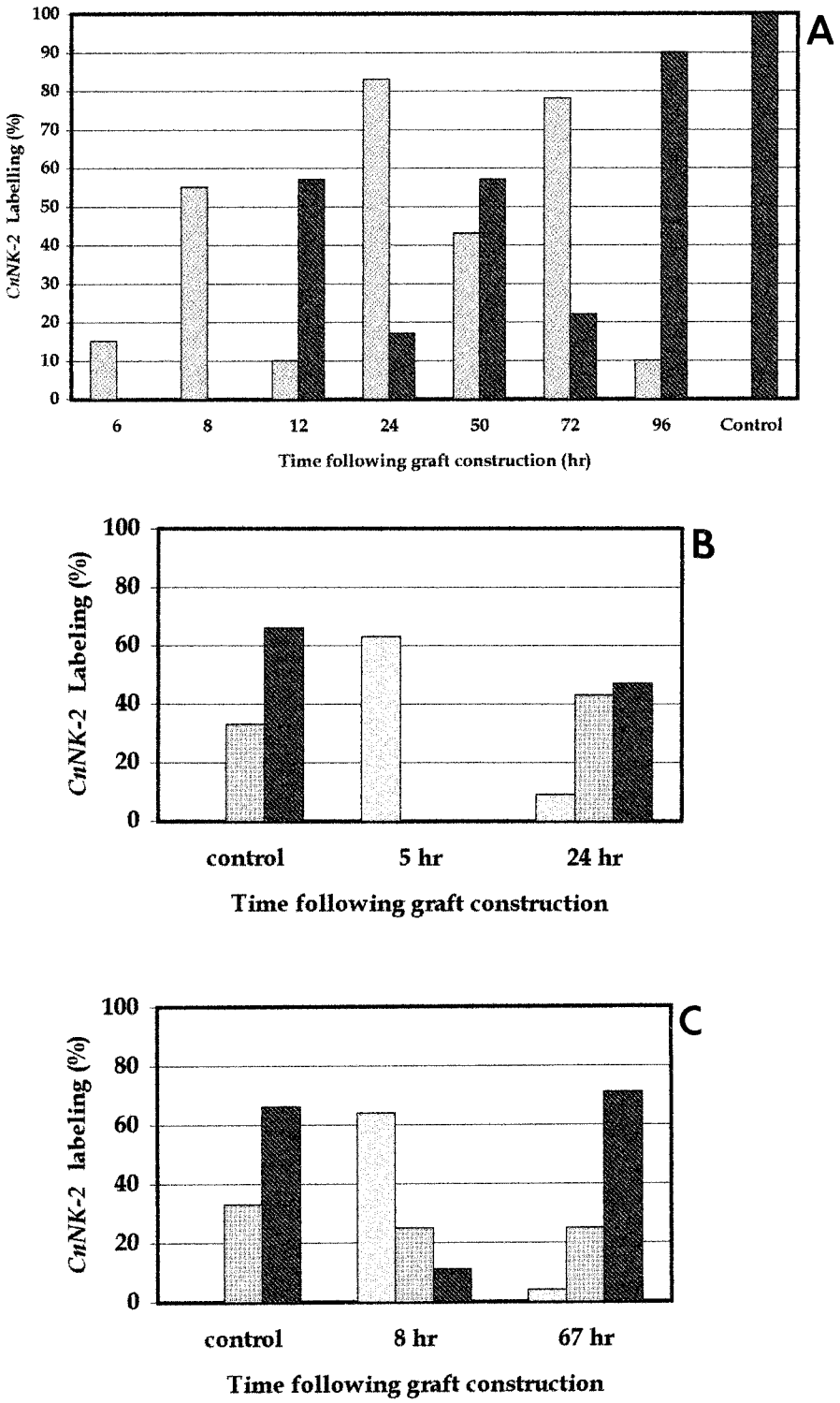
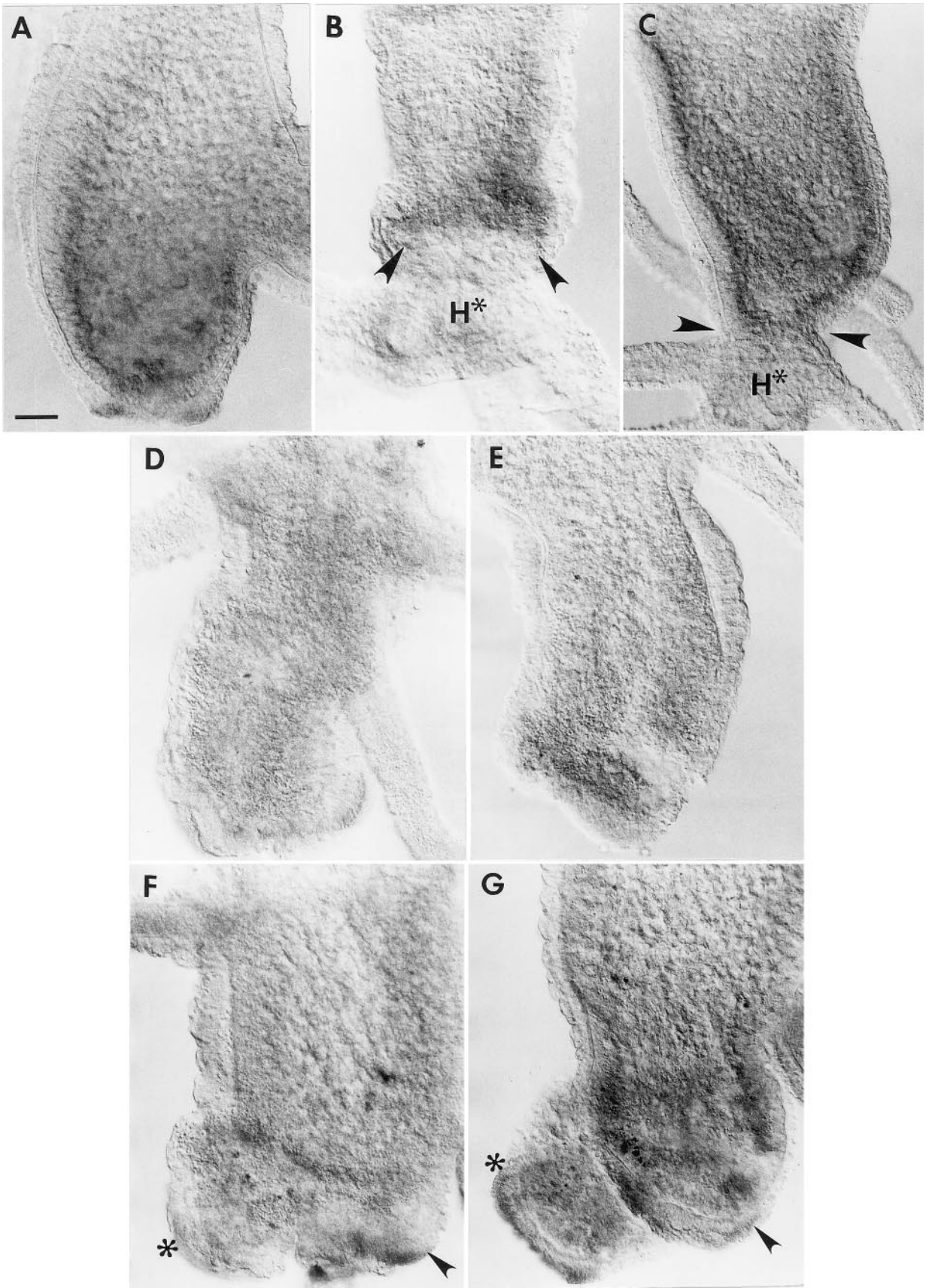


FIG. 7. Analysis of *CnNK-2* labeling in the graft constructs. (A) For H12/H* constructs, labeling was defined as extending throughout the body column (lighter bars) or localized to the 2-region (dark bars). Control animals uniformly had label localized to the peduncle. For each time point, $N = 23–29$. Labeling in H1/PF (B) and $(PF)^2$ (C) constructs was arbitrarily assigned to categories of light, medium, or heavy as indicated by the shading of the bars. For each time point, $N = 22–27$ in (B) and $N = 28$ in (C).



The close proximity of the head to the foot after axial grafting initially down-regulated basal gene expression, but it recovered to control levels within 24 h. Basal positional value in the H1/PF animals was evaluated using *CnNK-2* and expression changed dramatically. Animals were fixed at early, intermediate, and late time points following graft construction, hybridized with *CnNK-2,* and compared to control animals. Five hours after graft construction, roughly 60% of the animals displayed some light label in the peduncle-foot tissue while the majority of the control animals displayed a heavy label (Figs. 7B and 8D). Twenty-four hours after graft construction, *CnNK-2* label in the H1/PF animals was similar to the label seen in controls (Figs. 7B and 8E). This suggested that there was an initial down-regulation of *CnNK-2* expression during the first 5 h following graft construction. To determine whether the early loss of *CnNK-2* expression was due to an injury effect, sham grafts were hybridized with *CnNK-2* 5 h after construction. The sham grafts displayed *CnNK-2* labeling at control levels. Therefore, the initial down-regulation of *CnNK-2* in the H1/PF construct was not due to the injury incurred during graft construction.

Basal positional value in H1234B(PF)^2 animals initially declined, but then returned to normal. The morphology of the H1234B(PF)^2 animals was stable for more than a week. The animals were examined daily to ensure that the graft was not resorbed or lost. Only animals that maintained the (PF)^2 morphology with two functional basal discs (inverted Y-shape) were used for further analysis.

Since apical changes in PV were maximal 8 and 67 h following graft construction, *CnNK-2* expression was examined at 8 and 67 h also. Eight hours following graft construction, *CnNK-2* label was greatly reduced compared with control animals. The majority of control animals displayed heavy label in the PF tissue, whereas the majority of graft constructs displayed only light label in both host and donor PF tissue (Figs. 7C and 8F). This loss of gene expression was not due to injury incurred during grafting as sham control animals at 8 h displayed *CnNK-2* labeling levels equivalent to those of control animals.

Sixty-seven hours following graft construction, *CnNK-2* label was similar to that in control animals. *CnNK-2* was present in both the host and the grafted tissue and the intensity of label was similar in both tissues (Figs. 7C and 8G).

DISCUSSION

The classical approach to studying pattern formation in hydra has involved perturbing the normal pattern by removing differentiated structures or grafting donor tissue onto host animals. These manipulations result in the regeneration or induction of ectopic heads or feet (e.g., Browne, 1909; Wilby and Webster, 1970; Shostak, 1972; Wolpert *et al.,* 1972). The analysis of many different such experiments revealed the presence of developmental gradients of head and foot formation and inhibition, supporting the hypothesis that positional information might be maintained by gradients of diffusible substances emanating from the head and/or foot (Wolpert *et al.,* 1972; MacWilliams, 1983a, b). Theoretical models of pattern formation based on diffusion and gradients were formulated to describe patterning events (Gierer and Meinhardt, 1972; MacWilliams, 1982; Meinhardt, 1982; Müller, 1995; Sherratt *et al.,* 1995). While these models are mathematically complex, they describe simple, isolated events such as head or foot formation. Much of the experimental work has continued to focus on the induction of individual structures such as the head, foot, or bud. With the advent of more numerous markers for specific body regions, hydra is now a good model system for investigating more global aspects of pattern events.

Very little is understood about how the head, foot, and bud patterning systems interact. The head and bud patterning systems have been demonstrated to cross-react as the presence of heads and that of buds mutually inhibit each other (Shostak, 1974). Activating and inhibiting substances isolated from hydra that affect the head also affect budding (Schaller, 1973; Berking and Gierer, 1977). Most recently, the presence of an intact foot patterning system was demonstrated to be necessary for budding (Schiliro *et al.,* in press). Influences of the head patterning system on the foot also have been documented. Several experimental approaches have demonstrated that the presence of a head increases the rate of basal differentiation (Ando *et al.,* 1989; Müller, 1990; Javois and Frazier, 1991; Grens *et al.,* 1996). Newman (1974) demonstrated a close-range interaction when he simultaneously grafted apical and basal tissues to the same host site and found that the patterning systems interacted and inhibited one another. However, effects of the foot patterning system on the head have not been observed. Clearly there are long-range interactions between the head–bud and head–foot patterning systems, which

FIG. 8. *CnNK-2* labeling in the graft constructs. (A) Control budding adult illustrating *CnNK-2* labeling restricted to the peduncle of the adult. (B) H12/H* construct illustrating localized *CnNK-2* labeling in the 2-region near the grafted head (H*) at 24 h; arrows indicate graft junction. (C) H12/H* construct illustrating more generalized *CnNK-2* labeling extending from the 2-region near the grafted head (H*) toward the 1-region at 72 h; arrows indicate graft junction. (D) Loss of *CnNK-2* labeling in the H1/PF construct 5 h following graft construction. (E) Return of *CnNK-2* labeling in the H1/PF construct 24 h following graft construction. (F) Decreased *CnNK-2* labeling in grafted (asterisk) and host (arrow) peduncles of the (PF)^2 construct 8 h following grafting. (F) Return of labeling to the grafted (asterisk) and host (arrow) peduncles of the (PF)^2 construct 67 h following grafting. Scale bar, 100 μm.

emphasizes the global nature of patterning events. This study was undertaken to further investigate foot–head interactions.

Basal Influences on Apical Positional Values

Results of experiments presented here demonstrate two novel findings: manipulating the foot patterning system can alter apical pattern events and the PV gradient can undergo rapid changes. Rapid changes are arbitrarily defined as those occurring within the first 10 h following the manipulation while more long-lasting effects are defined as those occurring after 20 h. All three manipulations in this study resulted in a rapid change in the ability of the apical-most gastric tissue to regenerate a head. When a foot was induced to form in close proximity to the head, head regeneration was abolished, while moving basal tissue closer to the head or doubling the basal tissue resulted in a slower rate of head regeneration. Since rapid rates of head regeneration are correlated with high apical PVs, these results suggest that PVs of the upper gastric region were lowered. Lowering of apical PVs proved to be a long-lasting effect following foot induction or moving of a foot closer to the head. However, the effect was more transient following the doubling of basal tissue as head regeneration rates increased dramatically over the next 50 h to more than twice that of controls. Here the long-term effect was apparently an increase in apical PVs. Previous transplantation data (MacWilliams, 1983a, b) evaluated PV changes in animals with nearly normal proportions and documented relatively slow rates of change. From this study, it can be concluded that when grossly misproportioned constructs are made, tissue can undergo PV changes much more rapidly.

The *ks1* gene is expressed in apical tissue at the base of the tentacles and intertentacle zone. The down-regulation of *ks1* expression normally observed in the gastric region has been explained by the presence of inhibiting nuclear protein factors specific for the 1.5-kb *cis*-regulatory sequence of *ks1*. Endl *et al.* (1999) have shown that the amount and complexity of nuclear proteins from lower gastric tissue bound to the *ks1* regulatory elements are much greater than those from the 1-region (tentacle formation zone; Hobmeyer *et al.*, 1990) or head tissues. Nothing is known about the function of the *ks1* gene product.

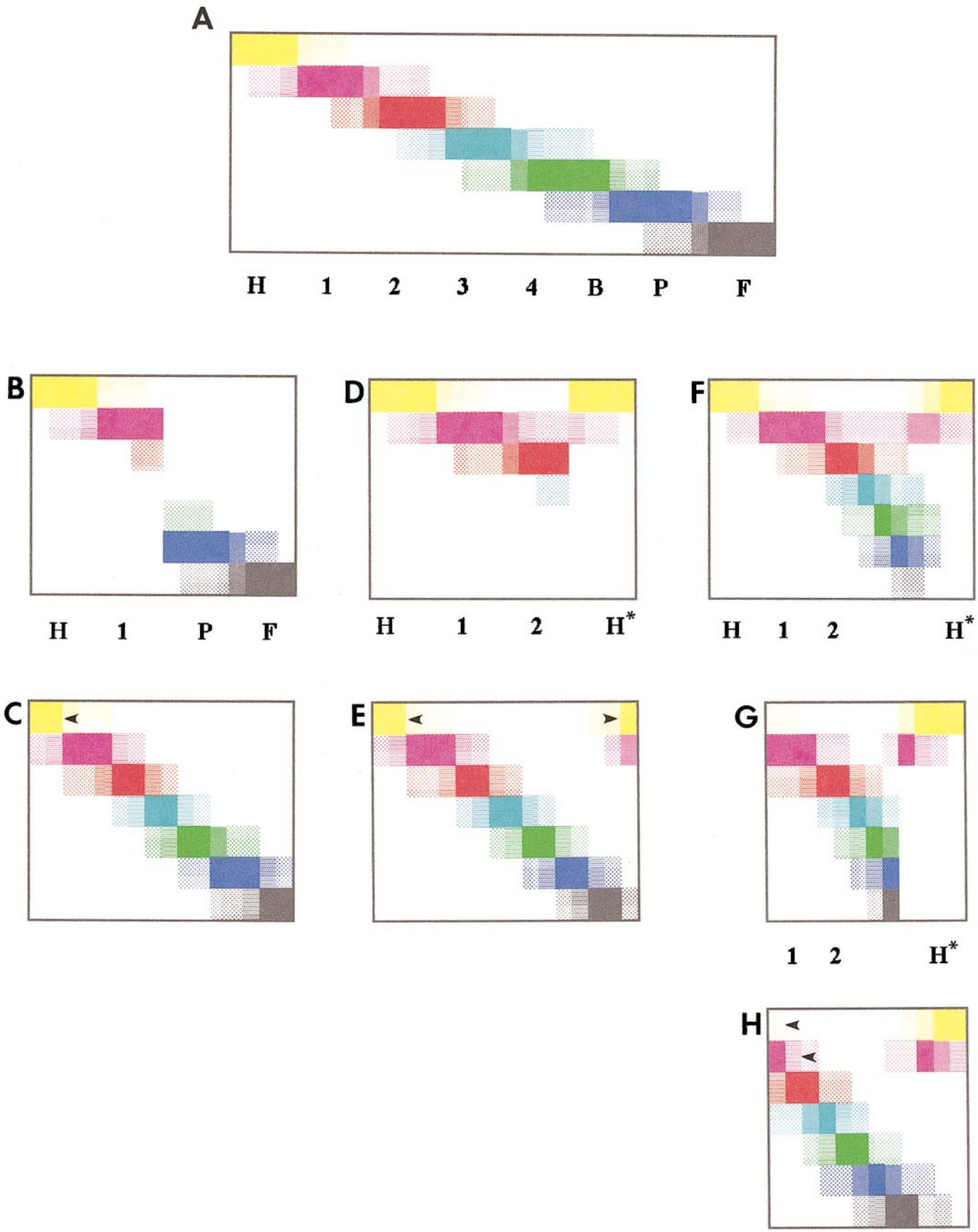
Ks1 expression responded rapidly to manipulations of basal tissue. The initial up-regulation of *ks1* expression may have been due to the loss of inhibitory proteins normally present in gastric tissue that was removed in making the H12/H* and H1/PF constructs. The long-term loss of apical PVs did correlate with the ultimate loss of gene expression. This would be consistent with an increased production of inhibitory proteins, as PVs decreased during regulation and reproportioning. Since the function of the *ks1* gene product is not known, it is hard to speculate on the long-term significance of lost *ks1* expression in intact heads.

In the presence of double the normal basal tissue and an intervening gastric region, there was a rapid, moderate down-regulation of *ks1* expression that correlated with a slower rate of head regeneration and loss of apical PVs. However, both of these effects were transient and perhaps reflected increased production of transcriptional inhibitors of *ks1* gene expression following the doubling of basal tissues. In the long-term, apical regeneration rate increased and was accompanied by an up-regulation of *ks1* expression, reflecting a loss of transcriptional inhibition consistent with tissue of higher apical PVs. With this construct, the long-term increase in apical PVs over many hours followed by the return to normal values might reflect the reproportioning known to occur in hydra (Bode and Bode, 1980). This is further evidenced by the fact that eventually the grafted PF is displaced basally and lost.

Apical Influences on the Basal Patterning System

The experimental results reported here confirm and extend to the molecular level the ability of the head to influence the foot patterning system. When two heads were placed in very close proximity, rapid induction of a foot occurred between the two heads. Basal gene expression (*CnNK-2*) was induced. Basal disc formation was influenced by the continued presence of the host head as its removal

FIG. 9. Computer simulation of the changes in the source density gradient following construction of the H1/PF and H12/H* axial grafts using the model of Meinhardt (1998), modified as described in the text. (A) The steady-state apical to basal distribution of regions of gene expression reflecting the underlying source density gradient. The apical to basal sequence of structures (H1234BPF) have been arbitrarily assigned to regions of gene expression. (B) Regions of gene expression reflecting the underlying source density gradient in the H1/PF graft at the time of construction. (C) The steady-state apical to basal distribution of regions of gene expression achieved after 6300 iterations of the program. Note that while the apical to basal pattern is reestablished, the range of expression for each gene is severely restricted compared to the normal animal (e.g., arrow indicating reduced apical-most gene expression). (D) Regions of gene expression reflecting the underlying source density gradient in the H12/H* graft at the time of construction. (E) The steady-state apical to basal distribution of regions of gene expression achieved after 6300 interactions of the program. Note the reduction in apical-most gene expression in both H and H* (arrows). Assuming that the complete simulation is equivalent to the first 24 h following graft construction, then the 3- to 4-h point following graft construction would occur following 900 iterations as illustrated in (F). The host head was removed at this point (G) and the simulation allowed to run to completion (H). At the steady state, note that the apical-most gene is no longer expressed and the next most apical gene has a severely restricted range of expression correlating with a loss of head regeneration (H, arrows). Abbreviations as in Fig. 1.



during the first 24 h prevented morphological foot differentiation. These findings are in agreement with earlier studies establishing the ability of the head to increase the rate of basal differentiation.

When basal tissue was physically moved closer to the head, *CnNK-2* gene expression was down-regulated rapidly. The same phenomenon was observe in animals with double the normal basal tissue. In both cases this response was transient and *CnNK-2* expression returned to normal. Both findings suggest that the head has an inhibitory influence on the foot patterning system. These apparently contradictory influences of the head on basal patterning events may, in part, be explained by the circumstances underlying the constructs. The presence of a head can positively influence the induction of a new foot; however, in cases where a foot already existed, manipulating the relationship between the foot and the head can have a negative impact on ongoing basal patterning processes.

Apical and Basal Patterning Systems Maintain the Positional Value Gradient

Since the proximity of the foot alters apical PVs and the proximity of the head alters basal PVs, potentially, both organizing centers of the animal play an active role in maintaining the slope of the PV gradient from head to foot. The head would maintain the high end of the gradient while the foot end would maintain the low end. Manipulations that alter the position of the head relative to the foot would be expected to alter the slope of the PV gradient. For example, if this distance were decreased, one would expect the slope of the gradient to be steeper in the resulting animal. In addition, the regions with apical and basal PVs would be reduced or compressed. Apically, this would correlate with a loss of head regeneration ability and a decreased in *ks1* gene expression due to increased production of transcription inhibitors. Basally, this model would predict reduced or more restricted *CnNK-2* expression. Experimentally, both moving apical tissue closer to the PF and doubling the PF resulted in a rapid down-regulating of *CnNK-2*. While the details of *CnNK-2* transcriptional regulation are not known, the removal of gastric tissue during H1/PF construction may result in loss of the signal regulating activation of *CnNK-2*. Likewise, doubling the PF tissue relative to a single gastric region may have resulted in an insufficient signal for transcriptional activation of *CnNK-2* in the $(PF)^2$. In both situations, long-term regulation of the PV gradient would result in a return of *CnNK-2* transcription.

Computer Simulations Predict the Observed Changes in Positional Value Following Alteration of the Distance between the Head and the Foot

Turing's (1952) reaction-diffusion model is based on an autocatalytic activator molecule of short-range diffusion and an inhibitor molecule of long-range diffusion. The activator activates its own production and production of its inhibitor, while the inhibitor inhibits the activator. Gierer and Meinhardt (1972) applied Turing's reaction-diffusion model to patterning in hydra. They proposed that the head would act as an organizing center in hydra and be a source of its own activation and inhibition. The short-range diffusion of activator would result in a peak of activation in the head. Inhibition would also be highest in the head, but the long-range diffusion of inhibitor would keep the peak of inhibition lower than activation. Therefore, the head would by definition be that region where activator level was above the threshold level of inhibition.

Meinhardt's (1993) recent version of the reaction-diffusion model includes a source density gradient and interaction between the two ends of the hydra. He proposed that separate activator–inhibitor systems exist for each end of the animal and that these systems are coupled by a nondiffusible gradient of source density. The source is defined as a cellular property that is present in a graded fashion along the body column with the highest end of the gradient apically where head activation actively maintains a high source level. The source is lowest at the basal end of the animal where the foot acts as a sink to lower the source density gradient. Therefore, this version of the reaction-diffusion model proposes an active role for both the head and the foot in maintaining a graded property that can be equated with PV. Meinhardt's (1993) version of the reaction-diffusion model involves the use of three activator–inhibitor systems. A stable pattern is achieved because structures mutually activate but locally exclude one another. By extending the number of states and using six activator–inhibitor systems, source density may be represented by the activities of different genes directly without a primary gradient (Meinhardt, 1998; Fig. 9). In this case, the activation of a structure is particularly stable if the correct neighbors border it. During regeneration, the sequence of remaining structures leads to regeneration of the correct pattern. This model exhibits good size-regulation, although the experimentally observed influence of the head on foot patterning is not yet incorporated. As a working hypothesis, we have used this model to simulate the H12/H* and H1/PF axial graft constructs (Fig. 9). Simulating the lateral graft construct used to create the $(PF)^2$ animals would require using full cylindrical geometry, and such a model is not available.

Figure 9A illustrates the apical to basal array of hypothetical genes expressed in a steady-state hydra simulated by interactions among six activator–inhibitor systems (Meinhardt, 1998; see GT12-6c for a version with four activator–inhibitor systems; Meinhardt, personal communication, for a version with six activator–inhibitor systems). The apical to basal regions of the hydra have been arbitrarily assigned to correspond to various genes by dividing the field into eight roughly equal regions. Figure 9B illustrates the H1/PF graft at the time of construction, and Fig. 9C illustrates the pattern of gene expression following completion of the simulation. While the steady-state apical

to basal pattern of gene expression was restored, note that the regions of gene expression were compressed proportionally in the smaller animal. Monitoring *ks1* and *CnNK-2* gene expression in these constructs revealed that both genes were down-regulated as a result of placing apical and basal tissue in close proximity to each other.

When the H12/H* construct was simulated (Fig. 9D), basal gene expression was rapidly induced in the 2-region near the graft border (Fig. 9F). When the simulation was run to completion, the complete apical to basal pattern of gene expression was evident within the former H12-region, and notably, apical gene expression was restricted to a much smaller area (Fig. 9E). These events correlated with the observed rapid induction of *CnNK-2* gene expression and foot differentiation localized to the former 2-region, as well as the down-regulation of *ks1* gene expression in both the host and the grafted heads.

Removal of the host head from the H12/H* construct as soon as 3 h after graft construction resulted in the lack of host head regeneration, suggesting the 1-region tissue no longer possessed high enough apical PVs to sustain head regeneration. This interpretation was supported by computer simulations (Fig. 9F–9H). The initial simulation was stopped after 900 interactions (Fig. 9F), the host head was removed (Fig. 9G), and the simulation was allowed to run to completion (Fig. 9H). The final pattern of apical to basal gene expression patterns revealed a dramatic loss of apical-most gene expression, with a reduction in expression of the next most apical gene, too (cf. Figs. 9F and 9H). Loss of tissue with these most apical PVs would equate with loss of head regeneration.

While it is not yet possible to simulate the lateral graft construct used to create the $(PF)^2$ animals, what has been learned from the axial grafting computer simulations may be applied to this situation. The immediate effect of doubling the PF tissue was a loss of head regeneration ability and down-regulation of *ks1* and *CnNK-2* gene expression. These observations are compatible with the rapid propagation and changes in gene expression from the $(PF)^2$ toward the head and the subsequent readjustment of the PV gradient. The observed apical and basal changes are compatible with a steeper gradient. These constructs appeared to continue regulating and reproportioning over the next several days as head regeneration rate increased before returning to normal. Gene expression also returned to normal. These events correlated with the gradual basal displacement of the grafted PF. Eventually, the graft PF will be displaced basally and lost, resulting in a correctly proportioned animal.

The only discrepancy observed between the simulations and the experiments had to do with the initial down-regulation of *CnNK-2* gene expression in the H1/PF constructs before the expression pattern stabilized at normal levels. This change in gene expression was not a result of the injury incurred during graft construction. Therefore, these changes must reflect a response to the loss of intervening gastric tissue and/or the proximity of the head even though the current version of the model does not confirm this observation. With regard to the increased apical *ks1* expression in the H1/PF and H12/H* constructs, labeling intensified up into the tentacles. The simulations consider patterning only along the main body axis and do not incorporate tentacle patterning. Therefore, the model cannot be expected to describe this phenomenon.

Computer simulations using reaction-diffusion mechanisms to establish positional information reflected the observed changes in gene expression, head regeneration, and foot formation to a large degree. This, in turn, supports the hypothesis that a mechanism involving multiple activator–inhibitor systems that mutually activate and locally exclude one another may underlie the patterning events. The evidence also suggests that any theoretical model used to describe these patterning events should include a role for the foot in pattern regulation of hydra.

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