NK-2-class homeobox genes have been identified in a variety of metazoans, from sponges to arthropods and vertebrates, and have been shown to play roles in a variety of cell and tissue specifications. Here we describe the characterization of the NK-2 homolog CnNK-2 from Hydra vulgaris, a freshwater cnidarian. CnNK-2 expression is restricted to the endodermal epithelial cells of hydra and is primarily in the peduncle, the lower end of the body column. In some species it is graded along the apical–basal axis with a maximum in the basal tissue of the lower peduncle, adjacent to the foot. CnNK-2 expression invariably precedes foot formation as part of the normal tissue dynamics of the adult as well as during asexual reproduction by budding, foot regeneration, or ectopic foot formation. Manipulations which alter the gradient of positional value along this axis affect CnNK-2 expression in a manner which indicates that expression of this gene is closely linked to the gradient. The normal and altered patterns of expression of this gene extend the understanding of the regulation of foot formation in hydra.

INTRODUCTION

Axis formation and the specification of body regions continue to be central issues in the elucidation of the patterning processes governing the development of an organism. Accumulating information indicates that the same molecules and mechanisms are used for the patterning processes in animals of very different phyla. This raises the important question as to the extent which they have been conserved throughout evolution. Hydra is a member of the Cnidaria, a group of animals that arose early in metazoan evolution. Should hydra use many of the same molecules and mechanisms for these processes that animals do which arose later in evolution use, this would provide support for their early origin and conservation. There is already some evidence that this is so.

Several homeobox genes have been isolated from hydra and other coelenterates (Schierwater et al., 1991; Murtha et al., 1991; Schummer et al., 1992; Naito et al., 1993; Shenk et al., 1993a; Kuhn et al., 1996). The expression patterns of some of these genes indicate that they play roles in patterning along the apicobasal axis. Hydra is radially symmetrical and thus has only one axis. The main structures to be patterned are the head at the apical end consisting of hypostome and tentacles, the body column, and the basal disk or foot (Fig. 1). Two Hox genes, Cnnox-2 and Cnnox-3, appear to be involved in specifying body column and tentacle-forming tissue, respectively (Shenk et al., 1993a,b, in preparation). A gene of the forkhead family, Budhead, is expressed in the hypostome (Martinez, D., et al., submitted). The subset of forkhead genes to which it belongs is expressed in organizer regions of vertebrate embryos such as the dorsal lip of amphibian embryos (Lai et al., 1993; Kaufmann and Knochel, 1996). Similarly, the hypostome in hydra is a region with organizing capacity. Perhaps the best example of such conservation is CnASH, the hydra homolog of the achaete–scute family of basic-helix-loop-helix transcription factors (Grens et al., 1995). CnASH protein will form heterodimers with Drosophila Daughterless, the normal dimerization partner for the fly Achaete and Scute proteins, and these dimers recognize and bind to the appropriate DNA targets in a sequence-specific manner. When the CnASH gene is introduced into Drosophila under the control of a Drosophila hsp70 promoter, the hydra homolog exhibits the same effects of ectopic expression as do those of the achaete–scute family of this organ-
ism (Rodriguez et al., 1990; Brand et al., 1993; Dominguez and Campuzano, 1993; Hinz et al., 1994). The CnASH gene will also rescue Drosophila mutants with defective achaete and scute genes.

Normally axis formation and specification of regions occur only during embryogenesis. In hydra these patterning processes also occur in three other circumstances. One is in the adult. The tissues of a hydra are in a steady state of production and loss of cells (Otto and Campbell, 1977a). Cells of the two epithelial layers of the body column, the ectoderm and endoderm (Fig. 1), are continuously in the mitotic cycle (Campbell, 1967a; David and Campbell, 1972). As new cells are added, the tissue is displaced apically into the head or basally toward the foot (Campbell, 1967b). To maintain the size of the animal, cells are lost by sloughing at either extremity or by displacement into developing buds. Because most body column cells ultimately change their identity to either head cells or foot cells, patterning processes must be continuously operating to provide them with positional information as to their fates. A second circumstance which involves patterning is bud formation, hydra's asexual form of reproduction. Here an evagination of the body column elongates into a protrusion and develops a head and foot at the ends. The third circumstance is regeneration. Bisection of the animal leads to the regeneration of the missing extremity on each half. Also, isolation of a piece of the body column results in the regeneration of the head at the original apical end and a foot at the original basal end. The polarity is provided by a gradient of positional value.

Studies at the cell and tissue level indicate an overlapping, but not necessarily congruent set of patterning processes for each of the three situations (e.g., Ando et al., 1989). Hence, it is of interest to determine to what extent the molecular basis for axis formation and specification of regions is similar or different among them.

Here we describe a hydra homolog of the NK-2 class of homeobox genes, CnNK-2, whose expression pattern suggests that it is involved in the specification of tissue to become a foot. It is expressed in the endodermal epithelial cells, primarily in the peduncle, the basal portion of the body column (Fig. 1). Manipulations of body tissue show that CnNK-2 expression always precedes foot formation in ectopic locations, as well as during budding and foot regeneration. Treatments which alter the gradient of positional value also alter the axial extent of CnNK-2 expression. Observations on the initiation, maintenance, and repression of CnNK-2 expression provide insight into the controls involved in the formation and maintenance of the basal end of the hydra axis.

**MATERIALS AND METHODS**

**General Molecular Biology Procedures**

Molecular biology procedures not detailed below were carried out by standard procedures as described in Sambrook et al. (1989). The cDNA library was constructed by Stratagene (La Jolla, CA) using poly(A)+ RNA isolated from adult Hydra vulgaris (UCI strain), as described in Sarras et al. (1994). Southern blots were prepared and probed for CnNK-2 by standard procedures (Sambrook et al., 1989) using the cDNA shown in Fig. 2A as a probe. Blots were washed at 50°C in 0.5 \( \times \) SSC (75 mM NaCl, 7.5 mM Na-citrate), 0.1% SDS (sodium dodecyl sulfate), a moderate stringency.
| A | TGGATACCTGCACCTGGGACTCTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGAC

| B | CnNK-2 (Hydra) | L Y E A H I P H Y | RKKPRFLFLSQQSFQMFLGKKFDQYLSEREDQIANKLNLTPQVKHPWNQNYKCKKQT

| Nkx2.5 (mouse) | -R---V-----A--Y--ERR--Q-R--P----E-LV-K-S--R-----------R-----R-R 70%
| Dth-2 (Planaria) | -R---V-----A--I--ERR--Q-----P--EHL-LI--H--------H-----R-AH 68%
| Nkx2.3 (mouse) | -R---V-----A--F--ERR--Q-----P--EHL-SS--K-R----R--------R-R 67%
| Nkx2.4 (mouse) | -R---V-----A--Y--ERR--Q-----P--EHL-SM--H--------H-----R-AH 65%
| TTF-1/Nkx2.1 (rat) | -R---V-----A--Y--ERR--Q-----P--EHL-SM--H--------H-----R-AH 63%
| Lox 10 (leech) | -R---V-----A--F--ERR--RQQ-----P--EHL-TFG--H--------H-----T-SK 63%
| NK-4/tin (Drosophila) | KR---V-----A--L--ERC-LR-----TGA-IE--Q---SA----------R-----S-RGD 62%
| Nkx2.6 (mouse) | QR-S-V-----A--LA--ERR--Q-R--T-P--EHL-SA-Q--S----------R-----S-R 60%
| prox-1 (sponge) | KRR-A-----A--HA--Y--ERR-AV--T-H-QSKL-TV--E----------R-----R-S-R 56%
| ceh-22 (C. elegans) | KR-R-V-----TAA-TY--ERR-RS-----P--EAL-NQIR----------H-----T-SH 57%
| Dth-1 (Planaria) | KR-V-----XX-LIL--ERR-RK-----P--EHL-LG--S----------H-----R-AH 55%
| XenK2 (Xenopus) | KR-V-----XX-KA--ERR-RQ-----P--EHL-SLIR----------H-----R-RAR 55%
| Nkx2.2 (mouse) | KR-V-----XX-TA--ERR-RQ-----P--EHL-SLIR----------H-----R-RAR 55%
| NK-2 (Drosophila) | KR-V-----XX-TA--ERR-RQ-----P--EHL-SLIR----------H-----R-RAR 55%
Hydra Culture

Several species and strains of hydra were used in the work described here: Hydra vulgaris, Basel strain (obtained from T. Holstein), H. vulgaris, Zurich strain L2 (T. Sugiyama), Hydra magnipapillata, strain 105 (T. Sugiyama), and Hydra oligactis, England strain (L. Javols). Hydrea were grown in hydra medium (HM), which consisted of either 1 mM CaCl₂, 1.5 mM NaHCO₃, 0.1 mM MgCl₂, 0.08 mM MgSO₄, and 0.03 mM KNO₃, or 1 mM CaCl₂ in Arrowhead spring water. The medium was changed daily, and animals were fed three times per week with larvae of Artemia salina. All strains were cultured in the same manner. Except where otherwise noted, the animals used were the Basel strain of H. vulgaris. When distance is indicated as percentage of body column length, animals were measured from directly below the tentacles (0%) to the base of the foot (100%). For all experiments, 20–25 animals were used per sample, per time point, or per concentration.

LiCl, Diacylglycerol, and Hydroxyurea Treatments

For LiCl treatment, H. vulgaris (Zurich strain L2) were grown in HM continuously supplemented with 0.5 mM LiCl, which was changed daily. For diacylglycerol (DAG) treatments, H. magnipapillata (strain 105) were treated on a daily basis with freshly sonicated 0.1 mM 1,2 dioctanoyl-sn-glycerol, a diacylglycerol, and 0.1 mM arachidonic acid (AA) in HM as described by Muller et al. (1993). Animals were treated with 3 ml of DAG/AA solution in a 60-mm petri dish. On the first day of treatment animals were exposed to DAG and AA for 30 min, and on all subsequent days they were exposed for 2 hr.

In Situ Hybridization and Detection

Digoxigenin-labeled RNA probes corresponding to the sense and antisense strands of the CnNK-2 cDNA shown in Fig. 2A were prepared using the Boehringer-Mannheim RNA labeling kit for in vitro transcriptions. Methanol and allowed to destain overnight in methanol. For LiCl treatment, solution was made of 0.08 M NaCl, 0.1 M Tris, pH 9.5, 0.01 M MgCl₂, 0.1% Tween-20 in 3 ml of DAG/AA solution in a 60-mm petri dish. On the first day of treatment animals were exposed to DAG and AA for 30 min, and on all subsequent days they were exposed for 2 hr.

RESULTS

Characterization of the Hydra NK-2 Homolog

A H. vulgaris (UCI strain) cDNA library was screened with a fully degenerate oligonucleotide encoding the amino acid sequence KIWF(Q/K)NRR, the highly conserved amino acid sequence in the third helix of a wide variety of homeobox proteins. Among the clones obtained was one containing a partial cDNA of a hydra NK-2 homolog, CnNK-2. Subsequent library screening yielded four additional CnNK-2 cDNAs, one of which was the full-length clone whose sequence is shown in Fig. 2A. The cDNA encodes a protein of 328 amino acids, with a predicted molecular weight of 38.3 kDa. All the clones had identical sequence in the regions of overlap, and Southern analysis indicated that there is only a single gene of this class in hydra (data not shown).
Three aspects of the sequence indicate that CnNK-2 is a member of the NK-2 class of homeobox genes. (1) The homeodomain of the hydra CnNK-2 gene product has significant amino acid sequence identity with other members of the NK-2 class (Fig. 2B). The hydra NK-2 protein is 50-70% identical with other NK-2 proteins across the region. The extent of identity of the homeodomain with other classes of homeobox proteins is less than 50%. (2) Within the homeodomain there are 9 amino acid residues which are considered indicative of the NK-2 class (indicated in Fig. 1B). The hydra CnNK-2 homeodomain contains 4 of these 9 amino acids and two conservative substitutions. Further, 1 of the 4 is the Y at amino acid 54 of the homeodomain which is unique to the NK-2 class. (3) Two other motifs in CnNK-2 are diagnostic of the NK-2 class. In vertebrate NK-2 proteins there is a conserved decapptide near the N-terminus (Guazzi et al., 1990; Price et al., 1992; Lints, et al., 1993; Saha et al., 1993; Tonissen et al., 1994); the hydra NK-2 homolog shares 5 of the 10 conserved residues (indicated in Fig. 2A). The other motif referred to as the conserved peptide is a 17-amino-acid sequence on the C-terminal side of the homeodomain found in the NK-2 gene of Drosophila and vertebrate homologs (Price et al., 1992). CnNK-2 contains 6 of the 7 amino acids in the core of this motif (underlined in Fig. 2A).

Expression of CnNK-2 Is Endodermal and Located Mainly in the Peduncle

As shown in Fig. 1, the tissue of the animal consists of two epithelial layers, the ectoderm and the endoderm, separated by a basement membrane. In situ hybridization on whole mounts using probes specific for hydra CnNK-2 demonstrates that this gene is expressed in the endoderm and not in the ectoderm (Fig. 3). This was corroborated by RT-PCR analysis, which also showed expression of CnNK-2 to be restricted to the endodermal cells and not expressed in the ectodermal cells (data not shown).

In situ hybridization on whole mounts of three species of hydra shows CnNK-2 expression to be strongest in the peduncle (Fig. 3). The kinetics of the staining reaction reveal a somewhat graded distribution. Staining first appears in the lower half of the peduncle adjacent to the foot. With longer staining times the stain in the lower peduncle intensifies and spreads to the upper peduncle. However, as seen in Fig. 3, the degree to which expression extends beyond the peduncle into the body column differs among the three species examined. After prolonged staining, expression in H. vulgaris is detectable throughout the entire budding zone and then fades out in the basal part of the gastric region (Fig. 3A). Staining was never observed in the upper part of the body column or head. A similar graded pattern of expression for H. vulgaris was observed when CnNK-2 RNA was assayed by RT-PCR (data not shown). In H. magnipapillata expression above the peduncle decreases rapidly, extending only about a quarter of the way into the budding zone (Fig. 3B; the light staining is associated with the bud, as described below). H. oligactis, which has a longer peduncle than the other two species, shows expression which is restricted to the peduncle (Fig. 3C). In all species the stain lightens and vanishes in the foot, indicating that CnNK-2 expression ceases as peduncle cells become foot cells (Fig. 3D).

CnNK-2 Expression Is Correlated with Foot Regeneration

If the animal is bisected at almost any point along the body column, the apical piece containing the head will regenerate a foot at its basal end. This provides an opportunity to examine the relationship between expression of CnNK-2 and the formation of a foot at novel locations. Foot regeneration was examined at different axial levels in all three species for which the CnNK-2 in situ hybridization pattern had been determined. Animals were bisected at one-third or two-thirds body length or in midpeduncle. Foot regeneration occurred at all three levels in both H. vulgaris and H. magnipapillata (Figs. 4A and 4B). The kinetics were similar, and as previously described (Mookerjee and Bhattacharjee, 1967), the rate of regeneration increased the lower the axial level of bisection. In contrast, foot regeneration in H. oligactis was restricted to animals bisected in the peduncle (Fig. 4C). H. oligactis bisected at more apical levels failed to regenerate feet. Further, the rate of regeneration in the midpeduncle was significantly slower than in the other two species.

The pattern of CnNK-2 expression was examined during foot regeneration at all three axial levels in two of the species. Variations were found that correlated closely with the ability to form a foot as well as with the rate of foot formation. In H. vulgaris CnNK-2 expression preceded morphological foot formation at each level (Figs. 5A–5D). The initial appearance of the gene was sooner in animals bisected in the midpeduncle compared to animals bisected at more

FIG. 3. Regional pattern of expression of CnNK-2 in whole mounts of three species of hydra as indicated by in situ hybridization. (A) Basal strain of H. vulgaris, (B) 105 strain of H. magnipapillata, (C) England strain of H. oligactis. Magnification, 10×. (D) Pattern of expression of the gene in the foot of H. vulgaris. Magnification, 120×.

FIG. 5. Changes in CnNK-2 expression during foot regeneration in H. vulgaris of animals bisected at one-third body length (A–D) and at midpeduncle (E–H). Samples were stained (A) immediately following bisection, (B) after 2 days of foot regeneration, (C) after 3 days, and (D) after 5 days when a foot had regenerated. For animals bisected in the midpeduncle, samples were analyzed (E) immediately following bisection and 6 hr, (G) 12 hr, and (H) 24 hr after removal of the lower peduncle and foot. Magnification of all panels, 25×.
apical axial levels (Fig. 6). In addition, the pattern of recovery of CnNK-2 staining was the same at all three levels. CnNK-2 expression was first detectable in the most basal portion of the regenerating tissue between 12 and 24 hr (Figs. 5B and 6). With longer regeneration times, CnNK-2 staining became more intense in the most basal tissue and the apical border of expression moved up the body column (Fig. 5C) until a proportionally normal extent of CnNK-2 expression was achieved (Fig. 5D). A morphologically identifiable foot had regenerated by 7 days in most cases.

Just as the foot regeneration behavior of _H. oligactis_ differed from that of _H. vulgaris_, so did the patterns of CnNK-2 expression. _H. oligactis_ bisected at midpeduncle regenerate feet more slowly than do _H. vulgaris_. Similarly, CnNK-2 staining became more intense in the most basal tissue and the apical border of expression moved up the body column (Fig. 5C) until a proportionally normal extent of CnNK-2 expression first appeared about 3 days after bisection in _H. oligactis_ compared to 12–24 hr in _H. vulgaris_. In animals

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**FIG. 4.** Rate of foot regeneration at three axial levels (one-third body column length, two-thirds body column length, and midpeduncle) in three species of hydra.

**FIG. 9.** CnNK-2 expression during ectopic foot formation in double-headed animals. (A, B) Double-headed _H. magnipapillata_ generated by treatment with DAG and excision of the upper body column. (A) An early stage after head regeneration and before ectopic foot formation has begun. (B) After foot formation has begun. (C) Early stages of foot formation in a double-headed _H. vulgaris_ generated by grafting. Magnification, 16×.

**FIG. 10.** Changes in the pattern of CnNK-2 expression during budding. Bud stages are as defined in Otto and Campbell (1977b). (A) Bud stage 3; tissue beginning to evaginate; (B) stage 4c; (C) stage 5c; (D) stage 5d; (E) stage 5e; (F) stage 6; (G) stage 7; and (H) stage 9, fully formed bud.

**FIG. 11.** Effect of LiCl treatment on CnNK-2 expression in Zurich L2 strain of _H. vulgaris_. Patterns of expression after (A) 0 days, (B) 5 days, (C) 10 days, and (D) 15 days of treatment.
FIG. 6. Kinetics of the development of CnNK-2 expression (solid symbols) and of foot regeneration (open symbols) in H. vulgaris following bisection. Animals were bisected at (A) one-third body column length, (B) two-thirds body column length, or (C) midpeduncle.

Bisected at one-third and two-thirds body length, no CnNK-2 expression was detected, although the animals were examined up to a week following bisection (Fig. 7 and other data not shown). Thus, in addition to the correlation of CnNK-2 expression with foot regeneration, we observed that tissue which does not express CnNK-2 does not regenerate foot structures.

Treatment of some species of hydra with LiCl can lead
FIG. 7. Effect of the treatment of LiCl on the kinetics of the development of CnNK-2 expression (solid symbols) and of foot regeneration (open symbols) in H. oligactis. Animals were bisected at two-thirds body length after pretreatment with LiCl (diamonds) or without pretreatment (circles).

to ectopic foot formation along the body column (Hassel and Berking, 1990). In the England strain of H. oligactis treatment with LiCl restores the ability to regenerate a foot above the peduncle to some extent. Following 15 days of LiCl treatment, CnNK-2 was detected in ~80% of the animals bisected at two-thirds body length, and ~40% had regenerated a foot within a week of bisection (Fig. 7). Neither CnNK-2 expression nor foot regeneration was observed in animals bisected at one-third body length, even after prolonged LiCl treatment. Again, the correlation was observed that tissue which induced CnNK-2 expression went on to regenerate a foot while tissue which did not express CnNK-2 also did not regenerate.

The Lower Peduncle and the Head Influence Expression of CnNK-2

The experiments presented above also provided data that the expression of CnNK-2 was influenced by the most basal regions of the animal. When H. vulgaris was bisected at one-third body length, no residual CnNK-2 expression was present in the apical portion which regenerated a foot (Figs. 5A and 6A). However, bisection at two-thirds body length and midpeduncle took place in tissue still expressing CnNK-2 (Figs. 5E and 6B and 6C). In these animals CnNK-2 RNA was transiently lost from expressing cells following the injury. This loss was relatively rapid, occurring within 3-12 hr following bisection (Figs. 5F and 6B and 6C). H. vulgaris bisected at midpeduncle also showed the same rapid loss of CnNK-2 RNA. These results suggested that CnNK-2 expression might be correlated with the presence of the existing foot. To test this idea, only the basal disk of H. vulgaris animals was removed, which left the peduncle largely intact. Unexpectedly, there was no decline in CnNK-2 expression (data not shown). From this we conclude that the effect on CnNK-2 expression is associated with the lower portion of the peduncle and is not due to the influence of the foot itself.

To determine whether the head influences expression of CnNK-2, animals were decapitated just below the tentacles. Decapitated animals were allowed to regenerate for up to 3 days and then analyzed for CnNK-2 expression. Samples were fixed for in situ hybridization at 2-hr intervals for the first 16 hr following decapitation and at half-day intervals thereafter. The extent and intensity of CnNK-2 staining remained unchanged throughout the period of analysis (data not shown). The absence of a change indicates that the head most likely has no direct effect on CnNK-2 expression in the presence of an intact peduncle and foot.

However, when animals were simultaneously decapitated and bisected at two-thirds body length, a distinct role for the head was observed. In animals which were bisected but not decapitated, the time course of the appearance of CnNK-2 transcripts was similar to that observed previously (Fig. 8, compare to Fig. 6B). In contrast, animals which were regenerating both a head and a foot showed a marked delay in the expression of CnNK-2 (Fig. 8). Thus, the presence of the head does affect the initiation of CnNK-2 expression while the foot is developing.

CnNK-2 Expression Is Correlated with Ectopic Foot Formation

In addition to regeneration at basal cut surfaces, animals can be manipulated so that a foot will form in locations other than at the basal end. We examined CnNK-2 expression in animals forming an ectopic foot under two conditions and again found that tissue which is forming a foot expresses CnNK-2, irrespective of position in the animal. Treatment with DAG will eventually result in the forma-
FIG. 8. Effect of the presence of the head on the kinetics of development of the CnNK-2 expression pattern during foot regeneration. H. vulgaris were bisected at two-thirds body column length. Half of the animals were also decapitated (solid symbols), while the other half were not (open symbols).

In H. vulgaris, CnNK-2 expression extends throughout the budding zone (Fig. 3A). Budding begins with an evagination of the adult body wall. This tissue, which will form the apical portion of the bud, ceases expression of CnNK-2 (Figs. 10A and 10B; stages 3 and 4, Otto and Campbell, 1977b). A short time later, as the bud elongates, a ring of expression appears in the parent body column (Fig. 10C; stage 5). As evagination continues, this tissue ring moves to the base of the bud (Fig. 10D) and gradually into it (Figs. 10E–10G; stages 6–8). As the head forms, the developing bud constricts at the basal end, and CnNK-2 is expressed mainly in the area which will become the peduncle. Eventually the basal disk forms and the pattern of CnNK-2 expression resembles that of the adult (Fig. 10H). The appearance of the ring of CnNK-2 expression suggests that tissue is already becoming patterned to form a peduncle and/or foot before leaving the adult body column.

Alteration of Positional Value Alters the Range of CnNK-2 Expression

Of the patterning processes in the adult hydra, a centrally important one is the positional value gradient, which is maximal in the head and decreases monotonically down the column (Bode and Bode, 1984a). High levels of positional value lead to head formation, while low levels lead to foot formation. As cells are displaced basally toward the foot, they will experience a reduction in positional value. At the same time they experience an increase in the level of CnNK-
FIG. 12. Changes in the apical border of CnNK-2 expression following treatment with (A) LiCl or (B) DAG for varying lengths of time. In (A), lightly shaded bars represent the extent of all detectable staining, while heavily shaded bars indicate the apical border of intense stain.

2 expression. This suggests that expression of the gene may be directly correlated with level of positional value. If so, one would expect treatments that alter positional value to alter the expression pattern of the gene.

Treatment of some strains of hydra with LiCl shifts the gradient of positional value toward more basal values. Prolonged treatment results in a decrease in head formation capacity (Maggiore and Bode, in preparation) and occasionally the formation of ectopic foot structures in the lower portions of the body column (Hassel and Berking, 1990). H. vulgaris were treated with LiCl for 15 days and assayed periodically to determine the extent of CnNK-2 expression along the body column. As shown in Figs. 11 and 12A, with increasing length of treatment the apical border of CnNK-2 expression moved up the body column. Treatment beyond 10 days did not extend expression any further apically, but the level of expression continued to increase as indicated by more intense staining in the midbody column. (Figs. 11C and 11D and 12A).

Treatment of hydra with DAG has the opposite effect of LiCl, causing a shift of positional value to more apical values (Muller, 1990). H. magnipapillata were treated with DAG for 15 days and assayed for CnNK-2 expression by in situ hybridization. CnNK-2 expression was progressively restricted to the most basal tissue in the animal (Fig. 12B). After 15 days of DAG treatment, only ~50% of the region which had expressed CnNK-2 in untreated animals was still capable of expressing detectable levels of CnNK-2 RNA. Thus, both LiCl and DAG treatments, which shift tissue to either more apical or more basal positional values, are accompanied by inverse changes in CnNK-2 expression.

DISCUSSION

Evolutionary Comparisons

Analysis of the hydra CnNK-2 gene shows that it is clearly a member of the NK-2 class of homeobox genes, based on the three criteria described under Results. Although 50-70% identity in the homeodomain may not seem particularly high, it is reasonable considering that the Cnidaria arose very early in metazoan evolution. The Cnidaria have been separated from other phyla of animals for a longer period of time than have these phyla from one another. In addition, a phylogenetic comparison indicates that the hydra CnNK-2 gene is more closely related to the NK-2 class than to any other class of homeobox genes (data not shown).

One other interesting aspect concerns the two conserved motifs outside the homeodomain. One of these motifs is the decapeptide near the N-terminal end, no other invertebrate NK-2 homolog shares more than 3 of these residues. For the NK-2 protein of Drosophila, 60% of the region hydra NK-2 gene is expressed in the endoderm, suggesting that when triploblastic animals appeared during evolution, expression of the genes of the NK class
spread to this new tissue. In nematodes the single described NK gene, ceh-22, is also expressed in mesodermal tissue, specifically pharyngeal muscle (Okkema and Fire, 1994). Groups that appeared later, the vertebrates and arthropods, have several members of the NK-2 class. In these organisms, the range of tissues in which NK-2 genes are expressed has expanded to include not only endodermal derivatives and muscle, but also the central nervous system and other tissues (Guazzi et al., 1990; Price et al., 1992; Lints et al., 1993; Nardelli-Haeﬂiger and Shankland, 1993; Saha et al., 1993; Tonissen et al., 1994).

It is plausible that the larger numbers of NK-2 homologs found in species that appeared later in evolution could simply reﬂect a more extensive search for this class of genes in vertebrates and Drosophila. However, the overall experience is that for any class of genes hydra has fewer members, consistent with the simplicity of the organism. Thus, the increase in number of NK-2 homologs in a species could readily reﬂect the increase in complexity that occurred through metazoan evolution.

Finally, the type of role CnNK-2 plays in hydra has not been shown with NK-2 genes in other organisms. Here it is involved in specifying the most basal portion of the body axis. The evidence thus far shows that most NK-2 genes are active in later steps of development, not in axis formation.

CnNK-2 Expression Is Correlated with Foot Formation during Normal Growth and Budding

A variety of experiments show a consistent correlation between the expression of CnNK-2 and the development of the foot in hydra. In an adult animal, tissue of the body column is converted into tissue of the foot in three different situations: during normal tissue growth, development of the bud, and regeneration. In each case, CnNK-2 expression precedes foot formation.

As part of the steady state of production and loss of cells in an adult hydra, tissue in the lower part of the body column is continually displaced basally through the peduncle onto the foot. Once in the foot, cells cease division (Holstein et al., 1991). In the ectoderm, they differentiate, changing their morphology drastically as well as expressing several antigens specific to the basal disk (e.g., Hoffmeister and Schaller, 1985). In the endoderm, no cytological or immunological changes have been described. However, CnNK-2 expression, which increases in intensity as the tissue is displaced through the peduncle, is shut off in the foot. This cessation of expression provides the ﬁrst marker to distinguish the foot endoderm from that of the peduncle. Thus, CnNK-2 is associated with tissue that is preparing to form a foot, but not with the foot itself.

In the steady state, most tissue is lost by funneling it into developing buds. Fate-mapping experiments have shown that a circle of tissue in the budding zone evaginates to form the bud (Otto and Campbell, 1977b). The center of the circle will form the head while its outer ring will form the foot. This rough "bull's-eye" pattern is reﬂected in the expression of genes associated with patterning in hydra. Budhead and Cnnox-3, both of which are expressed in the head, are active in a circular area before evagination begins (Martinez et al., in preparation; Schenk et al., in preparation). Later, as the bud becomes a protrusion, CnNK-2 expression appears as a ring at its base, in tissue which still lies within the parent. Thus, CnNK-2, as well as the two head genes, is expressed in tissue fated to become either foot or head but has not yet become part of the bud axis.

CnNK-2 Expression Is Also Correlated with Experimentally Induced Foot Formation

Foot formation also occurs as a result of a variety of tissue manipulations. For H. vulgaris and H. magnipapillata, bisecting an animal always results in the regeneration of a foot at the basal end of the upper part. In H. vulgaris, CnNK-2 expression was measured during regeneration and in all cases precedes foot formation. Further, the timing of CnNK-2 expression correlates with the differences in rates of foot regeneration at three positions along the body axis (Mookerjee and Bhattacharjee, 1967). The more basal the position, the faster the rate and the sooner the initial appearance of CnNK-2. Conversely, if no foot forms, no CnNK-2 expression is observed. When H. oligactis is bisected in the midgastric region it does not regenerate a foot nor is CnNK-2 expressed.

When animals are created with heads at both ends of the body column, they regulate by ﬁrst forming a foot at some point in between Muller (1990, 1995). This, in turn, is invariably preceded by CnNK-2 expression at the location where the foot will form. Thus, expression is associated with development of the foot, irrespective of whether it takes place along the body column or at its basal end.

These experiments, as well as observations during budding, show that the expression of CnNK-2 can be induced de novo in any tissue of the body column. This includes tissue in the upper part of the column which normally would be displaced into the head and would never express CnNK-2. CnNK-2, therefore, is tightly coupled to foot formation and is not restricted to specialized basal tissue in or near the peduncle.

Evidence for Signals from the Basal and Apical Ends of the Animal Affecting CnNK-2 Expression

How is expression of CnNK-2 regulated? Foot formation occurs in several different circumstances in hydra. To determine the effect of location relative to the foot and head, CnNK-2 expression was compared in the presence and absence of these structures.

Removal of the lower peduncle and foot results in the rapid loss of expression of the gene in the lower part of the body column in H. vulgaris. Removal of the foot alone has no effect on the expression pattern. These results indicate that the lower peduncle produces a signal that is transmitted up the column resulting in CnNK-2 expression. The
rapid loss of expression following removal of the lower peduncle also indicates that both the signal and the CnNK-2 mRNA have short half-lives. Further, the graded distribution of CnNK-2 expression suggests that the signal, plausibly a diffusible substance, has a graded distribution with an effective range of 25–30 cell diameters in H. vulgaris. A shorter steeper gradient would be consistent with the more limited extent of CnNK-2 expression in H. oligactis and H. magnipapillata.

This type of signal provides a means for maintaining the steady-state distribution of CnNK-2 expression in the context of the continuous tissue movements. Cells displaced basally into the lower half of the body column are exposed to increasing levels of the signal, thereby expressing the gene at increasingly higher levels. Once in the lower peduncle, a region where the tissue is known to be committed to foot formation (Bode and Bode, 1984b), the cells begin producing the signal for CnNK-2 expression and transmitting it up the column. In essence, this situation resembles an autoregulatory loop that maintains the constant pattern of CnNK-2 expression during the steady-state tissue displacement. Finally, upon displacement into the foot, cells differentiate into basal disk cells, and both the production of the signal and the expression of this gene cease.

The head, which is an organizing region, is a source of signals, both positive and negative. Several lines of evidence suggest that the head or developing head transmits a signal that initiates or enhances ectopic foot formation (e.g., Ando et al., 1989; Müller, 1990). Induction of an ectopic foot in the two-headed animals described above is another example. At an early stage during budding, the apical tip, which is committed to head formation (Li and Yao, 1945), can induce foot formation. This probably represents the normal course of events in the initiation and development of the bud foot. Finally, foot regeneration proceeds more rapidly in the presence of a head than in its absence (Muller, 1995). In all three cases CnNK-2 precedes foot formation, and, hence, expression of the gene appears to be affected positively by a signal or signals emanating from the head region.

On the other hand, when the head is removed from H. vulgaris whose peduncle and foot are intact, no change in the extent of the CnNK-2 pattern is observed. This suggests that head inhibition, a labile signal from the head which extends to the budding zone in a graded fashion (MacWilliams, 1983a), does not affect expression of this gene.

CnNK-2 Expression Is Inversely Correlated with the Positional Value Gradient

As described in earlier sections, the regeneration properties of bisected hydra or isolated segments of the body column are tightly correlated with axial position. This is also true for the kinetics of foot formation as well as of CnNK-2 expression during regeneration. The basis for the differences along the body column resides in the gradient of positional value (Wolpert et al., 1971), a stable property (MacWilliams, 1983b; Bode and Bode, 1984a) associated with the epithelial cells of both layers (Wanek et al., 1986; Nishimiya et al., 1986). The gradient is maintained primarily by signals from the head (Wilby and Webster, 1970; Herlands and Bode, 1974) which are transduced by the PKC and the PI cycle (Muller et al., 1993).

Prolonged treatment of animals with agents which interfere with this second messenger pathway results in changes in positional value. The positional value can be raised in the body column by treatment with DAG (Muller, 1990) and lowered with LiCl (Maggiore and Bode, in preparation). These agents provide a means for determining how positional value affects expression of CnNK-2. Raising the positional value by treating with DAG contracted the area of expression by about 50%. Conversely, lowering the positional value with LiCl expanded the range of expression of CnNK-2 far up the body column. Both results suggest that a signal associated with the positional value gradient acts in an inhibitory manner on CnNK-2 expression.

Two Plausible Roles for CnNK-2

The consistent correlation of CnNK-2 expression with the imminent formation of a foot suggests that the gene product is an integral part of the specification of the basal region of the animal. However, the data thus far cannot distinguish whether the gene acts upstream or downstream of foot determination. In either case, this gene provides the first marker for examining early stages in the process of foot formation.

If upstream, CnNK-2 could be part of the process that commits tissue of the lower peduncle to foot formation. If so, CnNK-2 and the signal produced there that leads to expression of the gene would be part of an autoregulatory loop for the maintenance of the foot. Further, the inhibitory effects associated with the positional value gradient could keep the source of the signal restricted to the basal end of the column in the lower peduncle.

If downstream, the gene could be involved in specifying the entire peduncle, a morphologically distinct area. In this case, the signal from the lower peduncle affecting CnNK-2 expression would be involved in peduncle, but not foot, formation. Any other signals would have only an indirect effect on the expression of the gene.

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