

Control of foot differentiation in *Hydra*: Phylogenetic footprinting indicates interaction of head, bud and foot patterning systems

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Abstract

Homeodomain transcription factor *CnNK-2* seems to play a major role in foot formation in *Hydra*. Recently, we reported in vitro evidence indicating that *CnNK-2* has autoregulatory features and regulates expression of the morphogenetic peptide pedibin. We proposed that *CnNK-2* and *pedibin* synergistically orchestrate foot differentiation processes [Thomsen, S., Till, A., Wittlieb, J., Beetz, C., Khalturin, K., Bosch, T. C., 2004. Control of foot differentiation in *Hydra*: in vitro evidence that the NK-2 homeobox factor *CnNK-2* autoregulates its own expression and uses *pedibin* as target gene. *Mech. Dev.* 121, 195–204]. Here, we further analyzed the regulatory network controlling foot formation in *Hydra*. By phylogenetic footprinting we compared the *CnNK-2* 5'-flanking sequence from two closely related species, *Hydra vulgaris* and *Hydra oligactis*. Unexpectedly, we detected a highly conserved binding site for *HNF-3β*, a vertebrate Forkhead transcription factor, in the *CnNK-2* 5'-flanking region. The *Hydra HNF-3β* homolog *budhead* is predominantly expressed in the apical region of the body column and early during budding. *Budhead* is absent from tissue expressing *CnNK-2* and thought to be involved in determining tissue for head differentiation [Martinez, D.E., Dirksen, M.L., Bode, P. M., Jamrich, M., Steele, R.E., Bode, H.R., 1997. *Budhead*, a *fork head/HNF-3β* homolog, is expressed during axis formation and head specification in *hydra*. *Dev. Biol.* 192, 523–536]. By electrophoretic mobility shift assays we demonstrate an in vitro interaction between recombinant *budhead* protein and the interspecific conserved *HNF-3β* binding motif in the *CnNK-2* 5'-flanking region. Our results strengthen the view of *CnNK-2* as an important regulator during foot patterning processes. Furthermore, they point to *budhead* as a candidate for a transcriptional regulator of *CnNK-2* and to an interaction of foot and head patterning processes in *Hydra* on the molecular level. © 2005 Elsevier Ireland Ltd. All rights reserved.

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1. Introduction

Hydra, a member of the most basal eumetazoan phylum Cnidaria, is notable for its simple body plan, its ability to regenerate missing body parts and for being a model system to trace the evolutionary conservation of bilaterian developmental pathways. The body plan consists of a bilayered radially symmetric gastric column with two terminal structures, the head and the foot. The head is defined by a ring of tentacles surrounding the hypostome, which contains the mouth opening. The foot is made up of a peduncle and a basal disc, which allows attachment of the polyp to

the substrate. The developmental processes governing the formation and maintenance of this body plan are well understood at the histological and cellular level (Bosch and Fujisawa, 2001; Steele, 2002; Bode, 2003). The continuous cell proliferation of epithelial and interstitial cells in both cell layers of adult *Hydra* polyps results in permanent tissue displacement towards the extremities. Morphogenetic mechanisms, therefore, are thought to be continuously active in adult polyps and required for (i) the remarkable regeneration capacity; (ii) the maintenance of body shape and proportions and (iii) the establishment of a new body axis by budding, the primary mode of reproduction in *Hydra*. Theoretical models based on transplantation and tissue manipulation experiments accurately describe these processes (Meinhardt, 1993; Meinhardt and Gierer, 2000; Berking, 2003). In brief, these models propose that pattern formation in *Hydra* features a local autocatalytic activator

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that produces a long-ranging inhibitor. This, in turn, antagonizes the self-activation (Meinhardt, 2004).

Within the last few years, the theoretical models of axial patterning controlled by head or foot activating and inhibiting diffusible substances have been complemented by molecular data (reviewed in Steele, 2002; Bosch and Khalturin, 2002; Bosch, 2003; Bode, 2003). One example is *CnNK-2*, an ortholog of the NK2 class of homeobox transcription factors, which has been shown to be involved in the specification of foot tissue. *CnNK-2* is strongly expressed during foot formation and responds to changes in the morphogenetic gradient that controls axial patterning (Grens et al., 1996). In particular, *CnNK-2* is responsive to *pedibin*, a peptide which is synexpressed with *CnNK-2* in the peduncle and enhances the foot formation potential of *Hydra* tissue (Hoffmeister, 1996). In vitro studies indicate that *CnNK-2* maintains its own transcription by autoregulation and directly regulates *pedibin* expression (Thomsen et al., 2004). Several genes have been discovered that are expressed whenever a head is formed. One of them is the *Brachyury* homolog *HyBra1*, which is expressed in the hypostome. Its transcriptional activation correlates with the changes of the head activation level (Technau and Bode, 1999) and, therefore, may be an essential part of the organizer activity. Other genes expressed early during head formation include genes of the Wnt signalling cascade (Hobmayer et al., 2000), homeobox gene *prdl-a* (Gauchat et al., 1998) and the novel peptide *Heady* (Lohmann and Bosch, 2000). Genes which appear to be involved in the formation of the *Hydra* head organizing center also appear to be crucial for the initiation of budding. Evidence that the early bud protrusion is committed to head formation is the onset of *HyBra1* expression, which can be detected in a small spot in the region where the next bud will appear (Technau and Bode, 1999). One of the first genes activated in the bud field is the *Hydra forkhead* homolog *budhead* (Martinez et al., 1997). *Budhead* expression in early buds is correlated with the disappearance of *CnNK-2* transcripts (Grens et al., 1996).

There is compelling evidence that head, foot and bud patterning systems in *Hydra* interact and influence one another. For example, buds always appear in distinct distance to the parental head and foot, indicating that buds form only in regions outside the influence of head and foot inhibitory signals. Evidence that the head patterning system interacts with the bud patterning system comes from the observation that heads and buds mutually inhibit each other (Shostak, 1974) and that budding is enhanced by removal of the head (Tardent, 1972). The head system also influences the foot since foot differentiation is enhanced by the presence of a head (Javois and Frazier-Edwards, 1991; Müller, 1995; Forman and Javois, 1999). Moreover, transplantation experiments have shown that an intact foot patterning system is necessary for budding (Schiliro et al., 1999). Consistent with these findings, a peptide isolated from *Hydra* foot tissue that enhances foot

differentiation also enhances bud formation (Hoffmeister-Ullrich, 2001). Thus, the head, bud and foot patterning systems appear to be connected by long-range interactions.

Very little is known about the molecules mediating this crosstalk. In an attempt to unravel the transcriptional regulatory network controlling foot specific gene expression, we analyzed the *CnNK-2* 5'-flanking sequence by phylogenetic footprinting using orthologous sequences from two closely related species, *Hydra vulgaris* and *Hydra oligactis*. Unexpectedly, the *CnNK-2* regulatory region was found to contain a highly conserved binding site for *HNF-3 β* , a Forkhead transcription factor. By EMSA experiments with nuclear extract and recombinant protein we show that the *Hydra HNF-3 β* homolog *budhead* can specifically bind to this motif. As *budhead* has been proposed to play a critical role in head formation processes, our results indicate a molecular interaction of foot and head patterning processes during axis formation in *Hydra*.

2. Results

2.1. The 5'-flanking region of *CnNK-2* in two closely related *Hydra* species contains conserved binding sites for Forkhead class transcription factors

Cis regulatory sequences hardwired into the genome represent an important part of the regulatory logic underlying the control of development. Given that many developmental processes are strikingly similar across species borders, it is reasonable to expect important sequences to be strongly conserved at the nucleotide level, since their potential for mutation is constrained by their function. Thus, such sequences can be identified by comparison of the promoters of closely related organisms. We used such a phylogenetic footprinting approach to identify evolutionary conserved *cis* regulatory elements in the *Hydra CnNK-2* promoter. Approximately 1 kb of 5'-flanking sequence of the *CnNK-2* gene was analyzed from *H. vulgaris* and *H. oligactis* using the web based ConSite platform. Due to the relatively high overall identity of the 5'-flanking regions (69% within 989 bp) compared to approx. 90% (582 bp) within the coding region (data not shown), we chose a high conservation cut off of 90% (Fig. 1A, shaded). Two conserved regions, named Regions I and II (Fig. 1A), partially exceeding the conservation cut off, were identified and subjected to transcription factor binding site prediction (Fig. 1B,C). ConSite scores the matrix profiles using a uniform base composition (Lenhard et al., 2003). As *Hydra* has an A+T-rich 'biased' genome composition several cycles of analysis were performed with increasing transcription factor score thresholds (TFST), thus modulating the stringency of the sequence analysis.

We first used the ConSite program to screen the *CnNK-2* promoter for the presence of the previously described (Thomsen et al., 2004) *CnNK-2* autoregulatory site.

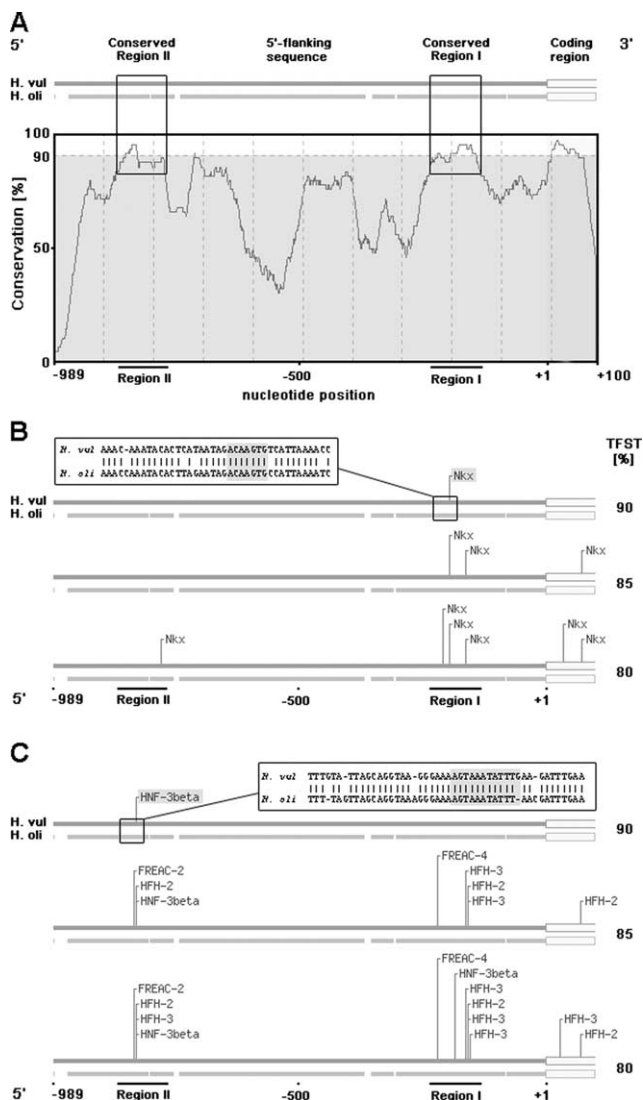


Fig. 1. Phylogenetic footprints in the *H. vulgaris* and *H. oligactis* *CnNK-2* 5'-flanking regions. (A) Conservation profile of *H. vulgaris* and *H. oligactis* *CnNK-2* 5'-flanking regions. Detection of two conserved regions partially exceeding the conservation cut off (90%, shaded in gray). (B) Previously characterized (Thomsen et al., 2004) autoregulatory *CnNK-2* binding site. The *H. vulgaris* sequence corresponding to NK-2 (1/2) oligonucleotide from our previous study is boxed. The conserved *Nkx2.5* binding motif is shaded. TFST, transcription factor score threshold. (C) Conserved *HNF-3 β* binding motif. The *H. vulgaris* sequence corresponding to BH_1 oligonucleotide used in EMSA (Fig. 3) is boxed. The conserved *HNF-3 β* binding motif is shaded (graphics modified from <http://phylofoot.org/>).

The search was performed with the implemented *Nkx* weighted matrix profile based on the binding specificity of murine *Nkx2.5* transcription factor (Chen and Schwartz, 1995). The same matrix had been used in our previous study to predict potential binding sites for cnidarian *CnNK-2*, which shows 70% identity (on amino acid level) within the homeodomain of the aforementioned murine gene (Grens et al., 1996). In both conserved regions (Fig. 1A), potential *Nkx*-binding sites were detected (Fig. 1B). When the TFST was raised to 90%, only the previously described (Thomsen

et al., 2004) autoregulatory *CnNK-2* binding motif remained detectable (Fig. 1B, Box). The interspecific conservation of the *CnNK-2* element, which can be bound by *CnNK-2* protein in vitro, supports our view that it plays an important role in autoregulation of *CnNK-2* transcription in vivo.

The phylogenetic footprinting approach also revealed a number of conserved potential binding sites for Forkhead class transcription factors (Fig. 1C). When using very stringent TFST of 90% or even 99% (data not shown), one potential binding site for murine *HNF-3 β* (*Rattus norvegicus*) remained detectable (Fig. 1C, Box). The *Hydra* Forkhead transcription factor *budhead* is 78% identical with *HNF-3 β* (*R. norvegicus*) at the amino acid level within the conserved Forkhead DNA-binding domain. *Hyfkh2* and *Hyfkh3*, the two other Forkhead transcription factors known so far in *Hydra* (Martinez et al., 1997), show a weaker sequence identity to rat *HNF-3 β* . Since *budhead* in *Hydra* is predominantly expressed in head tissue and upregulated during budding when transcripts for the gene *CnNK-2* disappear, detection of this site in the 5'-flanking region of a foot specific gene was unexpected.

2.2. *CnNK-2* and *budhead* show complementary expression patterns

To assess the nature of a potential interaction between the head specific *budhead* protein and the foot specific *CnNK-2* gene, we first re-examined the expression patterns of both genes in *H. vulgaris* with emphasis on the budding process. In agreement with previous observations (Grens et al., 1996; Martinez et al., 1997), *budhead* is predominantly expressed in the apical part of *Hydra* while *CnNK-2* shows a foot specific expression. *CnNK-2* transcripts are most abundant in the endoderm of the peduncle. They are also present in the budding zone but absent in the more apical region of the body column (Fig. 2E). In contrast, *budhead* is strongly expressed in the lower half of the hypostome and the tentacle zone, while *budhead* mRNA is not detectable in the tentacles (Fig. 2A). We note that *budhead* transcripts are not exclusively restricted to head tissue but also detectable in the gastric region. During budding (Fig. 2B–D), *budhead* transcripts are abundant from the earliest stages on. In later stages of budding, *budhead* transcripts are localized preferentially in the more apical region and are absent in tentacle anlagen (arrow in Fig. 2C) and tentacles (Fig. 2D). *CnNK-2* transcripts are absent from early buds (Fig. 2F) and are first detected in intermediate budding stages. The adult pattern (Fig. 2E) is established shortly before the mature bud detaches from the parent polyp (Fig. 2H). In sum, both genes encode transcription factors and are expressed in endodermal epithelial cells. However, they have a strikingly reciprocal expression pattern with *CnNK-2* mRNA conspicuously absent in tissue expressing *budhead*.

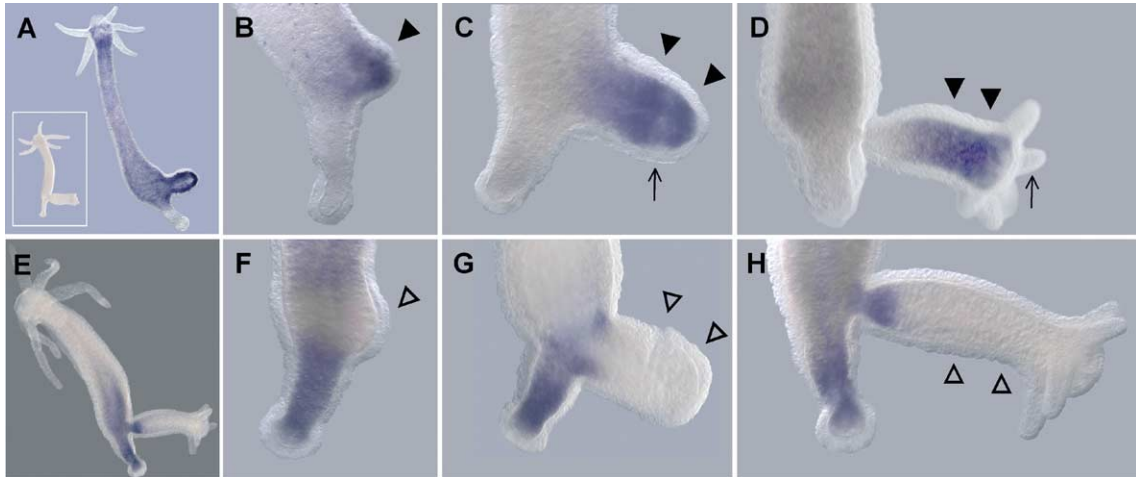


Fig. 2. Expression patterns of *budhead* and *CnNK-2* in budding *Hydra vulgaris*. (A) *budhead* expression. Box: sense control. (B–D) *budhead* expression in different budding stages. (E) *CnNK-2* expression. (F–H) *CnNK-2* expression in different budding stages. Budding stages 3 (B, F), 5 (C, G) and 8–9 (E, H; according to Otto and Campbell, 1977). Black arrowheads, *budhead* transcripts; open arrowheads, absence of *CnNK-2* transcripts; arrows indicate *budhead* transcript free prospective tentacle bases (C) or tentacles (D) of developing buds.

2.3. *Budhead* protein binds to the conserved forkhead binding motif in the *CnNK-2* promoter

To investigate whether the conserved *HNF-3 β* binding motif in the *CnNK-2* promoter can be considered as a binding site for *budhead*, we performed electrophoretic mobility shift assays (EMSA) using a 45 bp oligonucleotide (BH_1; Fig. 3A) corresponding to the predicted binding site in the *H. vulgaris* *CnNK-2* promoter (box, Fig. 1C) and *Hydra* nuclear extract or recombinant *budhead* protein. As shown in Fig. 3B, incubation of the oligonucleotide with nuclear extract from *Hydra* tissue leads to the formation of a specific DNA/protein complex. Specificity of binding was determined by competition experiments using both an unrelated oligonucleotide (C_u) and unlabeled oligonucleotide BH_1 (C_s).

To determine whether the *Hydra budhead* protein is binding to *CnNK-2* oligonucleotide BH_1, EMSA experiments were performed using recombinant *budhead* protein as shown in Fig. 3B. When comparing the binding of recombinant protein and nuclear extract to BH_1 on one gel, comigrating complexes were observed. This indicated that native *budhead* is the DNA-binding protein in the reaction with nuclear extract. As also shown in Fig. 3B, addition of unspecific competitor (C_u) slightly reduced the binding of recombinant *budhead* to BH_1. However, since only by addition of specific competitor the binding could be prevented completely, recombinant *budhead* protein specifically interacts with oligonucleotide BH_1.

To characterize this binding in more detail, we performed EMSA experiments with a mutated oligonucleotide (BH_1mu) in which the predicted *HNF-3 β* binding motif was disrupted (Fig. 3A). Using recombinant *budhead* and oligonucleotide BH_1mu (Fig. 3B), only a weak

unspecific binding could be detected. Moreover, when BH_1mu was used as unlabeled competitor in an EMSA experiment with BH_1 and recombinant *budhead* protein, BH_1mu acted like an unspecific competitor and did not prevent complex formation (data not shown).

Taken together, our data show that the *HNF-3 β* binding site in the *CnNK-2* promoter detected initially by phylogenetic footprinting can be bound by *budhead* in vitro. This finding suggests that *budhead* is interacting with the *CnNK-2* promoter in vivo and – due to the reciprocal expression patterns of both genes – may act as an inhibitor of *CnNK-2* expression in tissue committed to form a head.

CnNK-2 expression has been shown to be upregulated by signals which lower the positional value along the body axis and cause an increase in the foot forming potential, such as *pedibin* or lithium-chloride (Grens et al., 1996, 1999), the latter probably acting by either blocking GSK-3 in the Wnt pathway (Klein and Melton, 1996) or by disturbing the IP₃ pathway (Hassel et al., 1998). To test whether the effect of these foot stimulating signals on *CnNK-2* transcription is mediated by a change in *budhead* expression, we monitored the abundance of *CnNK-2* and *budhead* mRNA in response to LiCl-treatment by both RT-PCR and in situ hybridization (Fig. 4).

In agreement with previous reports (Hassel and Berking, 1990), the LiCl-treatment as described in Section 4 caused formation of ectopic foot structures along the lower body column (Fig. 4E). As described below, the efficiency of the LiCl-treatment varied in independent experiments resulting in different degrees of ectopic foot formation. Interestingly, this correlated with different changes in *budhead* expression. In all batches of incubated animals, the LiCl-treatment lead to an upregulation of *CnNK-2* and to an expansion of its expression domain towards more apical regions (Fig. 4B,C). In control animals, *CnNK-2* expression

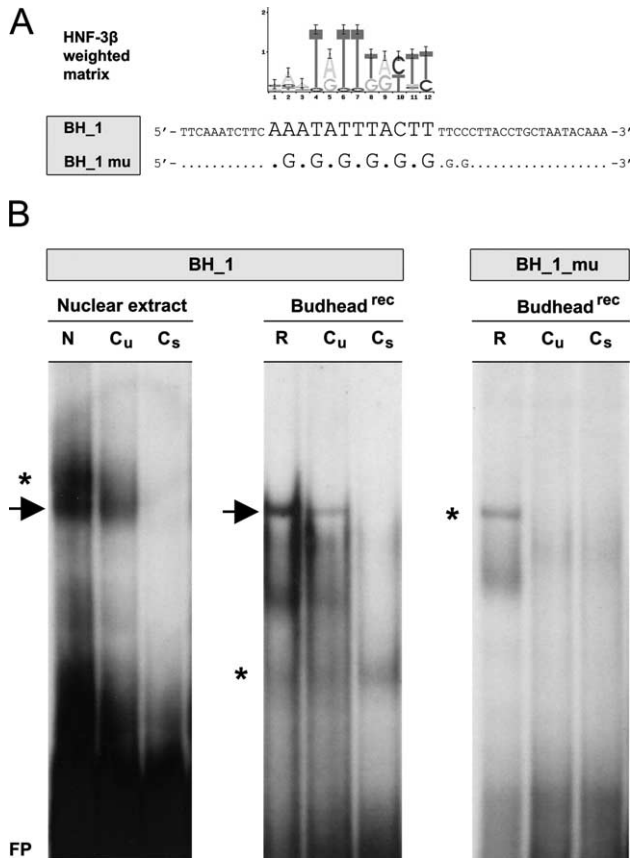


Fig. 3. DNA/protein interactions at a predicted *HNF-3 β* binding site in *H. vulgaris* *CnNK-2* 5'-regulatory region. (A) Graphical representation of *HNF-3 β* weighted matrix (JASPAR database, http://jaspar.cgb.ki.se/cgi-bin/jaspar_db.pl), BH_1 oligonucleotide with conserved binding motif and mutated version BH_1mu. (B) Nuclear protein extract as well as recombinant *budhead* protein specifically interact with oligonucleotide BH_1. Mutation of predicted *HNF-3 β* binding motif eliminates specific binding of recombinant *budhead* protein. Varying intensities of free probe (FP) are due to different exposure times. C_s, addition of specific competitor; C_u, addition of unspecific competitor; FP, free probe; N, nuclear extract; R, recombinant protein. Arrows indicate specific DNA/protein interaction; asterisks indicate unspecific DNA/protein interactions seen in all controls or competed by unspecific competitor.

was spatially restricted to the aboral end and could hardly be detected in gastric tissue.

Consistent with in situ hybridization data in untreated polyps (Fig. 2A), RT-PCR with control animals revealed *budhead* transcripts along the whole body in similar quantities (Fig. 4B,C). In batches of LiCl-treated polyps which developed only few or no ectopic feet, respectively (Fig. 4D), RT-PCR demonstrated that *budhead* expression was not affected (Fig. 4B). Consistent with that, in situ hybridization showed detectable levels of *budhead* transcripts throughout the body column and a local concentration above the tentacle zone (Fig. 4D). In batches of LiCl-treated polyps where more than 90% of the animals showed ectopic foot structures (Fig. 4E), RT-PCR revealed a drastically reduced *budhead* transcript level in comparison to control animals. Moreover, the amounts of *budhead* RNA

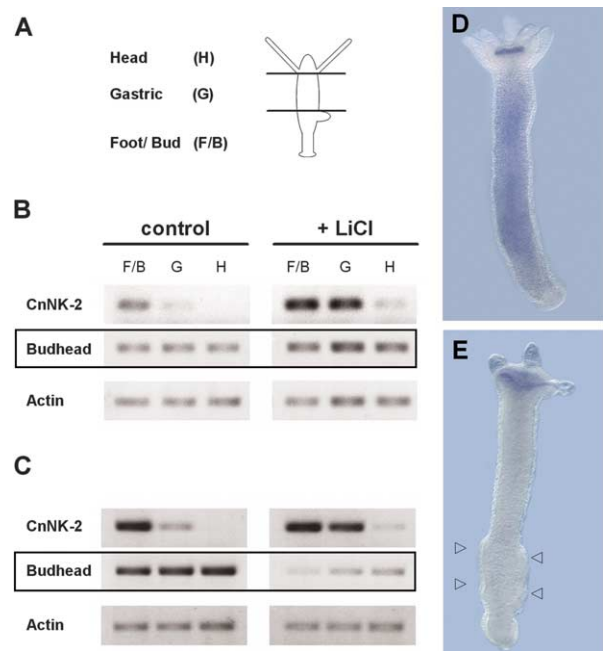


Fig. 4. Unlike *CnNK-2*, expression of *budhead* shows no immediate response to LiCl. (A) RT-PCRs were performed using cDNAs from different body regions. (B, C) Independent RT-PCR experiments using RNA from LiCl-treated animals with weak (B) or strong (C) induction of ectopic foot formation. (D, E) *budhead* in situ hybridizations. Independent LiCl-treatments lead to batches of polyps without (D) or with ectopic foot structures (E). Only in LiCl-treated animals with ectopic feet the expression of *budhead* is affected (C). Arrowheads indicate ectopic foot structures.

decreased from head to foot (Fig. 4C). This observation was supported by in situ hybridization showing no detectable amounts of *budhead* mRNA below the tentacle zone of LiCl-treated polyps (Fig. 4E).

Since *budhead* is downregulated only after ectopic feet have been formed (Fig. 4C,E) while *CnNK-2* is upregulated long before ectopic structures are visible (Fig. 4B), it seems unlikely that *budhead* is involved in the early modulations of *CnNK-2* expression in LiCl-treated polyps.

3. Discussion

3.1. *Budhead*, a possible link between head, bud and foot patterning systems

Pattern formation along the single body axis of *Hydra* involves developmental gradients originating from two organizing centers, the apical head and the basal foot (Fig. 5A). Reciprocal interactions between these organizing centers and the bud patterning system have long been known (Schiliro et al., 1999 and references within; Forman and Javois, 1999). The molecular code of this crosstalk, however, has not been deciphered yet. We have examined the 5'-regulatory sequences of *CnNK-2* in two species of *Hydra* and identified a small number of highly conserved

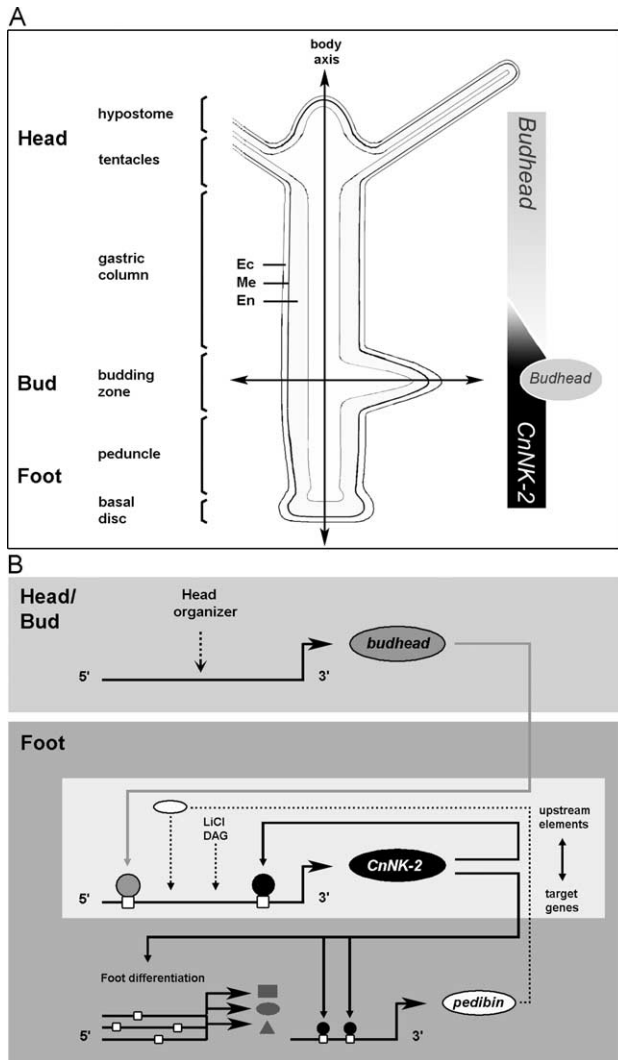


Fig. 5. A model describing the molecular interaction of head, bud and foot patterning systems in *Hydra*. (A) Morphology of *Hydra* and schematic expression patterns of *CnNK-2* and *budhead* along adult and developing body axes (see Fig. 2). (B) Molecular interactions in *Hydra* basal patterning events (see Section 3 for details). Ec, ectoderm; Me, mesogloea; En, endoderm.

regions. One of them contains the previously characterized putative autoregulatory *CnNK-2* element (Thomsen et al., 2004). The other conserved region contains a *HNF-3 β* binding motif. *HNF-3 β* belongs to the Forkhead class transcription factor family. In *Hydra*, three members of this family are known so far (Martinez et al., 1997): *budhead*, *Hyfkh2* and *Hyfkh3*. By electrophoretic mobility shift assays, we could show the specific in vitro binding of (i) *Hydra* nuclear extract and (ii) *budhead* recombinant protein to this motif. *Budhead* shows a 78% identity at the amino acid level with *HNF-3 β* within the DNA-binding Forkhead domain. In contrast, the Forkhead domains of *Hyfkh2* and *Hyfkh3* are only 44 and 48% identical to *HNF-3 β* suggesting a reduced ability to bind the conserved *HNF-3 β* -motif in comparison to *budhead*. In addition, the deduced molecular

weights of *Hyfkh2* and *Hyfkh3* (>48 and 27 kDa, respectively; Daniel Martinez, pers. comm.) significantly differ from *budhead* (37 kDa). As the DNA/protein complexes formed with nuclear extract and recombinant *budhead* are of the same size (Fig. 3B), an involvement of *Hyfkh2* and *Hyfkh3* in this complex formation appears to be unlikely. Since the Forkhead gene family in other organisms includes numerous members and since the ongoing *Hydra* EST- and genome projects might reveal additional Forkhead genes in *Hydra*, we note that the observed DNA/protein complexes with nuclear extract (Fig. 3B) could be also based on the binding of a yet unknown Forkhead factor with a molecular weight and a binding specificity similar to *budhead*. Based on the current state of knowledge, the observed DNA/protein interactions on the interspecific conserved *HNF-3 β* -motif seem to be specific for *budhead*, the *Hydra* Forkhead protein used in this study.

Agents like diacylglycerol (DAG), arachidonic acid (AA) and LiCl interfere with patterning events in *Hydra*. They lead to formation of ectopic head or foot structures and are discussed to raise (DAG, AA) or lower (LiCl, prolonged treatment at low concentrations) the gradient of positional value (Müller, 1990; Hassel and Berking, 1990; Hassel et al., 1993). Several genes, among them *CnNK-2*, with a proposed role in axial patterning events respond to DAG and LiCl with complementary shifts of their expression domain along the body column of *Hydra* (Grens et al., 1996; Bridge et al., 2000). This has been interpreted as a coupling of their expression to this gradient. In contrast, previous works have shown that the expression of *budhead* is not altered by DAG-treatment (Martinez et al., 1997). Consistent with that, our observations indicate that *budhead* shows no immediate response to LiCl, as no changes are observed prior to ectopic foot formation.

The dynamic, complementary expression patterns of *CnNK-2* and *budhead* during budding (Figs. 2,5A) are consistent with a negative regulation of *CnNK-2* by *budhead* during the formation of a new axis. However, the results of the LiCl-treatment followed by RT-PCR and in situ hybridization suggest that the regulation of *CnNK-2* along the adult body axis is more complex and that the restriction of its expression to the basal pole of *Hydra* is mediated by several independent factors in a combinatorial, context dependent manner.

On the basis of the data presented in this study, we propose *budhead* as a candidate for a transcriptional regulator of *CnNK-2*. The in vivo binding of *budhead* to the conserved motif and its functional relevance remain to be shown. As *budhead* is predominantly expressed in head tissue (Martinez et al., 1997) and has been proposed to be associated with the *Hydra* head organizer (Broun and Bode, 2002), its in vitro interaction with the *CnNK-2* promoter region provides first evidence at the molecular level for an interaction of head, bud and foot formation processes in *Hydra*.

We recently summarized the available data on molecular interactions in the foot region of *Hydra* in a model based on

the in vitro binding specificity of *CnNK-2* (Thomsen et al., 2004) and the work of others (Grens et al., 1999). We suggested that the differentiation of foot specific cells occurs in response to a signalling gradient of peptides such as *pedibin* at the basal end of the axis (Thomsen et al., 2004). The hints for *budhead* as an additional component of the signaling network allows us to expand the model (Fig. 5B) and to propose a signal from tissue determined for head formation. Furthermore, we added the demonstrated coupling of *CnNK-2* expression to LiCl- and DAG-sensitive signalling cascades (Grens et al., 1996).

According to the interactions depicted in Fig. 5B, peptide *pedibin* is upstream and controls the expression of *CnNK-2*. *CnNK-2*, in turn, controls localized expression of *pedibin* and presumably also genes further downstream whose products are directly involved in foot differentiation. In addition, *CnNK-2* regulates its own expression by a feedback loop and, thus, shows features proposed for molecules mediating foot activation (Meinhardt, 2004). The diversity of signalling inputs integrated at the 5'-region of *CnNK-2* (Fig. 5B) supports our view of *CnNK-2* being an important 'interface' in the regulatory network underlying foot differentiation in *Hydra*. The observed in vitro interaction of recombinant *budhead* with an interspecifically conserved motif in the *CnNK-2* promoter is intriguing and indicates a molecular crosstalk between the head, bud and foot patterning systems and – together with our previous indications for autoregulation of *CnNK-2* (Thomsen et al., 2004) – adds experimental support to the idea (Meinhardt, 2004) that local self-enhancement and long-range inhibition, partially realized by interacting transcription factors, are essential components of patterning systems in *Hydra*.

3.2. Prospects for phylogenetic footprinting in *Hydra*

Our studies suggest that by comparing the regulatory regions from equivalent genes of different *Hydra* species, *cis* regulatory elements can be identified and additional components in regulatory networks can be detected. Regulatory elements reveal themselves by their high homology when compared to orthologous sequences from equivalent genes in different species because they are protected from random drift across evolutionary time by selection (Gumucio et al., 1992; Wasserman et al., 2000; Pennacchio and Rubin, 2001; Iwama and Gojobori, 2004). Regulatory elements usually lie outside coding sequences and are embedded in a highly variable sequence background. In *Hydra*, this background is extensive due to the large average genome size of 1.200 Mbp (Zacharias et al., 2004) making isolation of genomic sequences and identification of *cis* regulatory elements difficult. Phylogenetic footprinting represents one way to differentiate between functional regulatory elements and non-functional DNA, which is applicable to *Hydra*. In future, this approach will benefit from the availability of bacterial artificial chromosome (BAC) libraries from different

Hydra species (Hemmrich et al., in prep.). The detection of binding sites for *Hydra* transcription factors with weight matrices based on the binding specificities of their bilaterian orthologs is intriguing. The results of this and a former study (Thomsen et al., 2004) are supported by several other examples of transcription factors, which have retained their binding specificities during the course of evolution as demonstrated by in vitro binding of *Hydra achaete scute* and *CREB* homologs to bilaterian consensus motifs (Grens et al., 1995; Galliot et al., 1995) or in vivo by heterologous expression of *Hydra gooseoid* and *brachyury* genes followed by specific inductive effects (Broun et al., 1999; Marcellini et al., 2003).

Phylogenetic footprinting appears to be widely applicable in *Hydra* and may be used to address a number of important questions. For example, beside *CnNK-2*, several other genes have been found to be involved in foot specific differentiation in *Hydra* including signalling molecules like the morphogenetic peptide *pedibin* (Hoffmeister, 1996; Grens et al., 1999), the transcription factor *manacle*, the receptor tyrosine kinase *shin guard* (Bridge et al., 2000), potential target genes like the metalloproteinase *HMP-2* (Yan et al., 2000) and the peroxidase *PPOD1* (Hoffmeister-Ullrich et al., 2002). A simple mechanism for the activation of a set of genes in a complex spatial pattern is to regulate their expression by transcription factors that bind to common promoter elements. Can, therefore, foot genes in *Hydra* be characterized and identified by a group of common *cis* regulatory elements? Such conserved promoter elements may be considered as 'foot specific signature' which may give hints to the molecular and evolutionary mechanisms involved in controlling position dependent gene expression in *Hydra*. Moreover, since key to understanding organismal complexity are the regulatory sequences (Levine and Tjian, 2003), phylogenetic footprinting may also be a valuable tool to understand the molecular basis of morphological diversity within the genus *Hydra*. In designing the phylogenetic footprinting experiments described here, we chose to compare *CnNK-2* in *H. vulgaris* and *H. oligactis*. Both species have a shared regulation of development but differ conspicuously in their foot morphology. An intriguing question therefore is whether the differences in foot morphologies in both species are correlated with differences in *cis* regulatory sequences of foot specific genes. Thus, 'reverse phylogenetic footprints', i.e. differences in 5'-flanking sequences of closely related species and potential regulatory elements within could account for differential regulation of foot shaping target genes.

In conclusion, as numerous developmental control genes are discovered and a wide spectrum of molecular techniques are applied to *Hydra*, a complex signalling system emerges (see also Endl et al., 1999; Thomsen et al., 2004), which is required to control the differentiation behavior of cells along the body axis. Rapid identification of regulatory elements in key developmental genes by the comparative approach described above will not only lead to more rapid progress in

understanding patterning in these simple metazoans but will also provide important information about ancestral components of the axial patterning system before bilaterality evolved.

4. Experimental procedures

4.1. Animals

We used *H. vulgaris* strain Basel and *H. oligactis* for all of the work presented here. The animals were cultured according to standard procedures at 18 °C.

4.2. Isolation of 5' regulatory regions of the *H. vulgaris* and *H. oligactis* *CnNK-2* gene

5'-flanking regions for *CnNK-2* in both species were isolated by a nested PCR-based approach with adapter-ligated genomic DNA fragments as described before (Thomsen et al., 2004). The identity of PCR products was confirmed by Southern hybridization using known coding or 5'-flanking sequence as a probe. PCR-fragments were ligated in vector pGEM-T (Promega) prior to sequencing. For *H. oligactis*, a nested PCR with genomic DNA, digested with *Sca* I or *Bcu* I and ligated to the splinkerette adapter, yielded a fragment of 1090 bp comprising 989 bp 5'-flanking sequence. The PCRs were performed combining *CnNK-2* specific primer 5'-GGG GAA CGT AGA GAG TCT-3' and splinkerette specific primer 5'-GAA TCG TAA CCG TTC GTA CGA G-3' in the first and specific inner primer 5'-TGA GAA AAA TCG CTC TGG TG-3' combined with inner splinkerette primer 5'-TAC GAG AAT CGC TGT CCT C-3' in the second amplification with 1:30 diluted first PCR-product as template. The 5'-flanking regions of *H. vulgaris* and *H. oligactis* *CnNK-2* genes are available at GenBank (Accession nos: AY927374, AY927375).

4.3. Phylogenetic footprinting, sequence analysis

Phylogenetic footprinting analysis was performed using the free web based ConSite platform (<http://phylofoot.org/>) which integrates transcription factor binding site prediction and analysis of sequence conservation (see Fig. 1A) in orthologous genomic sequences making use of the JASPAR collection of transcription factor DNA-binding preferences modeled as position specific weight matrices (Lenhard et al., 2003; Sandelin et al., 2004a,b). *H. vulgaris* and *H. oligactis* *CnNK-2* genomic sequences (−989 to +100 bp) and the *CnNK-2* cDNA sequence of *H. vulgaris* (Grens et al., 1996) were submitted.

Analysis was performed with ConSite default settings for window size (50 bp), a stringent 90% conservation cut off and an increasingly stringent transcription factor score threshold (TFST, see Fig. 1) from 80 to 90%. Here, we show

the results analyzing the whole sequences with either the implemented single *Nkx*-profile (Fig. 1B) or all Forkhead-domain profiles (Fig. 1C). Molecular weights of proteins were deduced using ExPASy (<http://www.expasy.org>).

4.4. In situ hybridization

In situ hybridizations for *CnNK-2* and *budhead* were performed as described before (Martinez et al., 1997), using riboprobes covering full-length cDNA sequences for both genes (Grens et al., 1996; Martinez et al., 1997).

4.5. Recombinant protein production

A *budhead* full-length cDNA clone obtained by RT-PCR was cloned into the pCR T7 TOPO TA expression vector (Invitrogen) and expressed in *Escherichia coli* strain BL21 (DE3) pLys. Recombinant *E. coli* were grown at 37 °C in Terrific Broth-medium (Difco)/34 µg/ml Chloramphenicol for 10 h to an optical density of 2 and induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation, resuspended in phosphate buffer (1 M K₂HPO₄/KH₂PO₄, pH 7.4) and broken by sonication. The His-tagged recombinant protein was purified from the cleared supernatant by absorption to Ni-NTA-Agarose (Qiagen), washing in 10 mM Imidazol in phosphate buffer and elution with 0.5 M Imidazol in phosphate buffer. The protein was stored in aliquots at −80 °C after changing buffer to ZKC (20 mM Hepes/KOH (pH 7.9), 420 mM KCl, 1.5 mM MgCl₂, 0.1 mM EDTA (pH 8.0), 20% glycerole, 4 mM Pefabloc) using a NAP column (Amersham).

4.6. Electrophoretic mobility shift assay (EMSA)

Nuclear protein extracts from *H. vulgaris* were prepared as described (Endl et al., 1999). For EMSAs, double-stranded oligonucleotides were end-labeled with γ³²P ATP. The binding reaction, with 1 µg nuclear protein or 0.4 µg recombinant protein, respectively, and approx. 100 pmol target DNA, was incubated for 60 min on ice in binding buffer (50 mM Tris/HCl pH 7.7, 2.5 mM EDTA, 5 mM MgCl₂, 250 mM NaCl₂, 20% glycerin, 2.5 mM DTT) containing 1 µg poly(dI-dC). Unlabeled competitor DNA was added to the reaction in 10-fold molar excess. Electrophoresis was carried out on a 6% native polyacrylamide gel and visualized by autoradiography. The sequence of the oligonucleotides used as labeled probes and specific competitors are shown in Fig. 3A. As unrelated competitor (C_u) we used oligonucleotide CREB (5'-TGG CCC ATC AAA TTA ATT TTT TTC TAA-3'). For autoradiography, longer exposure times have been chosen for reactions with nuclear extract to obtain optimal signal/background ratios, thus leading to more intense signals from the free probe (Fig. 3B).

4.7. Lithium-chloride treatment

Lithium-chloride treatment was performed as described before (Grens et al., 1996). *H. vulgaris* polyps were incubated for 15 days in Hydra medium supplemented with 0.5 mM LiCl. The medium was changed daily. Animals were fed daily.

4.8. RT-PCR

In two independent experiments 60 budding *H. vulgaris* polyps, either lithium-chloride pretreated or untreated, were cut under the tentacle ring and above the most apical bud or the peduncle, respectively. Single stranded cDNA was prepared after isolating RNA from the three tissue fractions (head, gastric, foot+buds, Fig. 4A) with Trizol (Invitrogen). For amplification of gene specific fragments, the following primer-sets, cycle numbers and a common annealing temperature ($T_A = 56^\circ\text{C}$) were used: (1) *CnNK-2* (*CnNK-2* forward 5'-CGT GTC GTG TTA TAG TAA CGT-3', *CnNK-2* reverse 5'-TTG AAG TCT CGC TCA GTT TCA G-3'; 30 and 29 cycles, respectively), (2) *budhead* (*budhead* forward 5'-AACAACATGATGGA-CACGGTT-3', *budhead* reverse 5'-GAG TTT TGC CAC CGT TGT TG-3'; 28 and 26 cycles, respectively). The cDNA-samples were equilibrated by PCR with *Hydra* Actin specific primers (*Actin* forward 5'-AAG CTC TTC CCT CGA AGA ATC-3', *Actin* reverse 5'-CCA AAA TAG ATC CTC CGA TCC-3'; 20 and 17 cycles, respectively). The first cycle number refers to the RT-PCR shown in Fig. 4B, the second to the one shown in Fig. 4C.

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