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# 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

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## Abstract

The foot of the simple metazoan *Hydra* is a highly dynamic body region of constant tissue movement, cell proliferation, determination and differentiation. Previously, two genes have been shown to participate in the development and differentiation of this body region: homeodomain factor *CnNK-2* and signal peptide *pedibin* [Dev. Biol. 180 (1996) 473; Development 126 (1999) 517; Development 122 (1996) 1941; Mech. Dev. 106 (2001) 37]. *CnNK-2* functions as transcriptional regulator and is responsive to changes in the positional value while the secreted peptide *pedibin* serves as 'extrinsic' positional signal. Exposure of polyps to *pedibin* increases the spatial domain of *CnNK-2* expression towards the gastric region, indicating that positional signals are integrated at the *cis*-regulatory region of *CnNK-2*. In the present study, to elucidate the molecular basis of the interaction of *CnNK-2* and *pedibin*, we characterized the 5' regulatory regions of both genes. Within the *CnNK-2* 5' upstream region, electrophoretic mobility shift assays showed that putative NK-2 binding motifs are specifically bound by both nuclear protein from *Hydra* foot and by recombinant *CnNK-2*, suggesting that *CnNK-2* might autoregulate its own expression. This is the first indication for an autoregulatory circuit in *Hydra*. In addition, we also identified NK-2 binding sites in the *cis*-regulatory region of the *pedibin* gene, indicating that this gene is one of the targets of the transcription factor *CnNK-2*. On the basis of these results, we present a model for the regulatory interactions required for patterning the basal end of the single axis in *Hydra* which postulates that *CnNK-2* together with *pedibin* orchestrates foot specific differentiation.

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**Keywords:** *Hydra*; NK-2 homeodomain gene; Autoregulation; Axial patterning; Morphogen; Peptide

## 1. Introduction

Since development of multicellular organisms is, to a large extent, dictated by transcriptional control of gene expression (Davidson, 2001), it is crucial, for a deeper understanding of animal development, to elucidate the transcriptional network that specifies when and where genes are expressed. Development in the evolutionary old metazoan *Hydra* leads to a simple body plan consisting of a two-layered radially symmetrical tube with two differentiated structures, head and foot, at the ends of a single body axis. *Hydra* tissue grows constantly, and as cells divide

they are displaced along the body column towards the head and foot (for recent review, see Bosch (1998)). Thus, during asexual growth an epithelial cell has a fundamental decision to make: whether to remain a gastric epithelial cell, or to differentiate into head or foot cells. There is good evidence that spatially localized signals inform *Hydra* cells of their relative positions along the body axis (for review, see Bosch (2003)) and that patterning is governed by a positional value gradient (Meinhardt, 1993; Meinhardt and Gierer, 2000).

Position-dependent differentiation at the apical end of the *Hydra* body axis is controlled by an head organizer located in the hypostome (Galliot, 2000; Broun and Bode, 2002; Steele, 2002). Several genes have been discovered to be expressed in tissue whenever a head is formed, including the brachyury homolog *HyBral* (Technau and Bode, 1999), genes of the WNT signaling cascade (Hobmayer et al., 2000), the homeobox gene *pdl-1* (Gauchat et al., 1998) and

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the novel peptide *Heady* (Lohmann and Bosch, 2000). In addition, downstream effector genes essential for head formation, like *ks1* (Lohmann et al., 1999), contain *cis*-regulatory elements with binding sites for a variety of transcription factors (Endl et al., 1999), suggesting that proper position-dependent differentiation is controlled by a complex network of interacting transcriptional regulators.

A framework for understanding aspects of position-dependent differentiation at the basal end of the *Hydra* body axis is provided by studies of Campbell (1967), Dübel et al. (1987), Hoffmeister (1996), Grens et al. (1996, 1999), and Kobayakawa and Kodama (2002) and is summarized in Fig. 4A. This body region below the budding zone, generally referred to as 'foot', consists of the peduncle and the basal disk (Campbell, 1967). When displaced towards the basal end, ectodermal epithelial cells are gradually determined along the peduncle to become highly specialized basal disk cells (Kobayakawa and Kodama, 2002). Two genes previously have been shown to be involved in patterning the basal end of the body axis. First, signal peptide *pedibin* stimulates foot formation and regeneration (Hoffmeister, 1996; Hoffmeister-Ullerich, 2001). *Pedibin* also downregulates *Farm1*, a matrix metalloprotease that is expressed in the gastric region and absent in apical and basal tissue (Kumpfmüller et al., 1999). The second gene is the NK-2 class homeodomain transcription factor *CnNK-2* (Grens et al., 1996). Expression of *CnNK-2* is upregulated during foot regeneration and is responsive to changes of positional values along the body axis (Grens et al., 1996). Grens et al. (1996, 1999) demonstrated an interaction between *CnNK-2* and *pedibin*, since expression of *CnNK-2* is influenced by *pedibin*. Both genes are coexpressed in entodermal epithelial cells of the peduncle. Expression of both genes attenuates in a gradient-like manner from the peduncle towards the gastric region. In polyps treated with *pedibin*, the *CnNK-2* expression is greatly extended towards the gastric region (Grens et al., 1999). Thus, the peptide appears to cause a decrease in positional value of gastric tissue leading to an increased spatial domain of *CnNK-2* expression.

Though important key players required for foot specific differentiation have been identified, our understanding of the regulatory events in this process is limited, since (1) no target genes for transcription factor *CnNK-2* are known; (2) the mechanisms by which *CnNK-2* expression is continuously maintained in peduncle specific tissue remains to be shown; (3) the functional relationship between the two foot specific genes has not been established. Notwithstanding these deficiencies, it seems probable that the 5' regulatory region of the *CnNK-2* gene constitutes a site at which many different signals are integrated. Analysis of this region, therefore, should provide insight into how positional signals promote foot specific differentiation.

To address this question, we have isolated and analyzed the 5' upstream regions of the *CnNK-2* and *pedibin* genes,

respectively. We identified and functionally tested potential binding sites for NK-2 class homeodomain transcription factors by electrophoretic mobility shift assays (EMSA) with nuclear extracts from *Hydra* foot and gastric tissue as well as with recombinant *CnNK-2*. We provide in vitro evidence that *pedibin* is one of the target genes of *CnNK-2*. In addition, we found putative *CnNK-2* binding sites in the regulatory region of the *CnNK-2* gene itself indicating that *CnNK-2* might regulate its own expression through an autoregulatory feedback loop. This is the first indication that autoregulation may be an important feature of the gene regulatory network in *Hydra*. A working model emerges in which the foot specific regulatory network includes interaction of the peptide *pedibin* and the homeodomain-containing transcription factor *CnNK-2* as well as autoregulatory feedback loop which makes maintenance of foot specific gene expression independent of the continuous presence of positional signals.

## 2. Results

### 2.1. The 5' flanking region of *CnNK-2* and *pedibin* contains putative NK-2 binding sites

To isolate 5' regulatory sequence of *CnNK-2* and *pedibin*, a PCR strategy with adapter-ligated restricted genomic DNA was used (see Section 4). The 5' flanking regions obtained are shown in Fig. 1. In the 1364 bp region upstream of the *CnNK-2* start codon (Fig. 1A), computer analysis identified putative binding sites for many transcription factors, including eight predicted high (site 1) and low (sites 2–8) affinity binding sites for murine homeodomain transcription factor *Nkx-2.5*. These sites, referred to as NK-2 sites, were located within 1150 bp upstream of the translation start site (Fig. 1A,C). In Fig. 1D, we compiled a collection of functionally defined NK-2 binding sites for three NK-2 genes known to be required for development in human, mouse and *Drosophila* and aligned them with the putative NK-2 binding sequences located in the 5' flanking region of *Hydra CnNK-2*. The sequence of the 872 bp genomic fragment upstream of the *pedibin* translation start is shown in Fig. 1B. Interestingly, and similarly to *CnNK-2*, in silico analysis revealed seven matches to binding sites for murine homeodomain transcription factor *Nkx-2.5* which are located within 650 bp upstream of the *pedibin* translation start site (Fig. 1B). As shown in Fig. 1D, putative NK-2 binding sites 1 and 4 in the *pedibin* 5' flanking region had the diagnostic 5'-C/TAAG-3' core sequence that is supposed (Harvey, 1996) to be recognized by most NK-2 homeodomain factors. In sum, the presence of conserved NK-2 binding sites in the two coexpressed *Hydra* genes points to a functional relevance of *CnNK-2* in regulating gene expression at the basal end of the *Hydra* body axis.

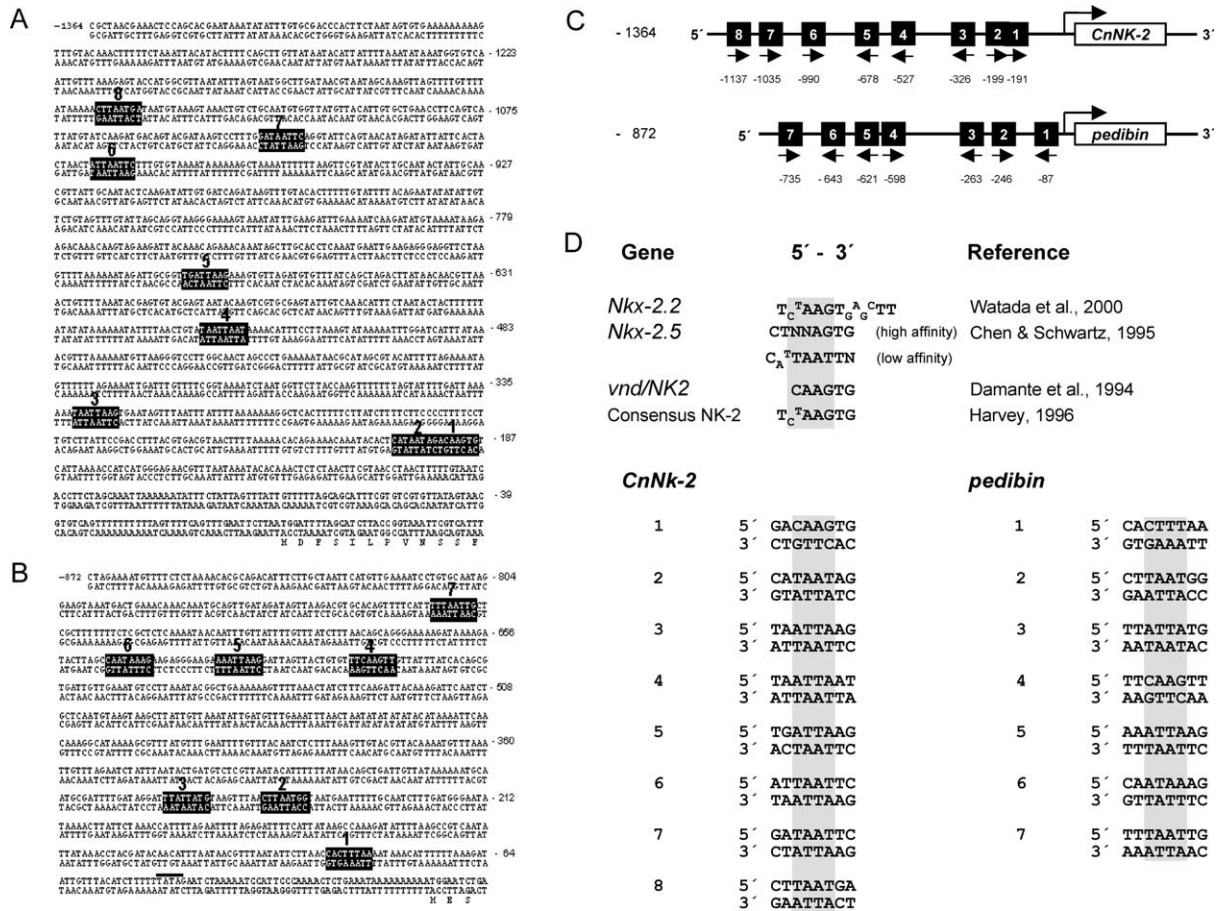


Fig. 1. Predicted NK2 binding sites in the 5' flanking sequence of *Hydra CnNK-2* and *pedibin*. (A) Nucleotide sequence of 1364 bp 5' upstream region of the *Hydra CnNK-2* gene. Putative NK-2 binding sites are indicated by boxes and numbers. (B) Nucleotide sequence of 872 bp 5' flanking region of *pedibin*. A putative TATA box is indicated by a bar. (C) Scheme of the *CnNK-2* and *pedibin* 5' regulatory regions. (D) Functionally defined NK-2 binding sites in human, mouse and *Drosophila* genes compared to predicted NK-2 binding sequences in the *Hydra CnNK-2* and *pedibin* regulatory region. NK-2 core binding sites are shaded.

2.2. Foot specific nuclear proteins bind to oligonucleotides containing putative NK-2 sites of the 5' flanking region of *CnNK-2*

To explore whether nuclear factors spatially restricted in foot tissue bind to the predicted NK-2 elements in the regulatory region of the *CnNK-2* gene, we performed EMSAs, using nuclear extract from foot or gastric tissue and an oligonucleotide covering putative NK-2 binding sites 1 and 2. As shown in Fig. 2A, incubation of this oligonucleotide, named NK-2(1/2) (see also Fig. 3), with nuclear extract from foot tissue resulted in the formation of two complexes (labeled I and II in Fig. 2), whereas only one specific complex was formed with nuclear extract from body column tissue (head plus gastric tissue). Specificity of binding was determined by competition experiments using the unlabeled specific oligonucleotide NK-2(1/2) or an unrelated oligonucleotide (see Section 4) in 10-fold molar excess. As shown in Fig. 2A, competition with unlabeled specific oligonucleotide resulted in the loss of the foot specific DNA binding, whereas the unrelated oligonucleotide had no effect

on DNA binding. Taken together, the observations indicate that oligonucleotide NK-2(1/2) forms a specific DNA/protein complex with both proteins from gastric and foot tissue (complex I) and that an additional specific complex (complex II) is formed in the presence of nuclear proteins from foot tissue.

2.3. The putative NK-2 binding sites in the *CnNK-2* 5' flanking sequence are essential for foot specific DNA/protein interactions

We next tested the importance of each of the NK-2 sites in oligonucleotide NK-2(1/2) for binding nuclear proteins from foot tissue by using mutated versions, named NK-2(1/2)-mu1 and NK-2(1/2)-mu2 (Fig. 2B). In NK-2(1/2)-mu1, the core binding site 5'-CAAG-3' was mutated from AA to GG and in NK-2(1/2)-mu2 the 5'-TAAT-3' core binding site was mutated from AA to GG (see Fig. 1D). As shown in Fig. 2B, both mutations prevented formation of foot specific complex II while complex I formation apparently was not affected. These observations suggest

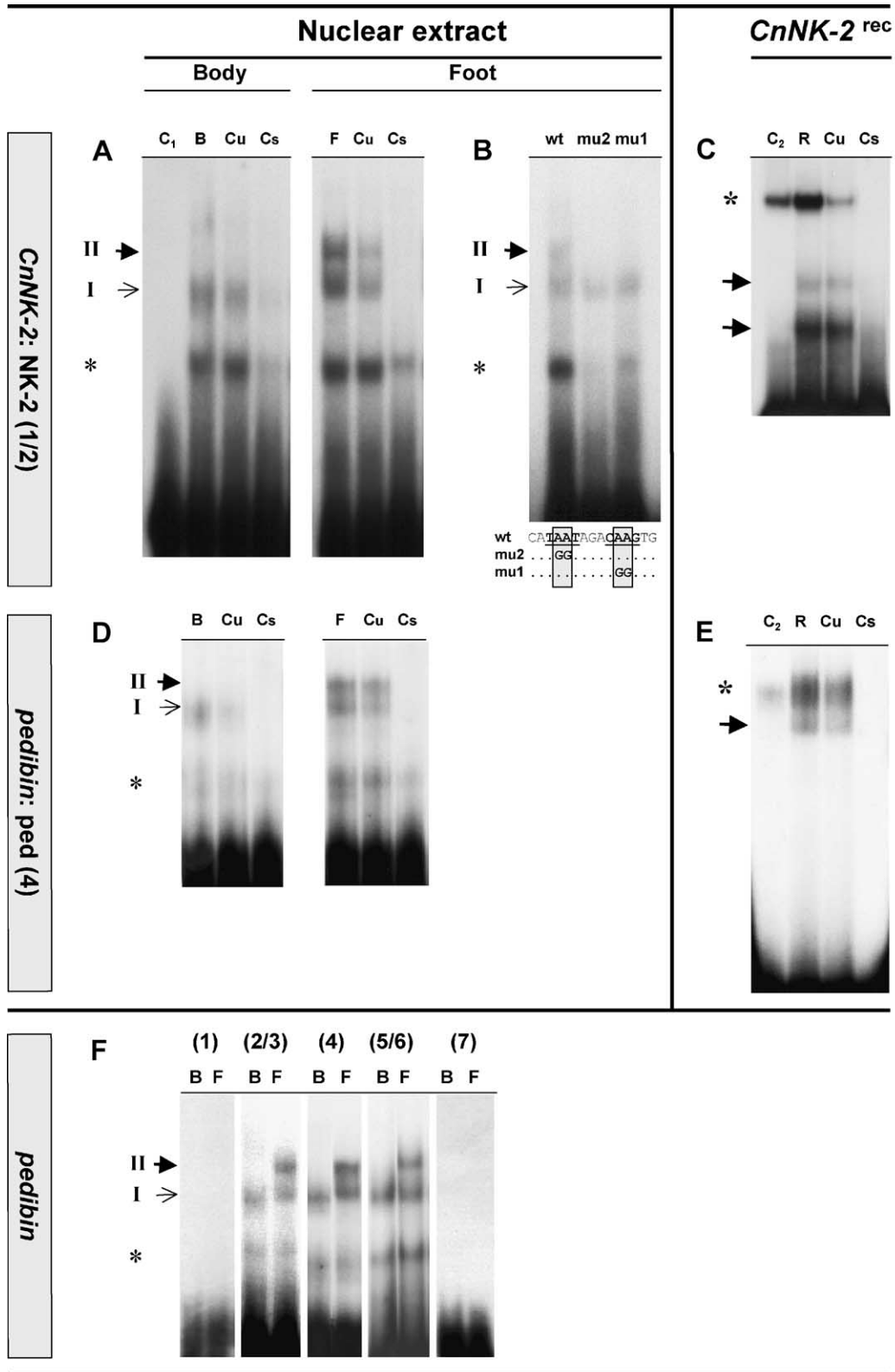


Fig. 2. DNA/protein interactions at putative NK-2 binding sites in the *CnNK-2* and *pedibin* 5' regulatory regions. (A) Nuclear protein extract from foot tissue specifically interacts with oligonucleotide NK-2(1/2). C<sub>1</sub>, control containing binding buffer only; B, nuclear extract from body column tissue; F, nuclear extract from foot tissue; Cu, addition of unspecific competitor; Cs, addition of specific competitor. Arrows indicate specific DNA/*CnNK-2* protein complexes. The asterisk indicates unspecific DNA/protein complexes seen also in controls. (B) EMSA using mutated versions of oligonucleotide NK-2(1/2) and nuclear protein extract from foot tissue. wt, NK-2(1/2) oligonucleotide; mu2 and mu1, mutated versions of oligonucleotide NK-2(1/2). (C) Bacterially expressed *CnNK-2* protein interacts with oligonucleotide NK-2(1/2). C<sub>2</sub>, control containing bacterial lysate only; R, lysate with recombinant *CnNK-2* protein and radiolabeled

Gene	Oligonucleotide			EMSA				
	Name	Sequence		Nuclear extract	<i>CnNK-2</i> <sup>rec</sup>			
<b><i>CnNK-2</i></b>	<b>NK-2 (1/2)</b>	AAACAAATACACT TTTGTATTATGTGA	<b>CATAATA</b> <b>GACAAGTG</b> <b>GTATTAT</b> <b>CTGTTTAC</b>	TCATTAAAACC AGTAATTTTGG	+	+		
		<b>Ped (1)</b>	GTTTAATATTCTTAAC CAAATTATAAGAATTG	<b>CACTTTAA</b> <b>GTGAAATT</b>	AATAAACATTTTTTAA TTATTGTAAAAAATT	-	n.d.	
<b><i>Pedibin</i></b>	<b>Ped (2/3)</b>	GATAGGAT CTATCCTA	<b>TTATTATG</b> <b>AATAATAC</b>	TAAGTTTAA ATTCAAAATT	<b>CTTAATGG</b> <b>GAATTACC</b>	TAATGAAT ATTACTTA	+	n.d.
		<b>Ped (4)</b>	GATTAGTTACTGTGT CTAATCAATGACACA	<b>TTCAAGTT</b> <b>AAGTTCAA</b>	GTTATTTATCAGCG CAATAAATAGTGTCCG		+	+
<b><i>Pedibin</i></b>	<b>Ped (5/6)</b>	TACTTAGC ATGAATCG	<b>CAATAAAG</b> <b>GTTATTTC</b>	AAGAGGGAAGA TTC1CCCTTCT	<b>AAATTAAG</b> <b>TTTAATTC</b>	GATTAGTT CTAATCAA	+	n.d.
		<b>Ped (7)</b>	GTGCACAGTTTTCATT CACGTGTCAAAAGTAA	<b>TTTAATTG</b> <b>AAATTAAC</b>	CTCGCTTTTTTCTCG GTGCGAAAAAAGAGC		-	n.d.
<b>Consensus</b>			<b>C<sub>A</sub><sup>T</sup>TAATTG</b>					

Fig. 3. Putative NK-2 binding sites in the 5' flanking sequence of *CnNK-2* and *pedibin* and their performance in EMSA with nuclear extract or recombinant *CnNK-2*. Predicted NK-2 binding sites are boxed; (+), oligonucleotides which bind to nuclear extract or recombinant *CnNK-2*; (-), oligonucleotides which do not bind to nuclear extract; (n.d.), not determined binding specificities.

that complex I is formed by factors abundant both in foot and body column tissue and binding to sequences outside the NK-2 core sites. In contrast, formation of complex II requires the presence of the NK-2 core binding sites. Since both NK-2 sites appear to be essential for complex II formation, it seems possible that it is caused by a protein, which binds to both adjacent NK-2 sites as dimer. We note that complex II formation is directly correlated to the localization of *CnNK-2* transcripts in foot tissue (Grens et al., 1996) and, therefore, may be involved in activation or maintenance of *CnNK-2* transcription.

#### 2.4. *CnNK-2* protein directly binds to oligonucleotides containing NK-2 sites

What is the nature of the proteins binding to the NK-2 sites? Is Hydra *CnNK-2* the transcription factor which is differentially binding to NK-2 elements in foot cells? In order to address this question, we performed EMSAs using

bacterially expressed *CnNK-2* protein and radiolabeled oligonucleotide NK-2(1/2). As shown in Fig. 2C, recombinant *CnNK-2* specifically bound to this oligonucleotide. Two specific complexes were formed. Both of them were abolished by competition with unlabeled NK-2(1/2) oligonucleotide ( $C_s$ ) indicating that binding was sequence specific. Presence of excess amount of an unrelated oligonucleotide ( $C_u$ ) had no effect on binding of the *CnNK-2* protein. Thus, recombinant *CnNK-2* protein appears to specifically bind to NK-2 elements at the *CnNK-2* 5' flanking region, indicating a direct feedback of *CnNK-2* on its own regulation.

#### 2.5. The *CnNK-2* protein binds to putative NK-2 sites in the cis-regulatory region of the *pedibin* gene

Having established that *CnNK-2* binds to its own regulatory region, and having identified putative target sites for *CnNK-2* in the cis-regulatory region of the *pedibin*

oligonucleotide NK2(1/2);  $C_u$ , lysate with recombinant *CnNK-2* protein, radiolabeled oligonucleotide NK2(1/2), and excess amount of unspecific competitor.  $C_s$ , lysate with recombinant *CnNK-2* protein, radiolabeled oligonucleotide NK2(1/2), and excess amount of specific competitor. Arrows indicate specific DNA/*CnNK-2* protein complexes. The asterisk indicates an unspecific DNA/protein complex seen also in controls. (D) Nuclear protein extract from foot tissue specifically interacts with oligonucleotide ped(4). B, nuclear extract from body column tissue; F, nuclear extract from foot tissue;  $C_u$ , addition of unspecific competitor;  $C_s$ , addition of specific competitor. Arrows indicate specific DNA/protein complexes. The asterisk indicates unspecific DNA/protein complexes seen also in controls. (E) Bacterially expressed *CnNK-2* protein interacts with oligonucleotide ped(4).  $C_2$ , control containing bacterial lysate only; R, lysate with recombinant *CnNK-2* protein and radiolabeled oligonucleotide ped(4);  $C_u$ , lysate with recombinant *CnNK-2* protein, radiolabeled oligonucleotide ped(4), and excess amount of unspecific competitor.  $C_s$ , lysate with recombinant *CnNK-2* protein, radiolabeled oligonucleotide ped(4) and excess amount of specific competitor. Arrows indicate specific DNA/*CnNK-2* protein complexes. The asterisk indicates an unspecific DNA/protein complex seen also in controls. (F) DNA/protein interactions at putative NK-2 binding sites of *pedibin*. Nuclear protein extract from foot tissue specifically interacts with oligonucleotides ped (2/3), ped (4) and ped (5/6). B, nuclear extract from body column tissue; F, nuclear extract from foot tissue. Arrows indicate specific DNA/protein complexes.

gene (Fig. 1B), we wanted to determine whether transcription factor *CnNK-2* is indeed binding to NK-2 *cis*-regulatory elements in the *pedibin* gene. As indicated in Fig. 2D, EMSA experiments with oligonucleotide ped(4) containing the putative NK-2 site 4 (see Figs. 1B and 3) and nuclear extract from foot and body column tissue yielded the same DNA/protein complexes as described for the NK-2 sites in the *CnNK-2* 5' flanking region. Specificity of binding was determined by competition experiments using unlabeled sequence specific or unrelated oligonucleotide in 10-fold molar excess. Fig. 2D shows that, similar to oligonucleotide NK-2(1/2), incubation of oligonucleotide ped(4) with nuclear extract from foot tissue resulted in the formation of two complexes (labeled I and II in Fig. 2D), whereas only one specific complex was formed with nuclear extract from body column tissue. To determine whether transcription factor *CnNK-2* is binding to the NK-2 elements in the *pedibin* 5' upstream region, we performed EMSAs using recombinant *CnNK-2* protein and radiolabeled oligonucleotide ped(4). As shown in Fig. 2E, recombinant *CnNK-2* specifically bound to this oligonucleotide and caused the formation of a specific DNA/protein complex. Interestingly, as shown in Fig. 1D, the core sequence of *pedibin* NK-2 site 4 is identical to the core sequence of NK-2 site 1 in the *CnNK-2* promoter which has been shown to be required for complex II formation (Fig. 2B). Beside ped(4), the *pedibin* 5' flanking sequence contains six more putative NK-2 sites (Fig. 1B). To examine these sites for foot specific DNA/protein interactions, EMSA experiments were performed using oligonucleotides ped(1), ped(2/3), ped(4), ped(5/6) and ped(7) and nuclear extract of foot and body column tissue (Fig. 2F). While oligonucleotides ped(1) and ped(7) yielded no shift, incubation of oligonucleotides ped(2/3), ped(4) and ped(5/6) with nuclear extract from foot tissue resulted in the formation of two complexes (labeled I and II in Fig. 2F), whereas only one complex was formed with nuclear extract from body column tissue. Specificity of binding was determined as described above by competition experiments using unlabeled specific oligonucleotides or an unrelated oligonucleotide in 10-fold molar excess (data not shown). Taken together, the data shown in Fig. 2 indicate binding of *CnNK-2* to several NK-2 sites in the *cis*-regulatory region of the *pedibin* gene. Thus, *pedibin* appears to be one of the target genes of transcription factor *CnNK-2*.

### 2.6. Towards a *CnNK-2* *in vitro* consensus binding motif in *Hydra*

In the 5'-regulatory region of two foot specific genes, *CnNK-2* and *pedibin*, initially 15 putative sites for murine transcription factor *Nkx-2.5* were identified (Fig. 1). Nine out of these 15 sites were tested by EMSA for foot specific DNA/protein interaction (Fig. 2). As shown in Fig. 3, seven out of these nine sites bound nuclear proteins from foot tissue. In addition, three out of these seven NK-2 sites bound recombinant *CnNK-2* (Fig. 2C,E). Alignment of

the sequences of these seven sites revealed a consensus sequence 5'-C<sub>A</sub><sup>T</sup>TAATTG-3' which corresponds to the intermediate/low affinity consensus binding motif 5'-C<sub>A</sub><sup>T</sup>TAATTN-3' of murine *Nkx-2.5* (Chen and Schwartz, 1995).

## 3. Discussion

A major challenge in analyzing position-dependent cell differentiation is first to understand how cells are informed of their relative positions within the organism and second, to elucidate how this positional information is translated into the precise spatial and temporal expression of key regulatory genes.

### 3.1. Peptides in *Hydra* are potent inducers for foot specific differentiation

In *Hydra*, there is good evidence that key signals for foot specific differentiation are conveyed by peptides. Previous studies have shown that *pedibin*, a 12 amino acid peptide identified from *H. vulgaris* (Hoffmeister, 1996) is a signaling molecule that fulfills a critical role during foot differentiation. A closely related peptide, *Hym-346*, has been identified in extract of *Hydra magnipapillata* (Takahashi et al., 1997). Both peptides are expressed in entodermal foot cells. *Pedibin* is also expressed in the tentacle bases of *Hydra* and was suggested to play a critical role in positioning of the tentacles (Hoffmeister-Ullerich, 2001). During foot regeneration, *pedibin/Hym-346* expression is activated rapidly in few cells at the basal end. Both peptides not only accelerate foot regeneration but also cause an increase in the foot activation potential in gastric tissue by lowering the positional value gradient (Grens et al., 1999). Two experiments demonstrated that both peptides are sufficient for foot induction (Grens et al., 1999). First, incubation of intact polyps in synthetic *pedibin/Hym-346* causes extra foot formation along the whole body axis. Second, the potential of *pedibin/Hym-346* to induce foot fate was shown in lateral grafting experiments. Using this assay, the morphogenetic activity of tissue can be tested without influencing cell proliferation effects. When polyps were continuously incubated in 10<sup>-6</sup> M peptide for 5 days, and then used as donors for homotopic gastric transplantation, a large proportion of peptide treated implants formed secondary axes with foot morphology. Thus, *pedibin/Hym-346* are potent and specific inducers of foot fate. Transplantation and regeneration experiments indicate that a third peptide, *Hym-323*, which shares no structural similarity to *pedibin/Hym-346* also affects the patterning processes involved in foot formation (Harafuji et al., 2001). In contrast to *pedibin/Hym-346*, *Hym-323* is expressed in epithelial cells along the body column, but not in foot and head tissue. It seems likely, therefore, that upon initiation of foot formation, the stored *Hym-323* peptide is released from

the epithelial cells and induces differentiation of basal disk cells of the foot (Bosch and Fujisawa, 2001; Harafuji et al., 2001; Fujisawa, 2003). Taken together, there is compelling evidence that peptides are important and specific inducers of foot differentiation in *Hydra*.

### 3.2. Integration of positional information by homeodomain factor *CnNK-2*

How the positional value gradient in *Hydra* is translated into changes in cell behavior is not understood nor are the molecular events known that trigger *pedibin* gene activation in foot specific cells. In *Hydra*, transcription factor *CnNK-2* is sensitive to foot specific positional signals and critically involved in foot specific differentiation (Grens et al., 1996). We, therefore, anticipated that a great deal of foot specific differentiation is manifested in the regulation of *CnNK-2* activity, or of genes downstream of *CnNK-2*. In this study, we found that regulation of *CnNK-2* activity might involve an autoregulatory feedback loop and that the positional signal *pedibin* is one of the targets for foot specific transcription factors. Thus, this is the first insight in how a potent positional signal might be regulated in *Hydra*.

Our data indicate in vitro binding of both foot specific nuclear protein and recombinant *CnNk-2* to motifs initially identified as potential binding sites for a murine NK-2 class transcription factor *Nkx-2.5*. Several observations indicate that these NK-2 binding sites are functionally significant and that the proteins binding to them function as transcriptional regulators: (i) The proteins binding to the NK-2 sites of the *CnNK-2* and *pedibin* promoter are asymmetrically distributed along the apical–basal axis with DNA–protein complex II restricted to foot tissue where *CnNK-2* is expressed (Fig. 2A,D,F). (ii) NK-2 elements in the regulatory region of *CnNK-2* are highly specific binding sites since use of mutated binding sites reduces binding activity completely (Fig. 2B). (iii) Recombinant *CnNK-2* is binding to NK-2 sites of the *CnNk-2* as well as the *pedibin* 5' flanking region in a sequence specific manner (Fig. 2C,E). Since a single foot specific NK-2/protein complex (complex II) is formed (Fig. 2A,D,F) and since two putative NK-2 binding sites in close proximity appear to be required for complex II formation (Fig. 2B), we presume that *Hydra CnNK-2* is acting as a dimer to form complex II as has been shown for the closest orthologue of *CnNK-2* in vertebrates, murine *Nkx-2.5* (Kasahara et al., 2001). (iv) Additional support for the view that NK-2 elements are significant binding sites comes from our observation (Thomsen et al., in preparation) that the regulatory region of a foot specific effector gene coding for a peroxidase also contains NK-2 elements. Taken together, the NK-2 elements identified appear to play a major role in foot specific regulation of gene expression in *Hydra*.

### 3.3. Autoregulatory control of *CnNK-2* expression in *Hydra* foot cells

A commonly used control feature in gene regulatory networks is autoregulation, wherein a protein modifies, directly or indirectly, its own rate of production. Whether such interactions embody positive or negative feedback depends on the details of the network dynamics (Hasty et al., 2002). Due to autoregulation of transcription factors, the response of a cell can become independent of the presence of positional signals. Therefore, such transcription factors can be considered as a molecular memory of prior signals (Scully and Rosenfeld, 2002). First evidence that control of expression of NK-2 related transcription factors involves autoregulatory activity comes from *Drosophila*. Functional dissection of genomic regions from the *vnd/Nk-2* locus indicated that the *vnd/NK-2* protein is required for maintenance of *vnd/NK-2* gene expression (Saunders et al., 1998). Furthermore, screening for DNA-binding proteins that mediate the Dpp response of *NK-4/tinman* revealed that *NK-4/tinman* autoregulation plays an important role in the Dpp response (Xu et al., 1998). Transcription factors of the NK-2 family contain both a N-terminal activation and a C-terminal inhibition domain to control interaction with cofactors and other nuclear proteins (Watada et al., 2000; Chen and Schwartz, 1995). Thus, binding of and regulation by NK-2 may not only involve the NK-2 protein itself but also other not yet identified proteins. Due to the interaction domains, non-linear control could be achieved either by dimerization of activating molecules or by binding to other nuclear factors. Our studies have uncovered NK-2 binding sites in the upstream sequence of the *Hydra* NK-2 related gene *CnNK-2* and specific binding of recombinant *CnNK-2* to this site. This strengthens the view that autoregulation is a major feature of control of expression of NK-2 transcription factors and indicates that this mechanism can be traced back in evolution to the basal metazoan *Hydra*.

Although autoregulation has long been considered to be an essential feature during *Hydra* patterning (Meinhardt, 1982, 1993), the molecular components of this system have not been elucidated. Based on reaction–diffusion models, pattern formation depends on the production of a long-range inhibitor, which is regulated by an antagonist, an autocatalytic activator (for recent review, see Meinhardt and Gierer (2000)). Is the autocatalytic regulation of the *CnNK-2* gene part of the predicted activator–inhibitor system in *Hydra*? We do not know the answer yet. Irrespective of it, however, our experiments have uncovered *CnNK-2* as a critical component in the foot patterning system in *Hydra*.

### 3.4. Interaction between the *Hydra* homeobox protein *CnNK-2* and the positional signal *pedibin*

Current views on foot formation in *Hydra* (for recent reviews, see Steele (2002), Bosch and Khalturin (2002), Bosch (2003) and Fujisawa (2003)) are based on a gradient

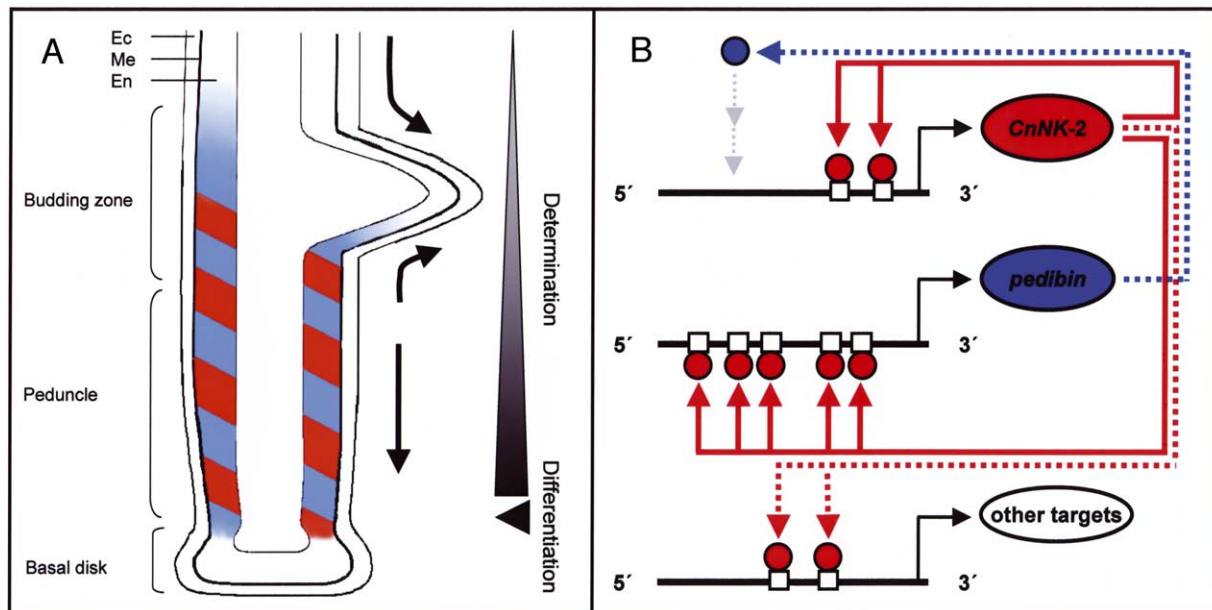


Fig. 4. Patterning at the basal end of the single body axis in *Hydra*. (A) Expression pattern of *CnNK-2* (red) and *pedibin* (blue), tissue movements, and spatially restricted cell differentiation events. For references, see Grens et al. (1996, 1999) and Kobayakawa and Kodama (2002). (Ec), ectoderm; (Me), mesogloea; (En), endoderm. (B) Model of the *CnNK-2* transcriptional regulatory activity in foot specific differentiation in *Hydra*. *CnNK-2* autoregulates its own expression and binds to the regulatory region of *pedibin* and possibly of other target genes. The mechanism of interaction between peptide *pedibin* and *CnNK-2* remains to be elucidated.

concept with peptides acting as extrinsic positional signal molecules and homeodomain containing genes as regulators of position-dependent gene expression. How does all work together? How do the multiple genes contribute to this specific differentiation event? The interactions we have characterized can be described in terms of a model (Fig. 4B) where the differentiation of foot specific cells in *Hydra* occurs in response to a signaling gradient of peptides such as *pedibin/Hym-346* at the basal end of the axis. The primary function of these peptides is to induce expression of *CnNK-2* (and interacting transcriptional regulators) in a temporally and spatially overlapping pattern. *CnNK-2*, in turn, controls localized expression of *pedibin* by either repression or activation. In addition, *CnNK-2* maintains its own expression by a feedback loop. In this view, *CnNK-2* and *pedibin* together synergistically regulate foot specific differentiation. *Pedibin* is upstream of, and controls, the expression of *CnNK-2*. The latter, in turn, presumably controls not only *pedibin* and its own expression but also genes further downstream whose products directly are involved in foot specific differentiation (e.g. foot specific peroxidases). The challenge now at hand is (i) to understand how inductive inputs, which include those mediated by *pedibin*, are integrated molecularly at the promoter level of the *CnNK-2* gene and (ii) to identify the corresponding effector genes. We anticipate that *CnNK-2* and *pedibin* are components of a complex regulatory network and that an understanding of cell decision making in basal metazoans such as *hydra* as in higher organisms requires an analysis of this 'network architecture' (Davidson, 2001).

## 4. Materials and methods

### 4.1. Animals

We used *Hydra vulgaris* strain Basel 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 *CnNK-2* and *pedibin* gene

To isolate the 5' flanking sequence of the two foot specific genes *CnNK-2* and *pedibin*, we applied a PCR-based approach with adapter-ligated restricted genomic DNA. DNA was digested with different restriction enzymes (producing GATC-, CTAG- or CG-overhangs or blunt ends) and subsequently ligated to 'splinkerettes', a special type of vectorette (Devon et al., 1995). Briefly, a top-strand (5'-CGAATCGTAA CCGTTCGTAC GAGAATTCGT ACGAGAATCG CTGTCCTCTC CAACGAGCCA AGG-3') and different bottom strands according to the overhangs produced by the restriction enzymes (5'-GATCCCTTGG CTCGTTTTTTT TTTGCAAAAA-3' for GATC-, 5'-CTAGCCTTGG CTCGTTTTTTT TTTGCAAAAA-3' for CTAG-, 5'-CGCCTTGGCT CGTTTTTTTTT TGCAAAAA-3' for CG-, 5'-CCTTGGCTCG TTTTTTTTTT GCAAAAA-3' for blunt ends) were designed and annealed. Following removal of excess adapter molecules, the genomic DNA-Splinkerette preparation was used as template for a nested PCR combining specific primers against known coding or 5'

flanking sequence and pointing towards the unknown 5' region. PCR was performed using Platinum Taq DNA Polymerase (Invitrogen) and a touchdown protocol for the first PCR. The identity of generated fragments 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 isolation of 5' flanking sequence of *CnNK-2*, a first nested PCR using outer primer 5'-CTTATTGAAG TCTCGCTCAG TTTC-3', inner primer 5'-TTTAATTTGC TAGAAGGTGA TTAC-3', and genomic DNA digested with *Bcl* I lead to the isolation of a 401 bp fragment, showing a short overlap with the *CnNK-2* 5'-UTR. With an additional specific primer derived from this new sequence, a second nested PCR was performed using outer primer 5'-TTTAATTTGC TAGAAGGTGA TTAC-3', inner primer 5'-CGTCACGTAA AGGTCGGAAT AAG-3', and genomic DNA digested with *Hin* 6I. The resulting 1130 bp fragment showed a 260 bp overlap with the first one. For isolation of 5' flanking sequence of *pedibin*, we used genomic DNA digested with *Xba* I and specific primers derived from the coding region (outer primer: 5'-TTCGGAATGA TTTGCAAGAG-3'; inner primer 5'-GGCGGCTAAT TTTTCTTGC-3') for nested PCR. The resulting 958 bp fragment contained 872 bp of 5' flanking region. For analysis of the 5' flanking sequence TFSEARCH version 1.3 and MatInspector were used.

#### 4.3. Electrophoretic mobility shift assay

Nuclear protein extracts from *H. vulgaris* were prepared as described (Endl et al., 1999). For EMSAs, double-stranded oligonucleotides were end-labeled with  $\gamma$ -<sup>32</sup>P ATP. The binding reaction, with 1  $\mu$ g protein and 5–10 fmol target DNA, was incubated for 60 min on ice in 5  $\times$  binding buffer (50 mM Tris/HCl pH 7.7, 2.5 mM EDTA, 5 mM MgCl<sub>2</sub>, 250 mM NaCl, 20% glycerin, 2.5 mM DTT) containing 1  $\mu$ g poly(dI-dC). Unlabeled competitor DNA was added to the reaction in 10-fold molar excess. Electrophoresis was carried out on a 6% non-denaturing polyacrylamide gel and visualized by autoradiography. The sequence of the oligos used as labeled probes and specific competitors is shown in Fig. 3. The sequence of mutated oligos NK-2(1/2)-mu1 and NK-2(1/2)-mu2 is shown in Fig. 2B. As unrelated competitor we used oligonucleotide CREB (TGG CCC ATC AAA TTA ATT TTT TTC TAA).

#### 4.4. Recombinant protein production

The *CnNK-2* cDNA coding for the full length protein was cloned into the pCR T7 TOPO TA expression vector (Invitrogen) and expressed in *Escherichia coli* strain BL21(DE3). Recombinant *E. coli* were grown at 37 °C to an optical density of 0.6–0.9 and induced by addition of isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG). Cells were harvested after 20 h of induction, washed in cold TEN

buffer (10 mM Tris–HCl, 1 mM EDTA and 100 mM NaCl), and resuspended in 50 mM Na<sub>2</sub>PO<sub>4</sub> pH 8.0, 300 mM NaCl, 10 mM Imidazole. Two protocols were used for preparation of the bacterial lysate containing recombinant protein. First, following addition of 1% NP40, 10 mM MgCl<sub>2</sub>, Pefablock, DNase, and lysozyme to 1 mg/ml, cells were incubated for 30 min on ice. After centrifugation, the supernatant was concentrated using Millipore Microcon Centrifugal Filter Devices. In the second approach, for cell lysis bacteria were incubated at 4 °C in 50 mM Tris/HCl pH 8.0, 1 mM EDTA, 100 mM NaCl, 50 mM phenylmethylsulfonyl fluoride (PMSF) and lysozyme. Following addition of DNase I (20  $\mu$ l of a 1 mg/ml solution per gram bacteria), cells were incubated at 37 °C for 15 min and centrifuged at 15 000g for 2 h at 4 °C. The supernatant containing the recombinant protein was precipitated with ammonium sulfate. Following resuspension in ZKS buffer (20 mM HEPES/KOH (pH 7.9), 420 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.1 mM EDTA (pH 8.0), 20% glycerin, and 4 mM Pefabloc), the protein solution was centrifuged at 6000g for 15 min. To remove residual ammonium sulfate and DNase I, the supernatant was ultrafiltered (Centriplus YM-30, Millipore) and resuspended in ZKS.

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