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Expression of *Cnox-2*, a HOM/HOX homeobox gene in hydra, is correlated with axial pattern formation

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SUMMARY

***Cnox-2* is a HOM/HOX homeobox gene that we have identified in the simple metazoan *Hydra vulgaris* (Cnidaria: Hydrozoa). *Cnox-2* is most closely related to anterior members of the Antennapedia gene complex from Drosophila, with the greatest similarity to *Deformed*. The *Cnox-2* protein is expressed in the epithelial cells of adult hydra polyps in a region-specific pattern along the body axis, at a low level in the head and at a high level in the body column and the foot. The expression pattern of *Cnox-2* is consistent with a role in axial pattern formation. Alteration of hydra axial patterning by treatment with diacylglycerol (DAG) results in an increase of head activation down the body column and in a coordinate reduction of *Cnox-2* expression in epithelial cells in 'head-like' regions. These results suggest that *Cnox-2* expression is negatively regulated by a signaling pathway acting through protein kinase C (PKC), and that the varying levels of expression of *Cnox-2* along the body axis have the potential to result in differential gene expression which is important for hydra pattern formation.**

Key words: hydra, Cnidaria, HOM/HOX, homeobox, diacylglycerol, pattern formation

INTRODUCTION

The homeobox genes of the HOM/HOX class appear to be involved in anterior-posterior patterning in all organisms where they have been studied. HOM/HOX genes share a highly conserved region, the homeodomain, which binds DNA in a sequence-specific manner to regulate the transcription of other genes (see reviews by Scott et al., 1989; McGinnis and Krumlauf, 1992). In addition to amino acid sequence conservation, the HOM/HOX genes also share a conserved genomic organization, in which the genes are linked in a gene complex. HOM/HOX gene complexes have been conserved over hundreds of millions of years in animals from the nematodes to the chordates (Akam, 1989; Kessel and Gruss, 1990; Burglin et al., 1991; Kenyon and Wang, 1991), suggesting that the HOM/HOX genes and the gene complex arose before the divergence of the nematodes from the metazoan mainline. The recent discovery of HOM/HOX genes in other members of phylum Cnidaria, one of the oldest animal phyla (Schierwater et al., 1991; Murtha et al., 1991; Schummer et al., 1992), prompted us to look for HOM/HOX genes in hydra and to explore the potential role of these genes in hydra pattern formation. An understanding of the function of HOM/HOX genes in hydra should add significantly to our understanding of the origins and functions of HOM/HOX-class genes.

We isolated fragments of three HOM/HOX homeobox genes, *Cnox-1*, *Cnox-2*, and *Cnox-3*, from *Hydra vulgaris*. We further characterized one gene, *Cnox-2*, and explored the expression and the potential role of this gene in hydra pattern formation. Hydra has a well-established axial patterning system, which has been the focus of a considerable amount of research (e.g. MacWilliams, 1983a,b; Bode and Bode, 1984). Adult hydra polyps consist of a body column with dissimilar ends, the head and the foot. Unlike many animals that only undergo pattern formation as embryos, the tissue dynamics of hydra require the animal to maintain its axial patterning system in the face of continuous tissue displacement during the life of the adult (Bode and Bode, 1984). Cells proliferate in the body column and displace the tissues toward the ends, where cells are lost by sloughing (Campbell, 1967). Additional loss of cells arises through the displacement of tissues into buds. In the face of continuous tissue displacement, the size and pattern of the polyp are maintained. As a consequence of this growth habit, the genes that control pattern formation must be expressed in the appropriate regions of the polyp even while cells are displaced into and out of those regions.

The expression pattern of the *Cnox-2* protein in hydra epithelial cells is consistent with a role in axial pattern formation. *Cnox-2* is expressed at a high level in the foot and body column, and at a low level in the head. If the function of the HOM/HOX homeobox genes has been conserved from the cnidarians to the chordates, then we would inter-

pret the *Cnox-2* expression pattern as specifying the region of the polyp which is 'not-head'. We tested this interpretation by changing the size of the region of the polyp that is head-like and assaying for coordinate changes in the expression of *Cnox-2*. We found that high-level *Cnox-2* expression is absent from the body column tissue that we had experimentally converted to head-like tissue. These data argue strongly that *Cnox-2* plays a role in hydra axial pattern formation and that the functions of HOM/HOX genes have been conserved over the hundreds of millions of years that separate the members of the cnidaria and the more recent animal phyla.

MATERIALS AND METHODS

Cloning of *Cnox* genes

Fragments of three *Cnox* genes were isolated by PCR using first strand cDNA prepared from adult *H. vulgaris* polyp poly(A)+ RNA or *H. vulgaris* whole-cell DNA as a template. First-strand cDNA was prepared as described previously (Fisher and Bode, 1989) and provided by Doug Fisher. Whole-cell DNA was isolated according to Davis et al. (1980). Primers for PCR were designed to recognize nucleotides 31-62 and nucleotides 139-171 in HOM/HOX homeobox sequences and were modified from primer sequences provided by Michael Murtha and Frank Ruddle (Yale University; Schierwater et al., 1991; Murtha et al., 1991). To facilitate cloning of the PCR products, a sequence containing *Pst*I and *Bam*HI restriction sites was included at the 5′ end of the 5′ primer. A sequence containing *Hin*dIII and *Eco*RI sites was included at the 5′ end of the 3′ PCR primer. The sequences of the two primers were as follows.

5′ primer, 5′-TACCAGAC(C/G)(C/T)TGGA(A/G)CTGGA-(A/G)AA(A/G)GA(A/G)TT(C/T)CA-3′;

3′ primer, 5′-AGAATTCAAGCTT(C/T)TTCCA(C/T)TTC-AT(C/G)C(G/T)NCG(A/G)TT(T/C)TG(A/G)AACCA(G/A/T)AT-3′. The primer concentrations and reaction buffers were the same as those used by Schierwater et al. (1991). The samples received an initial denaturation at 95°C for 5 minutes. The cycling conditions for the amplification reactions were as follows: denaturation 95°C 1 minute, annealing 37°C 1 minute, elongation 72°C 1 minute, for 5 cycles; denaturation 95°C 1 minute, annealing 45°C 1 minute, elongation 72°C 1 minute, for 30 cycles; followed by a final elongation at 72°C for 10 minutes. Products of the expected size (166 bp) were digested with *Bam*HI and *Hin*dIII to generate ends for ligation into the multiple cloning site of the phagemid vector pBS- (Short et al., 1988) and isolated from 3% NuSieve agarose gels (FMC) by electroelution. Single-stranded template production and chain termination sequencing were carried out as described previously (Bosch et al., 1989). Using sequence information from the initial PCR product, an additional clone was isolated by inverse PCR (Ochman et al., 1990) of DNA from an amplified *H. vulgaris* λgt11 cDNA library (Bosch et al., 1989) to generate a large DNA fragment to use as a probe in library screens. DNA was extracted from an aliquot of the library, digested with *Eco*RI to separate cDNA inserts from phage vector DNA and circularized by ligation at low concentration. Inverse PCR was carried out using primers corresponding to nucleotide sequences within the initial PCR product. Restriction sites were included at the ends of the primers to facilitate cloning. The samples received an initial denaturation at 95°C for 5 minutes. The conditions for PCR were: denaturation 95°C 1 minute, annealing 45°C 2 minutes, elongation 72°C 3 minutes, for 35 cycles; followed by a final elongation at 72°C for 10 minutes. Of the several PCR products that were obtained, an 800 bp product was cloned and confirmed by sequence analysis to be derived from the same gene as the initial PCR product. Screening of the cDNA library with a 500 bp fragment from the 800 bp inverse PCR product was used to isolate additional clones. One of these clones contained a 1 kb insert. This insert was subcloned and its complete sequence determined.

Northern and Southern analyses

2 μg poly(A)+ RNA from whole *H. vulgaris* polyps was fractionated by electrophoresis in a formaldehyde-containing agarose gel. *Xenopus laevis* ribosomal RNAs were included in the gel as size standards. RNA was transferred from the gel to a Gene Screen membrane (DuPont) and hybridized as suggested by the manufacturer. The probe, consisting of the 500 bp fragment used in the library screen described above, was labeled with [^{32}P]dCTP using the random primer method (Feinberg and Vogelstein, 1984). The particular probe fragment was chosen because it lacks the sequence encoding the homeodomain, reducing the likelihood of cross-reactivity with related genes. The portion of the membrane containing the ribosomal RNA standards was stained with methylene blue (Sambrook et al., 1989). Whole polyp DNA was isolated according to Davis et al. (1980). Aliquots of the DNA (20 μg) were digested with restriction enzymes, fractionated in an agarose gel, transferred to Gene Screen and probed with the same fragment as was used for the RNA hybridization using the protocol supplied by the manufacturer.

Synthesis of *Cnox-2* protein in *E. coli* and polyclonal antibody production

The 5′ 540 nucleotides of the *Cnox-2* cDNA were cloned into the T7 expression vector pET-3 (Studier et al., 1990), to produce the plasmid pT7Cnox-2 (Fig. 5). We used PCR to convert the sequence containing the start methionine of *Cnox-2* to an *Nde*I site and the sequence encoding the first amino acid of the homeodomain (amino acid #181; Fig. 3) into an in-frame stop codon followed by a *Bam*HI site for cloning into pET-3. The recombinant p20^{Cnox-2} encoded by pT7Cnox-2 contains only hydra protein sequence, it is not a fusion protein. *E. coli* BL21(DE3) cells were transformed with pT7Cnox-2 and induced to express p20^{Cnox-2} protein in the presence of IPTG (Studier et al., 1990). The cells were lysed in a French pressure cell and the p20^{Cnox-2} protein, which was contained in small inclusion bodies, was partially purified by centrifugation at 100,000 ***g***. SDS-PAGE gel slices containing p20^{Cnox-2} protein were emulsified and injected into three rabbits to produce anti-*Cnox-2* polyclonal antibodies. Immunizations and serum collections were performed commercially (Hazelton Laboratories, Denver, PA). IgG fractions of preimmune and immune sera were prepared by protein G chromatography (Björck and Kronvall, 1984; Åkerström and Björck, 1986), using a method adapted from Ey et al. (1978).

In vitro translation of *Cnox-2* RNA and immunoprecipitation

Synthetic *Cnox-2* mRNA was made by run-off transcription of the *Cnox-2* cDNA using a Riboprobe transcription system (Promega) and the protocols supplied by the manufacturer. The mRNAs were translated in a rabbit reticulocyte lysate (Promega) in the presence of ^{35}S-translabel (ICN) and immunoprecipitated with anti-*Cnox-2* using the method of Anderson and Blobel (1983).

Immunocytochemistry

In both whole-mount and macerate immunocytochemistry, we used 'standard polyps', i.e. adult animals with one old bud and one young bud. The buds were retained in whole-mount prepara-

tions, they were removed before regional dissections in macerate preparations.

In whole-mount immunocytochemistry, polyps of *Hydra vulgaris, H. magnipapillata* and *H. oligactis* were starved at least 48 hours before use. Polyps were relaxed in 2% urethane in Hydra medium (1 mM $CaCl_2$ in Arrowhead spring water) and fixed in Lavdowsky's fixative (50:10:4:40 ethanol:formaldehyde:glacial acetic acid:water) for 1 hour. Fixed polyps were given three 10 minute washes in PBS-Triton (0.25% Triton X-100 in PBS) and blocked for 1 hour in 10% neonatal calf serum in PBS (blocking solution) at 22°C. After blocking, the polyps were incubated in a 1:30 dilution of the protein G-purified anti-*Cnox-2* antibody in blocking solution at 4°C for 12 hours, followed by three washes in PBS-Triton. Incubation in the secondary antibody solution, a 1:100 dilution of a biotinylated goat anti-rabbit IgG (Zymed) in blocking solution, was for 1 hour at 22°C followed by three washes in PBS-Triton and a 1 hour incubation in 1:25 streptavidin-FITC (Zymed) in PBS. Polyps were counter-stained in 0.01% Evans Blue for 10 minutes to mask endogenous fluorescence (Wilby and Webster, 1970; Sugiyama and Fujisawa, 1978). The polyps were washed with PBS three to five times to remove excess Evans Blue and mounted in 1:10 PBS:glycerol containing 50 mg/ml n-propyl gallate to reduce bleaching.

Regional dissections of 48 hour unfed *Hydra magnipapillata* polyps were made to produce seven defined regions: head, four equal sized lengths of the body column (regions I-IV), peduncle and foot (Fig. 7D); no bud tissues were included in the macerates. The isolated regions were disassociated into individual cells in maceration fluid (1:1:26 glycerol:glacial acetic acid:water; David, 1973). These macerated preparations were distributed dropwise to microscope slides subbed with 5% gelatin and 0.5% chrome alum, the cells were spread on the slides with 1 drop of 1% Tween-80 in water and allowed to dry overnight at 22°C. The slides were washed twice in PBS for 10 minutes and incubated in blocking solution for 30 minutes. The macerates were incubated for 1 hour in a humid chamber with a 1:20 dilution of the anti-*Cnox-2* antibody in blocking solution followed by two washes with PBS. Staining was performed batchwise using a Vectastain Elite system (Vector Laboratories) and the protocol supplied by the manufacturer. The horseradish peroxidase color reaction was developed for 7 minutes and stopped by a 10 minute wash in tap water. The slides were dehydrated for 2 minutes each in 70, 95 and 100% EtOH and mounted in Euparal (Asco Laboratories). The slides were examined at a magnification of 400× with phase-contrast microscopy and 200 epithelial cells per slide were scored as having intense, light or no HRP stain. The slides were counted blind twice and the average difference between counts was 5.1%. The data shown are for the second count.

DAG treatment

Hydra magnipapillata, strain 105, polyps supplied by Tsutomu Sugiyama (National Institute of Genetics, Mishima, Japan) were treated periodically with 1,2-dioleoylglycerol-C8, a diacylglycerol (DAG), at a concentration of 33 μg/ml over 12 days (Müller, 1989). On day 1 the polyps were incubated in DAG for 30 minutes, on days 2-12 the polyps were incubated in DAG for 2 hours. After 12 days of treatment, the polyps were dissected into the same regions as normal polyps, macerated and stained with the anti-*Cnox-2* antibody at the same time, in the same solutions, as the normal regional macerates.

RESULTS

Isolation of *Cnox* genes from *Hydra vulgaris*

We used PCR with degenerate oligonucleotide primers to isolate HOM/HOX-related sequences from *Hydra vulgaris* adult polyp first-strand cDNA and whole-cell DNA. An initial survey of the DNA fragments isolated in this screen yielded fragments of three genes of this class (Fig. 1). *Cnox-1* and *Cnox-3* were only isolated from PCR with whole-cell DNA as a template, while *Cnox-2* was isolated from both whole-cell DNA and first-strand cDNA. *Cnox-1* and *Cnox-3* are similar to each other and they are related to the hydrozoan genes *Cnox-1* from *Eleutheria dichotoma* (Schierwater et al., 1991), and *SAox3* from *Sarsia* sp. (Murtha et al., 1991). *Cnox-2* is the homologue of *Cnox-2-Ed* from *E. dichotoma* and *Cnox-2-Hs* from *Hydractinia symbiolongicarpus* (Schierwater et al., 1991), *SAox2* from *Sarsia* sp. (Murtha et al., 1991), and *cnox2* from *Chlorohydra viridissima* (Schummer et al., 1992).

Southern hybridization of *Cnox-2* to hydra whole-cell DNA digested with three restriction endonucleases revealed a single band for each digest, demonstrating that *Cnox-2* is a single-copy member of the *Hydra vulgaris* genome (Fig. 2A). Northern analysis of *Cnox-2* hybridized to hydra adult polyp poly(A)+ RNA showed that *Cnox-2* encodes a single mRNA of 1.4 kb (Fig. 2B). Using the approach described in the Materials and Methods, we isolated a *Cnox-2* cDNA of 991 nucleotides from an adult polyp *Hydra vulgaris* cDNA library, which predicts a protein of 255 amino acids (Fig. 3).

Cnox-2 is related to the HOM/HOX-class homeobox genes

The predicted *Cnox-2* protein has a structure that is typical of homeodomain proteins of the HOM/HOX class; the homeodomain is near the carboxyl terminus and the protein is rich in serine and proline residues outside the homeodomain. Amino acid sequence comparisons between the predicted homeodomains of *Cnox-2* and members of the Antennapedia (ANT-C) and Bithorax (BX-C) gene complexes from *Drosophila melanogaster* show a remarkably high degree of sequence conservation given the evolutionary distance between the cnidaria and the arthropods (Fig. 4A). The *Cnox-2* homeodomain is more similar to members of the Antennapedia complex (ANT-C) than to members of the Bithorax complex (BX-C), and *Cnox-2* has the highest amino acid identity with the *Deformed* gene. Thus, we consider *Cnox-2* to be a relative of the anterior members of the *Drosophila* Antennapedia complex. Outside the homeodomain, *Cnox-2* lacks amino acid sequences which aid in identification, with the exception of the amino terminus. Comparisons of this region of *Cnox-2* with the amino termini of representative HOM/HOX-class genes reveals additional similarity between *Cnox-2* and the *Deformed*-subclass of genes; all of the sequences in the subclass except *Deformed* share the amino acid sequence SFLIXS near the amino terminus (Fig. 4B).

Cnox-2 expression is region-specific in adult hydra

To examine the expression pattern of *Cnox-2* in hydra, we raised antisera against a portion of the *Cnox-2* protein produced in *E. coli.* The homeodomain was excluded from the protein to avoid cross-reactivity with related proteins. A segment encoding amino acids 1-180 with compatible ends

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Antp     yqtlelekefhFNRYLTRRRRIEIAHALCLTERQIKiwfqnrrmkwkk
Cnox-1              --HF-KKE--T-LSKK-N-S-----
Cnox-2              N----S-L---Q--AI-D---K-V-
Cnox-3              --HF-KKE--A-L-KH-N-S-----
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Fig. 1. Predicted amino acid sequences of three *Cnox* gene fragments isolated from *Hydra vulgaris* whole-cell DNA or cDNA by PCR. The sequence of a portion of the *Antennapedia* homeodomain is given for comparison, dashes indicate identity with the *Antennapedia* sequence; the sequences encoded by the PCR primers are shown in lower case.

for cloning into the pET-3 (Studier et al., 1990) vector was generated by PCR from the original *Cnox-2* cDNA clone (Fig. 5A; see Methods). Bacteria containing the pT7Cnox-2 plasmid produced a doublet of proteins absent from cells containing the pET-3 vector without an insert (bands indicated by the arrows in Fig. 5B). The predicted relative molecular mass of the protein was 19.5×10^3 M_r; the two bands are 26.8 and 25.4×10^3 M_r. This anomalous mobility in SDS-PAGE is likely due to the large number of proline residues in the protein (Fig. 3). The lower (25.4×10^3 M_r) band may be due to proteolysis or premature termination. The two proteins could be recovered in a high speed pellet from lysed bacteria (Fig. 5B). Gel slices containing the two proteins were used to raise antibodies in three rabbits. To confirm that the antibodies recognize $p30^{Cnox\text{-}2}$, the complete protein was produced by translation in vitro and tested for cross-reaction with antiserum in an immunoprecipitation assay as described by Anderson and Blobel (1983). The IgG fraction of immune serum, but not the IgG fraction of preimmune serum, precipitated a protein of the expected size, 29.5×10^3 M_r (indicated by the arrow in Fig. 5C). The additional bands are probably due to premature translation termination, perhaps due to the unusual codon bias in hydra genes (Bosch et al., 1989; Fisher and Bode, 1989; Keppel and Schaller, 1991; Kurz et al., 1991).

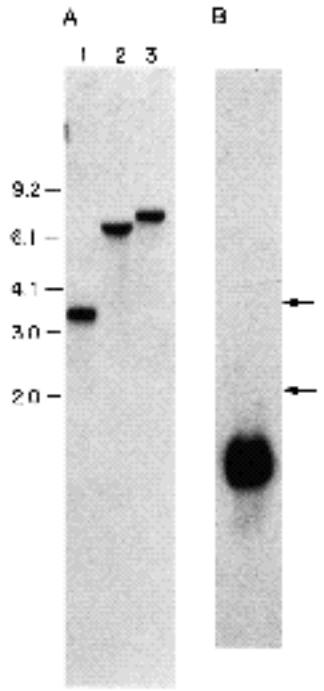

Fig. 2. Identification of *Cnox-2* DNA sequences and a *Cnox-2* transcript in *Hydra vulgaris*. (A) Hybridization to whole polyp DNA digested with *Eco*RI (lane 1), *Hin*dIII (lane 2), and *Pst*I (lane 3). (B) Hybridization to whole polyp poly(A)+ RNA; the arrows indicate the positions of *Xenopus laevis* ribosomal RNA standards. The 28S rRNA is 4110 nucleotides in length (Ware et al., 1983) and the 18S rRNA is 1825 nucleotides in length (Salim and Maden, 1981). The probe in both cases was a 500 bp fragment of *Cnox-2* excluding the homeobox.

As a further test of the specificity of the anti-*Cnox-2* antibodies, we used indirect immunofluoresence to determine whether the staining was confined to the nucleus, as expected for a transcription factor. In whole mounts of normal adult *Hydra vulgaris* polyps treated with immune sera from each of the three rabbits that were immunized, nuclei of epithelial cells and nuclei of large interstitial cells in the nematocyte differentiation pathway were stained (Fig. 6A, B). None of the corresponding preimmune sera gave any staining. The anti-*Cnox-2* antibodies also stained nuclei in *Hydra oligactis* (data not shown) and *H. magnipapillata* (see below). In all three species, we observed stained nuclei throughout the adult polyps and in developing buds; in each case, the body column of the polyp was intensely stained, with less intense staining in the head and especially in the tentacles. These data are consistent with the results of northern blots of RNA from the upper, middle and lower thirds of *Hydra vulgaris* polyps in which *Cnox-2* transcripts were detected in all three regions (data not shown). While it is still possible that the immune sera that we produced recognize some unknown hydra nuclear protein by chance, the combined data from the immunoprecipitation showing that antiserum recognizes the *Cnox-2* protein in vitro and the data from whole-mount immunocytochemistry showing that the three different antisera recognize a nuclear protein in three species of hydra, strongly suggest that the pattern of staining that we have observed reflects the distribution of the *Cnox-2* protein in hydra.

Pattern formation in hydra primarily involves epithelial cells (Rubin and Bode, 1982; Takano and Sugiyama, 1983, 1984; Nishimiya et al., 1986), for this reason, we focused our attention on the expression of *Cnox-2* in epithelial cells. To determine whether *Cnox-2* is expressed uniformly in epithelial cells along the body axis, we macerated defined regions of *Hydra magnipapillata* polyps. Maceration of hydra tissue yields a suspension of cells in which the cellular morphology is preserved so that individual cells may be identified (Fig. 6C, D; David, 1973). Before maceration, the polyps were dissected into seven regions: head, four body column segments of equal length (I-IV), peduncle and foot (Fig. 7D). The regional macerates were stained with the anti-*Cnox-2* antibody (see Methods), and the epithelial cells were counted blind and scored as having intense, light, or no nuclear stain (Fig. 6C, D).

The data from the analysis of the regional macerates show that the expression of *Cnox-2* is complex, with variation both in the intensity of staining of epithelial nuclei within regions and in the proportions of stained nuclei along the body axis. Essentially all of the epithelial cells of the foot and body column are stained (Fig. 7A), and up to 90% are stained intensely (solid line, Fig. 7A), indicating a high

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  1          M  S  T  S  F  L  I  D  S  L  I  H  E  K  E  K  Y     17
  1  GAATTCCGAATGTCTACTTCGTTTTTAATAGATTCTCTAATACATGAAAAAGAAAAGTAT   60

 18  K  I  R  Q  Q  P  G  T  S  F  L  F  R  E  S  S  P  P  D  R     37
 61  AAGATACGGCAGCAGCCTGGAACATCTTTTTTATTTCGTGAATCATCTCCTCCAGATCGA   120

 38  S  P  S  Y  S  P  G  A  S  M  I  R  Y  S  N  S  S  S  P  R     57
121  TCGCCGAGTTATTCACCCGGTGCGTCAATGATTAGATATTCCAATTCTTCTTCTCCAAGA   180

 58  S  L  D  S  P  I  N  P  L  D  R  H  P  L  E  R  V  H  Q  V     77
181  AGTTTAGATTCACCTATAAATCCATTGGATCGACACCCTCTTGAAAGAGTACATCAAGTA   240

 78  V  S  C  M  R  G  P  S  M  C  N  C  C  R  P  P  A  V  Q  P     97
241  GTTAGTTGTATGAGAGGACCTTCGATGTGTAATTGTTGTCGGCCTCCGGCTGTTCAACCT   300

 98  M  C  T  V  C  E  P  R  E  P  G  E  G  T  S  S  Q  Y  P  Y    117
301  ATGTGTACAGTATGTGAACCTAGGGAACCAGGTGAAGGTACCTCTTCACAATATCCTTAT   360

118  T  R  E  P  H  E  H  T  R  G  L  Y  G  N  D  R  S  R  L  F    137
361  ACCCGCGAACCTCATGAGCATACAAGAGGCTTGTATGGAAATGATAGATCAAGACTTTTT   420

138  P  I  L  S  P  L  H  G  Q  R  A  Q  F  S  P  N  Y  V  Y  D    157
421  CCAATATTATCACCTTTACACGGGCAAAGAGCGCAGTTTTCCCCGAATTACGTTTACGAT   480

158  L  E  L  R  H  S  R  Q  L  Q  L  Q  H  Q  E  H  E  T  D  L    177
481  TTGGAACTTCGTCATTCCCGTCAACTTCAACTGCAACACCAAGAACACGAAACAGATCTT   540

178  Y  G  K  S  K  R  I  R  T  A  Y  T  S  I  Q  L  L  E  L  E    197
541  TACGGAAAATCTAAACGCATTCGAACCGCGTATACTAGCATTCAGTTACTTGAACTTGAA   600

198  K  E  F  Q  N  N  R  Y  L  S  R  L  R  R  I  Q  I  A  A  I    217
601  AAAGAGTTTCAAAATAATCGTTATCTTTCGAGATTACGGAGAATCCAGATAGCTGCTATT   660

218  L  D  L  T  E  K  Q  V  K  I  W  F  Q  N  R  R  V  K  W  K    237
661  CTCGATCTAACAGAAAAACAAGTTAAAATATGGTTTCAAAATCGACGTGTAAAATGGAAA   720

238  K  D  K  K  G  Y  S  Y  S  P  T  G  S  P  Q  S  P  E  *       255
721  AAAGATAAGAAAGGATATAGCTATTCCCCTACTGGAAGTCCGCAATCTCCAGAATAACTA   780

781  CCGGTTTTCTTTCTCAAAAGTTCTTCTGCATCTTTCAAAACGAAGATATTTTTTTATAAA   840

841  AACAAACGATATTAAAATGATTTCCCATAGTCATTTTATAATATGTACATAAAAATTCAT   900

901  CAATAATTTATTCATCTTTTACGACATTTTTTTGTTTATCTTTGTATACTGAAACTTTAT   960

961  TTTCAGGTGAAGCGTAAGTTGTTCCGAATTC  991
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Fig. 3. DNA and predicted amino acid sequence of *Cnox-2*. The DNA sequence encoding the homeodomain is doubly underlined. The underlined nucleotides at the beginning of the sequence are derived from a synthetic *Eco*RI linker (GGAATTCC) added to the cDNA prior to cloning in λgt11. The underlined *Eco*RI site at the end of the sequence is not derived from a linker and is presumably an authentic *Eco*RI site in the clone which escaped methylation by *Eco*RI methylase during cDNA library preparation. The *Cnox-2* DNA sequence has been deposited in the GenBank/EMBL database under accession number M62870.

level of *Cnox-2* expression in the foot and body column. There is a sharp reduction of labeling in the head; the cells are either lightly stained (56%) or unstained (44%; Fig. 7A).

Diacylglycerol treatment alters *Cnox-2* expression along the body axis

If *Cnox-2* is expressed differentially in epithelial cells in the body column relative to cells in the head as our whole mounts and macerates of normal polyps have shown, then polyps in which body column tissue has been converted to head tissue should have an altered pattern of *Cnox-2* expression. Müller (1989) has shown that periodic treatment of *Hydra magnipapillata* polyps with diacylglycerol (DAG) results in the ectopic appearance of head structures, tentacles and complete secondary heads, in the body column of the polyps. In addition, excision of the gastric region of the body columns of treated animals results in double-headed regenerates, instead of regenerates with a head and foot in the same tissue orientation as the head and foot before excision (Müller, 1989). These data indicate that DAG treatment makes the excised tissue head-like.

We treated *Hydra magnipapillata* polyps with DAG to assay for altered expression of *Cnox-2*. After 6 days of treatment, 20 polyps were bisected below the head and above the peduncle and the resulting body column segments were assayed for altered head regeneration. 9 of 20 (45%) isolated body columns regenerated heads at both ends. After 12 days of treatment, many polyps had ectopic tentacles in the lower body column (region III; Fig. 7D), further indicating an effect of the DAG treatment on the axial patterning of the polyps. At the end of the DAG treatment, polyps that lacked ectopic tentacles were dissected into the same seven regions as normal polyps (see above), macerated and stained with the anti-*Cnox-2* antibody.

Our data show that the *Cnox-2* expression pattern is significantly altered by DAG treatment. Intensely stained cells (solid line, Fig. 7B) predominate in the lower body column; region IV, the peduncle and the foot. Above region IV, the majority of epithelial cells are either lightly stained or unstained. The sharp decrease in intensely stained nuclei in region III corresponds to the position of ectopic tentacles in some DAG-treated polyps (Fig. 7B). A comparison of the distributions of intensely stained cells in normal and DAG macerates indicates that DAG treatment has shifted the boundary of high-level expression of *Cnox-2* toward the foot (Fig. 7C). These data clearly show that the region of high *Cnox-2* expression is shifted toward the foot as the head-like region expands footward in DAG-treated polyps.

A

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Dfd       PKRQRTAYTRHQILELEKEFHYNRYLTRRRRIEIAHTLVLSERQIKIWFQNRRMKWKKDN
           || |||||  | |||||||  |||| | ||| ||  | | | | |||||||| |||||     42/60    ANT-C
Cnox-2    SKRIRTAYTSIQLLELEKEFQNNRYLSRLRRIQIAAILDLTEKQVKIWFQNRRVKWKKDK

p b       PRRLRTAYTNPQLLELEKEFHFNKYLCRPRRIEIAASLELTERQVKVWFQNRRMKHKRQT
            | |||||  |||||||||  | || | ||| ||| | ||| ||| |||||| | |      40/60    ANT-C
Cnox-2    SKRIRTAYTSIQLLELEKEFQNNRYLSRLRRIQIAAILDLTEKQVKIWFQNRRVKWKKDK

Scr       TKRQRTSYTRYQTLELEKEFHFNRYLTRRRRIEIAHALCLTERQIKIWFQNRRMKLKKEH
           || || ||  | |||||||  |||| | ||| ||  | ||| | |||||||| | ||     40/60    ANT-C
Cnox-2    SKRIRTAYTSIQLLELEKEFQNNRYLSRLRRIQIAAILDLTEKQVKIWFQNRRVKWKKDK

Antp      RKRGRQTYTRYQTLELEKEFHFNRYLTRRRRIEIAHALCLTERQIKIWFQNRRMKWKKEN
           || |  ||  | |||||||  |||| | ||| ||  | ||| | |||||||| ||||     40/60    ANT-C
Cnox-2    SKRIRTAYTSIQLLELEKEFQNNRYLSRLRRIQIAAILDLTEKQVKIWFQNRRVKWKKDK

lab       NNSGRTNFTNKQLTELEKEFHFNRYLTRARRIEIANTLQLNETQVKIWFQNRRMKQKKRV
              ||  |  || ||||||  |||| | ||| ||  | | | |||||||||| | ||     37/60    ANT-C
Cnox-2    SKRIRTAYTSIQLLELEKEFQNNRYLSRLRRIQIAAILDLTEKQVKIWFQNRRVKWKKDK

Abd-A     RRRGRQTYTRFQTLELEKEFHFNHYLTRRRRIEIAHALCLTERQIKIWFQNRRMKLKKEL
            | |  ||  | |||||||  | || | ||| ||  | ||| | |||||||| | ||     37/60    BX-C
Cnox-2    SKRIRTAYTSIQLLELEKEFQNNRYLSRLRRIQIAAILDLTEKQVKIWFQNRRVKWKKDK

Ubx       RRRGRQTYTRYQTLELEKEFHTNHYLTRRRRIEMAHALCLTERQIKIWFQNRRMKLKKEI
            | |  ||  | |||||||  | || | |||  |  | ||| | |||||||| | ||     36/60    BX-C
Cnox-2    SKRIRTAYTSIQLLELEKEFQNNRYLSRLRRIQIAAILDLTEKQVKIWFQNRRVKWKKDK

Abd-B     VRKKRKPYSKFQTLELEKEFLFNAYVSKQKRWELARNLQLTERQVKIWFQNRRMKNKKNS
              |  |   | |||||||  | | |   |   |  | ||| ||||||||| | ||     32/60    BX-C
Cnox-2    SKRIRTAYTSIQLLELEKEFQNNRYLSRLRRIQIAAILDLTEKQVKIWFQNRRVKWKKDK
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B

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Antp        MTMSTNNCESMT-SYFTNS…
Hox-1.1             MSSSYYVNA…
Xhox-36             MTSSYYVSA…
Quox-1              MSSSYYVNA…

Scr           MDPDCFAMSSYQFVNS…
Hox-2.1             MS-SYFVNS…
Hox-1.3             MS-SYFVNS…

Dfd                 MS-SFLMGY…
Hox-1.4           MTMS-SFLINS…
Hox-2.6           MAMS-SFLINS…
Xhox-1A           MRMS-SFLISS…
Cnox-2              MSTSFLIDS…
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Fig. 4. Comparison of *Cnox-2* protein sequences with other homeobox protein sequences. (A) Alignment of the homeobox sequence from *Cnox-2* with the homeoboxes of the genes from the *Drosophila melanogaster* Antennapedia (ANT-C) and Bithorax (BX-C) complexes. All of the sequences except that of *pb* are taken from Scott et al. (1989); the *pb* sequence is from Cribbs et al. (1992). Vertical lines indicate identical amino acids. The number of identities out of the 60 amino acids is indicated to the right of each alignment. (B) Comparison of the putative amino terminus of the *Cnox-2* protein sequence shown in Fig. 3 and the amino termini of the protein sequences predicted from several examples of *Antennapedia*-related (*Hox-1.1*, Xhox-36, Quox-1, *Antp*), *Scr*-related (*Scr*, *Hox-2.1*, *Hox-1.3*), and *Deformed*-related (*Dfd*, *Hox-1.4*, *Hox-2.6*, Xhox-1A) genes. A gap is introduced into some of the sequences to improve the alignment. The sources of the amino terminal sequences are as follows: *Scr* (LeMotte et al., 1989), *Hox-2.1* (Krumlauf et al., 1987), *Hox-1.3* (Oldenwald et al., 1987; Fibi et al., 1988), *Hox-1.1* (Kessel et al., 1987), Xhox-36 (Condie and Harland, 1987), Quox-1 (Xue et al., 1991), *Antennapedia* (Schneuwly et al., 1986), *Deformed* (Regulski et al., 1987), *Hox-1.4* (Galliot et al., 1989), *Hox-2.6* (Graham et al., 1988), and Xhox-1A (Harvey et al., 1986).

DISCUSSION

We have isolated a gene, *Cnox-2*, from *Hydra vulgaris* that is a relative of HOM/HOX-class homeobox genes; *Cnox-2* is most closely related to the anterior members of the Antennapedia complex (ANT-C) in *Drosophila*, and by extension *Cnox-2* is related to the homologous HOX genes in the vertebrates. Hydra is a member of the phylum Cnidaria, one of the oldest phyla of animals. The fact that HOM/HOX-class genes have been conserved over the hundreds of millions of years that separate the cnidaria and the chordates suggests that the functions performed by homeobox genes may be required by all multicellular animals.

It is not currently possible to address the question of whether the HOM/HOX gene complex has also been conserved from the cnidaria to the chordates. The fact that the gene complex arose before the divergence of the nematodes from the metazoan mainline (Burglin et al., 1991; Kenyon and Wang, 1991) suggests that the original gene complex arose early in animal evolution. We and other groups have isolated additional HOM/HOX-related gene fragments from a variety of cnidarians (Fig. 1; Schierwater et al., 1991; Murtha et al., 1991; Schummer et al., 1992); it is therefore possible that the cnidarian genes are contained in a gene complex. Additional data on the genomic organization of HOM/HOX genes in the cnidaria should be of considerable importance in understanding the origins and functions of HOM/HOX-class genes.

Many of the HOM/HOX genes are expressed in specific regions of developing embryos where they presumably mediate region-specific gene transcription. Our expression data suggest that *Cnox-2* may have a similar function in hydra. We have shown that the $p30^{Cnox\text{-}2}$ protein is expressed in a region-specific pattern along the body axis in adult hydra epithelia, with a high level of expression in the foot and body column and a low level of expression in the head. Our results differ from the only other study of cnidarian homeobox gene expression to date. Schummer et al. (1992) used a reverse-transcription PCR assay to determine the level of mRNA in regenerating heads and in the gastric region of a mutant strain of *Chlorohydra viridissima* for the *Cnox-2* homologue in this species. They found

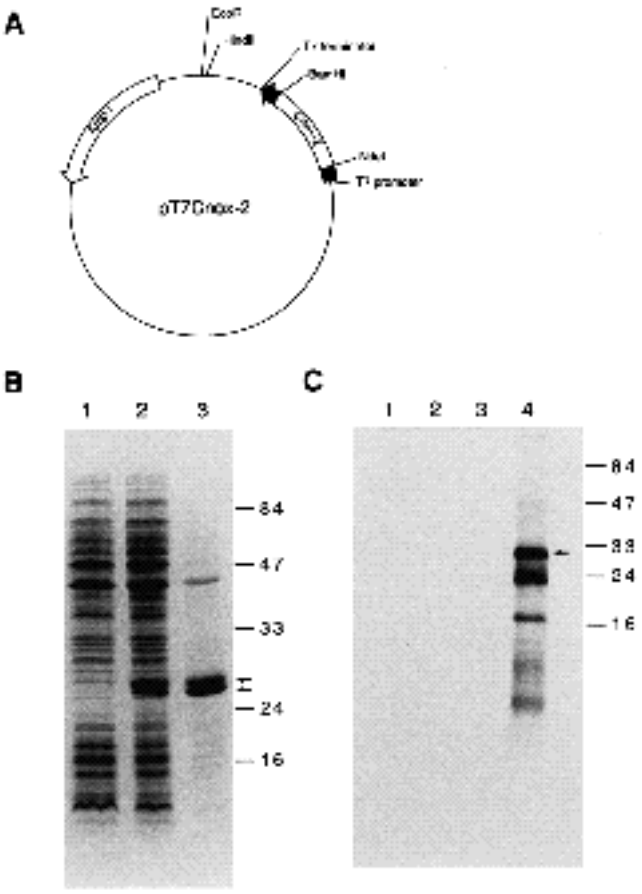

Fig. 5. Expression of *Cnox-2* protein in *E. coli* and immunoprecipitation of *Cnox-2* protein translated in vitro. (A) Structure of pT7Cnox-2, a plasmid containing a partial *Cnox-2* cDNA in the vector pET-3 (Studier et al., 1990). (B) Synthesis and partial purification of recombinant $p20^{Cnox\text{-}2}$ protein; the arrows indicate the *Cnox-2* protein. Lane 1, lysate from induced cells transformed with pET-3 plasmid without an insert; lane 2, lysate from induced cells transformed with the pT7Cnox-2 plasmid; lane 3, proteins in a 100,000 ***g*** pellet from lysate of induced cells transformed with pT7Cnox-2. (C) Immunoprecipitation of $p30^{Cnox\text{-}2}$ produced by translation of synthetic *Cnox-2* RNA in a rabbit reticulocyte lysate; the arrow indicates the *Cnox-2* protein. Lane 1, no RNA + preimmune IgG; lane 2, no RNA + immune IgG; lane 3, *Cnox-2* RNA + preimmune IgG; lane 4, *Cnox-2* RNA + immune IgG.

slightly higher levels of *Cnox-2* mRNA in the regenerating head than in the body column. Since it is difficult to compare directly mRNA levels in a regenerating polyp of one species to the distribution of protein in a normal adult polyp in another species, we are not currently able to reconcile these discrepant results. In addition, Schummer et al. used a mutant strain of hydra in which head patterning is altered. This further complicates comparison of our results with theirs. Future experiments on the distribution of the *Cnox-2* protein in regenerating polyps may help clarify this issue.

The maceration technique that we used to examine *Cnox-2* expression revealed unexpected complexity in *Cnox-2* expression within regions along the axis of polyps. Because maceration allows us to isolate, identify and quantitate the staining of individual cells, we were able to detect differences in the intensity of *Cnox-2* expression in epithelial cells. Within most regions of the polyp, there is a range of expression in epithelial cells from very high levels of *Cnox-2* in some cells to undetectable levels of *Cnox-2* in other cells. The regional pattern of expression which we have shown, reflects the proportions of cells of varying intensities in each region rather than uniform expression in all cells within a given region. *Cnox-2* is the only homeobox gene whose expression pattern has been examined at this level of resolution. Thus, we are unable to determine whether this level of complexity in expression might be a general feature of homeobox gene expression or might be unique to hydra.

The axial expression pattern of *Cnox-2* is similar to the broad zones of expression of HOM/HOX-class genes in the mouse and in nematodes. In nematodes, the *mab-5* gene is expressed in cells along the anterior-posterior axis from the middle of the animal to the posterior (Kenyon and Wang, 1991; Salser and Kenyon, 1992). In the mouse embryo, many of the HOX genes are expressed from the posterior end of the animal up to a unique anterior border for each gene (see reviews by Holland and Hogan, 1988; Kessel and Gruss, 1990). While the border at the upper end of the region of expression is not absolute in hydra as it is in the mouse, the expression pattern of *Cnox-2* is similar to the general pattern of the mouse genes: *Cnox-2* expression is high from one end, the foot, up to a sharp boundary in the upper body column, and expression is low in the head. The apparent conservation of broad patterns of homeobox gene expression in hydra, nematodes and vertebrates suggests that the ancestral HOM/HOX homeobox genes may also have been expressed in broad regions.

The expression of *Cnox-2* in adult polyps is dynamic, not static, because hydra polyps undergo pattern formation continuously. Cells in the body column are always dividing and the tissues are displaced from the mid-gastric region toward the head and the foot, and into buds (Campbell, 1967). The polyps do not increase in size as cells are either sloughed off at the extremities or displaced into buds. Despite this production and loss of cells, the axial pattern of the polyp is maintained; that is, the structures and the relative positions of the head and foot are fixed. As a consequence of this continual tissue displacement, every epithelial cell is constantly changing location either up or down the axis of the animal. Epithelial cells that are displaced into the head lose high-level expression of *Cnox-2* while cells that are displaced into the foot retain high-level *Cnox-2* expression. Hence, the level of *Cnox-2* in a given epithelial cell is not fixed, but rather is adjusted as the cell changes location.

Our experimental manipulation of hydra axial patterning with diacylglycerol (DAG) suggests that the regulation of *Cnox-2* expression is related to the axial patterning system of hydra. Treatment of *Hydra magnipapillata* polyps with DAG converts body column tissue into head-like tissue (Müller, 1989). Correspondingly, treated animals lack high-level *Cnox-2* expression in these head-like regions. After 6 days of DAG treatment 45% of the polyps that we assayed regenerated double heads and, after 12 days of treatment, greater than 70% of the treated polyps had ectopic tenta-

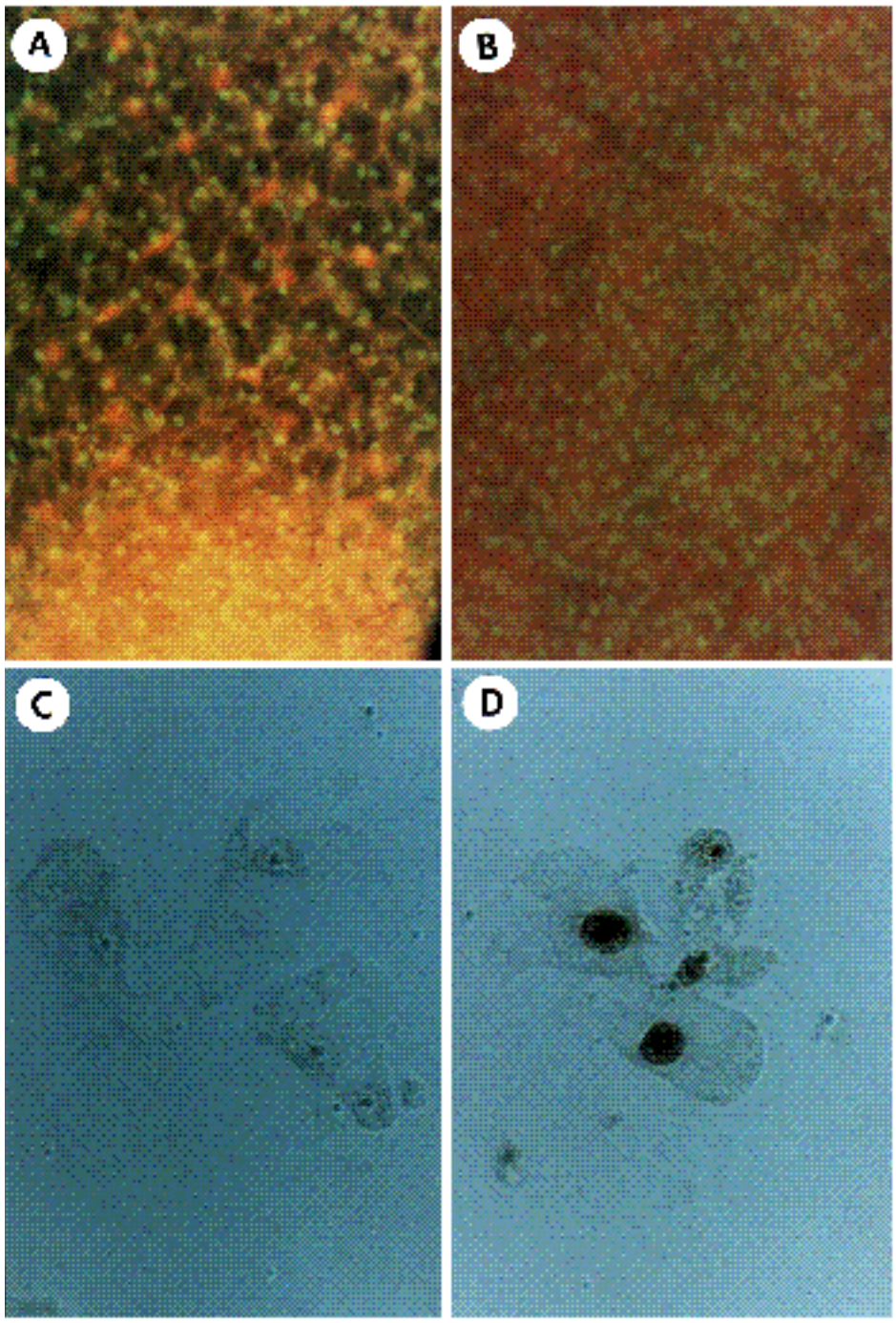

Fig. 6. Detection of *Cnox-2* protein in nuclei from *Hydra magnipapillata* whole mounts and macerates. (A) Representative view of indirect immunofluoresence in the foot and lower peduncle of a whole mount stained with protein G-purified anti-*Cnox-2* antibody. The specimen is oriented with the foot at the bottom of the panel; immunoreactive nuclei are stained green, non-reactive material is stained red. (B) Representative view of a whole-mount body column oriented and stained as in panel A. (C) Epithelial cells from a macerated polyp stained with preimmune serum and detected with horseradish peroxidase staining. (D) Epithelial cells from a macerated polyp stained with protein G-purified anti-*Cnox-2* antibody and detected with HRP. Three of the cells shown have intense nuclear staining, the cell at the upper right has light staining. Cells that lack the *Cnox-2* protein are indistinguishable from cells stained with preimmune serum, as in C.

cles in the III and IV region of the lower body column (Fig. 7D), indicating that the DAG treatment had produced an expansion of the head positional value to include the majority of the body column of the polyp. A comparison of the distributions of intensely stained epithelial nuclei, which indicates high-level *Cnox-2* expression, in normal and DAG-treated polyps shows that in DAG animals there is a low level of *Cnox-2* expression in the head and regions I, II and III in the body column, and normal levels of expression from region IV to the foot (Fig. 7C). In terms of *Cnox-2* expression, DAG-treated polyps have the following positional profile from head to foot: head, I, I/II, head, I, II/III, IV/foot. Clearly *Cnox-2* expression is regulated in response to axial positional cues.

Hydra axial pattern formation has been explained by a series of activation and inhibition gradients emanating from the head and the foot (MacWilliams, 1983a,b; Bode and Bode, 1984). A simple model to explain the axial expression pattern of *Cnox-2* based on developmental gradients requires two opposing signals, a positive signal from the foot and a negative signal from the head (Fig. 8). The positive signal from the foot would activate high-level *Cnox-2* expression and the negative signal from the head would repress *Cnox-2*, in opposition to the foot signal. To

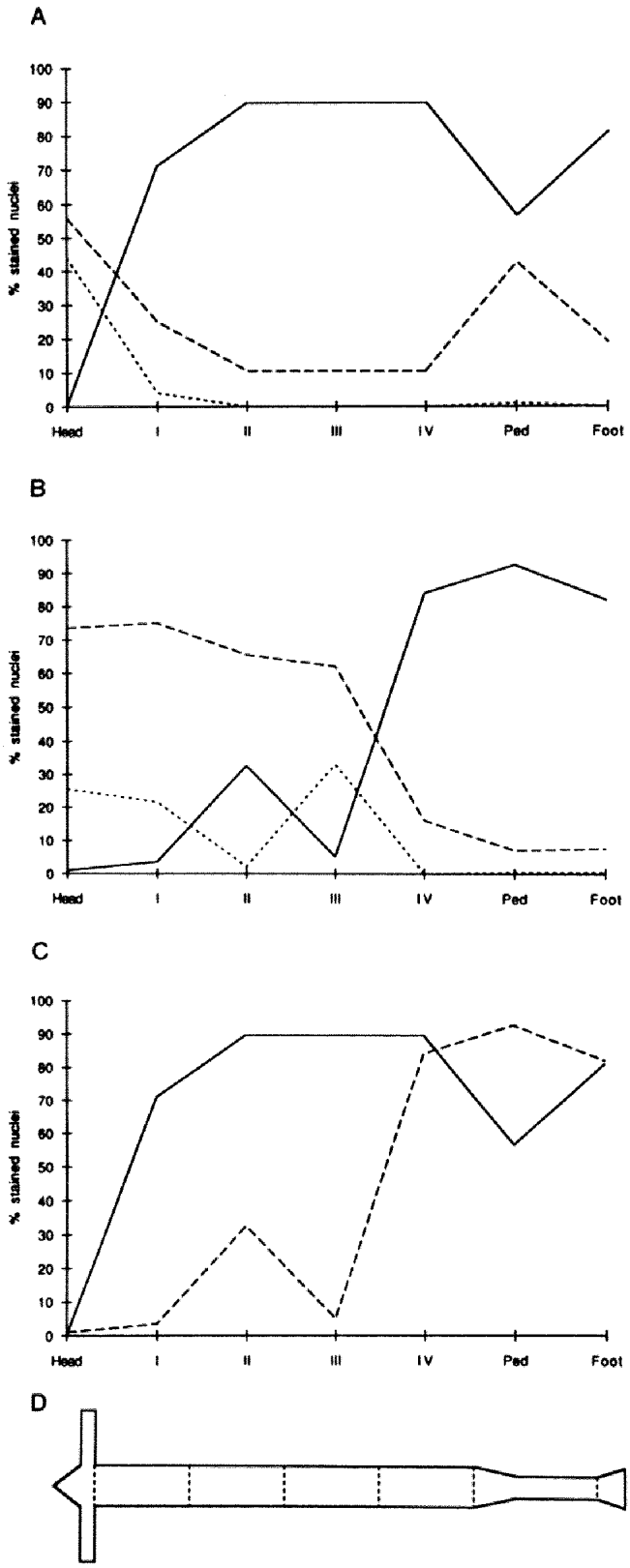

Fig. 7. Regional expression of the $p30^{Cnox\text{-}2}$ protein in normal and DAG-treated adult *Hydra magnipapillata*. Macerates were stained with anti-*Cnox-2* antibody and scored for intensity of staining. (A) Distribution of intensely stained (solid line), lightly stained (dashed line) and unstained (dotted line) epithelial nuclei along the body column of normal polyps. (B) Distribution of intensely stained (solid line), lightly stained (dashed line) and unstained (dotted line) epithelial nuclei along the body column of DAG-treated polyps. (C) Comparison of the distributions of intensely stained nuclei in normal (solid line) and DAG-treated (dashed line) polyps. (D) Diagram of an *H. magnipapillata* polyp showing the regions which were isolated and macerated.

achieve the sharp transition in *Cnox-2* expression levels between the upper body column and the head (dashed line, Fig. 8), we propose that the inhibitory signal has a threshold of activity; above the threshold the activation by the foot signal is repressed. Other examples of sharp boundaries in hydra polyps include the distributions of FMRFamide-like immunoreactive neurons and the expression pattern of the TS-19 antigen (Koizumi and Bode, 1986; Bode et al., 1988).

Our observations on the effects of DAG on *Cnox-2* expression suggest that the negative signal regulating *Cnox-2* is transduced by a pathway involving protein kinase C; we currently have no experimental evidence on the nature of the proposed positive regulatory signal. There is no way to determine from the present data whether the negative regulation of *Cnox-2* is direct or indirect. If the regulation is direct, the simplest hypothesis is that *Cnox-2* expression is regulated by a pathway in which a ligand binds to a receptor on epithelial cells, which stimulates protein kinase C, which in turn activates a pathway that turns off transcription of *Cnox-2*. The proposed *Cnox-2* pathway is the only homeobox gene regulatory pathway that appears to involve protein kinase C.

In conclusion, we have shown that *Cnox-2* is a cnidarian relative of HOM/HOX-class homeobox genes, that *Cnox-2* is expressed in adult polyps in a pattern consistent with a role in axial pattern formation, that *Cnox-2* expression is altered by experimental manipulation of hydra axial patterning and that *Cnox-2* expression may be regulated by a signal transduction pathway involving protein kinase C. Our results clearly show that the HOM/HOX

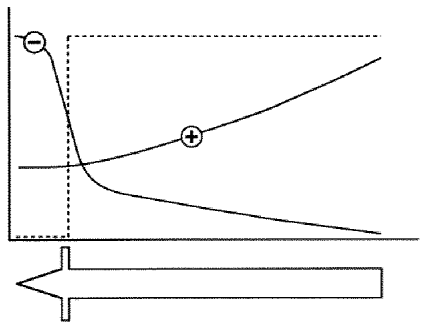

Fig. 8. A model of regulation of *Cnox-2* expression by developmental gradients. The dashed line indicates high-level *Cnox-2* expression. The curve with the plus sign indicates the distribution of a positive regulatory signal released by the foot. The curve with the minus sign indicates the distribution of a negative regulatory signal released by the head.

genes must have arisen very early in animal evolution, perhaps indicating that the genes are required by all multicellular animals. Our data on the expression pattern of *Cnox-2* in combination with the expression patterns of HOM/HOX genes in nematodes and chordates, suggests that the original function of HOM/HOX-class genes may have been to specify the identity of broad regions in developing embryos.

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