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prdl-a, a gene marker for hydra apical differentiation related to triploblastic *paired*-like head-specific genes

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SUMMARY

Two homeobox genes, *prdl-a* and *prdl-b*, which were isolated from a *Hydra vulgaris* cDNA library, encode *paired*-like class homeodomains highly related to that of the *aristaless*-related genes. In adult polyps, *prdl-b* is a marker for synchronously dividing nematoblasts while *prdl-a* displays an expression restricted to the the nerve cell lineage of the head region. During budding and apical regeneration, an early and transient *prdl-a* expression was observed in endodermal cells of the stump at a time when the head organizer is established. When apical regeneration was delayed upon concomittant budding, *prdl-a* expression was found to be altered in the stump. Furthermore, a specific anti-prdl-a protein immunoserum revealed that prdl-a was overexpressed in adult polyps of the *Chlorohydra viridissima* multiheaded mutant, with an expression domain extending below the tentacle ring towards the body column. Accordingly, prdl-a DNA-binding activity was enhanced in nuclear extracts from this mutant. These results suggest that *prdl-a* responds to apical forming signals and might thus be involved in apical specification. When a marine hydrozoan (*Podocorynae carnea*) was used, the anti-prdl-a antibody showed cross-reactivity with cells located around the oral region, indicating that *prdl-a* function is shared by other cnidaria. The ancestral role for *prdl-a*-related genes in the molecular definition of the head (or oral-surrounding region) is discussed.

Key words: *Paired*-like homeobox gene, Head development, Regeneration, Budding, Hydra, Cnidaria, Evolution

INTRODUCTION

Hydra is a freshwater cnidarian, which displays an apical to basal polarity that is either maintained through permanent cell differentiation and migration processes in adult polyps, or (re)established during budding, regeneration, reaggregation and sexual development. Hydra essentially consists of a tube with a mouth region, called the hypostome, at the apical end (head), surrounded by a ring of tentacles. At the opposite end (foot), the basal disk attaches the animal to the substrate. The body wall of the diploblastic animal is made up of two epithelial layers, the ectoderm and the endoderm, separated by a basement membrane, the mesoglea. Two main different stem cell populations are distinguished: the epithelial cells present in ectoderm and endoderm, which are the most abundant, and the interstitial cells, which constitute stem cells for nerve cells, nematocytes (stinging cells), gland cells and gametes in case of sexual reproduction. The apical to basal polarity results from a continuous process of differentiation initiated in the body column and finalised at the extremities. Thus, the gastric column consists mostly of undifferentiated cells, while the hypostome, tentacles and basal disk are composed of committed or terminally differentiated cells. Under laboratory culture conditions, most hydra species reproduce asexually by budding and display high potential for regeneration and reaggregation.

Homeobox-containing genes that are involved in developmental processes tend to display structural and functional features that are conserved throughout higher metazoans, thus suggesting that their roles during development are shared by all animals (e.g. Gehring, 1985; McGinnis and Krumlauf, 1992; Slack et al., 1993). In diploblastic animals, which are generally considered as representative of ancestral animals, a dozen homeobox-containing genes have been isolated so far, most of them encoding products that are clearly related to arthropod and vertebrate homeoproteins (Schummer et al., 1992; Miles and Miller, 1992; Miller and Miles, 1993; Shenk et al., 1993; Naito et al., 1993; Aerne et al., 1995; Kuhn et al., 1996; Grens et al., 1996; Sun et al., 1997). Whether or not this structural conservation correlates with related functions, however, remains an open question. Such genes are obvious candidates to respond to and/or regulate apical- and basal-determining signals in diploblastic animals and hence take a part in the establishment of their polarity. In this work, we have focused our analysis on the hydra homeobox gene, which displays the highest rate of conservation observed so far with its triploblastic counterparts, *aristaless (al)* (Schneitz et al., 1993; Miura et al., 1997) and *Cart1* (Zhao et al., 1993).

Expression analysis revealed that, in adult polyps, *prdl-a* was transcribed in nerve cells located in the ectoderm of the inner hypostome, the most apical area of the polyps surrounding the mouth opening. During budding and regeneration, *prdl-a* was first transiently expressed in the endoderm of the regenerating tip, at the time the head organizing activity was established. Subsequently, *prdl-a* was expressed in apical ectodermal cells, soon before tentacle buds appeared. These results suggest a role for *prdl-a* in cnidarian apical differentiation and raise the intriguing question of a functional conservation with related *paired*-like genes such as *aristaless (al)*, a gene initially expressed in embryonic head in *Drosophila* (Schneitz et al., 1993) and during brain development in vertebrates (Miura et al., 1997) or *Cart1* whose deficiency leads to acrania and meroanencephaly in vertebrates (Zhao et al., 1996).

MATERIALS AND METHODS

Culture of animals and regeneration

Hydra vulgaris (*Hv*) were cultured in 0.5 mM $NaPO_4$, pH 7.4, 1 mM $CaCl_2$, 0.1 mM KCl, 0.1 mM $MgCl_2$ and fed 6 days a week with hatched *Artemia nauplii*. Regeneration experiments were performed at 20°C, on budless animals, starved for 2 days and bisected at the mid-gastric position.

cDNA and genomic cloning

An *Hv* adult polyp cDNA library was screened using the guessmer oligonucleotide strategy (Galliot and Schummer, 1993). From a screening of 10^6 recombinants, a single clone for *prdl-a* was isolated. Introns located within the homeobox were identified by PCR on genomic DNA: sequences of oligonucleotides were GCGAGGAACTTGCTGTTCGGGT (forward) and GTACTGCACT-TGGTTCGGTTACAG (reverse).

Northern blot analysis

Poly(A)$^+$ RNAs were isolated from either 100 hydra or 200 apical or basal halves using a direct mRNA purification kit (Pharmacia). One quarter (about 1 μg) was electrophoresed on a 1% agarose-3.7% formaldehyde gel, electrically transfered on a Hybond N$^+$ membrane (Amersham) and treated as in Sambrook et al. (1989). Hybridization was performed overnight at 50°C with random-labelled DNA probes (10^6 cpm/ml). Washes were performed in 2× SSC, 1% SDS at 55°C. When Northern blotting was performed on mRNA from cell fractions obtained after elutriation, total hydra were first dissociated upon pronase treatment and resulting live cells were then separated according to their size upon combined differential flow through rates and centrifugal forces (Greber et al., 1992).

In situ hybridization on whole-mount and dissociated animals

In situ hybridizations using DIG-labelled riboprobes were performed on whole and pronase-dissociated hydra following the methods described by Schulte-Merker (1993) with modifications (Rosen and Beddington, 1993; Grens et al., 1996). For histological analysis, 7 μm sections were carried out after inclusion in paraffin. As the full-length cDNA antisense riboprobe provided strong unspecific signals when used on freshly bisected animals, a 730 bp cDNA probe, which lacks the 3′ untranslated region, was used on regenerating animals.

Anti-prdl-a antiserum

An N-terminal 6-HIS fusion protein containing prdl-a from residues 8-224 was obtained by inserting a 730 bp fragment of the *prdl-a* cDNA clone into the pQE31 vector (Qiagen). Prdl-a fusion protein was then expressed in M15(pREP4) bacteria and purified under denaturing conditions (guanidine-HCl). After acidic elution from a nickel-agarose column, prdl-a protein was dialyzed and lyophilized for storage. 100 μg was used for each injection in two rabbits. Purified (1/100 dilution) or unpurified (1/1000 dilution) sera from the 8th boost were used in most experiments.

Immunohistochemistry

Hydra were relaxed for 30 seconds in 2% urethane and fixed for at least 24 hours in 50% ethanol, 4% formaldehyde. After washing in PBS, animals were treated for 30 minutes with 2 N HCl and incubated overnight with the purified or unpurified anti-prdl-a antiserum in blocking solution. After washing in PBS, hydra were incubated for 2 hours in Cy3-conjugated anti-rabbit antibody (Dianova) and stained with DAPI (10 μg/ml) before mounting in 90% glycerol, 0.05% *n*-propyl gallate.

Western analysis and band-shift assays

For western analysis, up to 20 μg of nuclear extracts or whole cell extracts were denatured and separated by 12% PAGE. After electrophoresis and blotting, membranes were sequentially incubated with the anti-prdl-a antiserum and a peroxidase-conjugated secondary antibody. which was revealed with chemiluminescence (ECL, Amersham). The band-shift analysis was performed with either the P3 or P1 oligonucleotides (Wilson et al., 1993). The sequences of the mutated oligonucleotide, used for competition experiments, and the NFkB control oligonucleotide were ACGCTGTCACCTAAGGACGTA and GATCGAGGGGACTTTCCCTAGC, respectively.

RESULTS

prdl-a gene encodes a highly conserved *paired*-like homeodomain

Two distinct cDNAs encoding *paired*-like homeodomains (HDs), named *prdl-a* and *prdl-b*, were isolated from the *Hv* cDNA library (Fig. 1 and not shown). *Prdl-a* homeobox is mostly identical to the zebrafish *Arx* (69.4%), the human *Cart1*, the nematode *unc4*, the zebrafish *Pax6* (68.3%) and the flatworm *smox3* (67.8%) homeoboxes, but only 63.9% to that of the *Drosophila al* gene. In contrast, at the amino acid level, prdl-a HD showed highest similarity rates with aristaless sub-family members, reaching 90% when conservative substitutions are taken into account (Fig. 1B). The predicted prdl-a protein, 229 residues long (Fig. 1C), did not contain a paired domain (present in paired-type homeoproteins), but displayed within its HD sequence 13 residues out of the 16 that had initially been described as specific to the paired family (Bopp et al., 1986). At position 50, a glutamine residue was present, a unique feature of *paired*-like HDs, whereas a serine residue was found at this position in *paired*-type (PAX) HDs (Schneitz et al., 1993). Alignment of paired-type and *paired*-like HDs showed five additional residues shared by most members and absent in Antp-type HDs (see Fig. 1B): among these residues, His24 was specific to *paired*-like, Pax3 and Pax6 HDs. Tyr4 was only found in al-related HDs including prdl-a and prdl-b, which were assigned to the *al* family. In both *prdl-a* and *prdl-b* homeoboxes, we localized an intron between nucleotides 137 and 138 of the homeobox, at the position corresponding to the Q-VWF sequence (Fig. 1A,B). Introns with an identical position were also detected in most vertebrate or *Drosophila paired*-like and *paired*-type genes, although not specific to this class of genes (Bürglin, 1994).

prdl-a is an ectodermal apical marker in cnidaria

In adult polyps, mRNA in situ hybridization showed that *prdl-*

A

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   1 CAAATTTTACGCAAACTAAGCCAAAGCAAGTTAATACTGTTTTTCATCAAGCCAGATAATGACTTTGTTAGG
                                                               M  T  L  L  G       5
  73 TATGCTGCCAAGTAGTCAAGAGGAGAACAGCAATTTCTCTGACTTTAAAGAAATCAAGGGGGAAGAGTATCA
      M  L  P  S  S  Q  E  E  N  S  N  F  S  D  F  K  E  I  K  G  E  E  Y  Q      29
 145 AAACCAACGCTTTTCAAACGGAAAGCGAAACGAAAGAAGGTATCGCACTACCTTTACGCAGTTTCAACTAGA
      N  Q  R  F  S  N  G  K  R  N  E  R  R  Y  R  T  T  F  T  Q  F  Q  L  D      53
 217 TGAACTTGAAAGAGCATTTGATAAAACACACTATCCGGATGTTTTCATGCGCGAGGAACTTGCTGTTCGGGT
      E  L  E  R  A  F  D  K  T  H  Y  P  D  V  F  M  R  E  E  L  A  V  R  V      77
 289 TCACCTAACAGAAGCAAGAGTTCAGgtactgaaagtagttataaagtagaaataattttataaatttttact
      H  L  T  E  A  R  V  Q                                                      85
 361 atattaattataatttacaagaactattaaaagcagttacagtatagatatgtttatacacataactcacta
 433 ccaacatagaatgaaaaaaatttggtgtgattatgctactcgtttaaattttaataaacaaactaagttaaa
 505 tcacgtgttttgaaacttatttggcaacaaaaggttaccaaacacctggaaatttgaaaaaaataataataa
 577 ttatatgctatatagattctaagtaatctcttttcgatatataagaggcttaaataataaactcgtaattca
 649 gcggcttgtttttacgagtcggattgtttgatgataagaggccgtccaattaatatattttttttgccgtgtt
 721 atttacttcatccaatctgtcaaattaaaactcttttttttaattgtttcttatatacggcatcataaaaact
 793 ttctcagtaaagctcatagcagttgaatgcaaacctttagaaacatttgccgtgtataaaagattatagtt
 865 tgtattttgtgtttaagttaaaaatttttcaataaaactttttaccttactgttttgccactccggtttg
 937 cgtattgtagGTATGGTTTCAAAATAGACGTGCAAAATGGAGAAAGCGGGAAAAGTTAAGTTATAACGTTCA
               V  W  F  Q  N  R  R  A  K  W  R  K  R  E  K  L  S  Y  N  V  H     106
1009 TCAACAACAACATGGTTCAAATGGCGAAGTTTCAAGGCCTTACCATATATCTACTAGCCCAAAGATTCAGTC
      Q  Q  Q  H  G  S  N  G  E  V  S  R  P  Y  H  I  S  T  S  P  K  I  Q  S     131
1081 ACAGCAGAGACCAGTATCATCTCAAATGCCAACTCCTCTCTCTCACCATACTGCATTTCAACCTTGGGCTCA
      Q  Q  R  P  V  S  S  Q  M  P  T  P  L  S  H  H  T  A  F  Q  P  W  A  Q     154
1153 ATCTCCATACTGTCCGCAATCTACATCTCCTTATCCTCAGTACCCACATTACCAACAGTCTTCAATGATTGG
      S  P  Y  C  P  Q  S  T  S  P  Y  P  Q  Y  P  H  Y  Q  Q  S  S  M  I  G     178
1225 AAACTACGCTCGCCCATCATCATACCAATATATGTCTCATCATACAGGTTCAGTTATATCTTCCACACCTGA
      N  Y  A  R  P  S  S  Y  Q  Y  M  S  H  H  T  G  S  V  I  S  S  T  P  E     202
1297 AAATCTTTCACCTCAAGATTCTCAGTTTACGGGACATTCGTCATTTAACAATTTAAAGGGACAAAATCAGTT
      N  L  S  P  Q  D  S  Q  F  T  G  H  S  S  F  N  N  L  K  G  Q  N  Q  L     226
1369 AATTTTTGCATAAAAGTTTTATAAACTATATGTCAAATAAGACTAAAGAAGACTGTAACCGAACCAAGTGCA
      I  F  A  *                                                                 229
1441 GTACTTTACAGCGCAATAACTTTTGTGTGAGCGCAAAAGGAGGTAAATATAATTCCTCTAAAAGACTCCGCA
1513 AATAAGTTCAAAATGAACATTACTTTGTTGCAAGGTTTAATTTGTTCACGGCAAAGTTTTATCTAAATTAAA
1585 ACAATAATAATAACAAATTCAATTTGAAACAATGTGTAAATGAATTATTTACTTTTTAAATGTTATATAGAG
1657 CAGTTTTATATTTATGTTGTACTTCTTGTTTATAATCTTTTTTCTATTTAAATGCTAAATAATGTAAATAAT
1729 GAAAAATTGACTTCATAAAGTTATAGGATTTTTGCCG
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B

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Prdl-a Hv      ERRYRTTFTQFQLDELERAFDKTHYPDVFMREEIAVRVHLTEARVQVWFQNRRAKWRKRE                Y15515
al su          Q--------SY--E------C--------T---L-M--D--------------------- 85%, 90%    D85080
Arx zf         Q--------SY--E------Q--------T---L-M-LD--------------------- 83%, 90%    AB006104
al Ce          Q-------SA-------KV-AR-------T---L-T--Q----------------Y--Q- 78%, 85%    Z68008
al Dm          Q--------S---E---K--SR-------T---L-MKIG-----I-------------Q- 77%, 88%    L08401
Prdl-b Hv      K--------TH-------V-NR-----I-L---M--KLG-----I-------------NK 75%, 83%    Y15516
Cart1 hu       K--H-----SL--E---KV-Q-------YV--QL-L-TE--------------------- 75%, 83%    U31986
rax/Rx mu      H--N-----TY--H------E-S-----YS---L-GK-N-P-V--------------RQ- 72%, 83%    U73177
smox3 Sm       Q--I-----SL--K------QE-----IYT--DL-L-ID----------------F--T- 72%, 82%    M85303
Otp mu         QK-H--R--PA--N----S-A------I-----L-L-IG---S--------------K--K 72%, 82%    Y10413
Pax-3 mu       Q--S-----AE--E------ER-----IYT---L-Q-AK----------S--------QA 72%, 82%    P24610
Unc4 Ce        R--T--N-SGW--E---S--EAS---------AL-M-LD-L-S----------------- 70%, 80%    X64904
Phox2 rt       Q--I-----SA--K----V-AE-----IYT---L-LKID----------------F--Q- 70%, 80%    X75014
Drg11 rt       Q--N----ALQ--EA--AV-AQ-------T---L-MKIN-------------------T- 70%, 77%    U29174
MHox/Prx1 mu   Q--N----NSS--QA---V-ER-----A-V--DL-R--N----------------F-RN- 68%, 77%    Q02810
S8/Prx2 mu     Q--N----NSS--QA---V-ER-----A-V---L-R--N-S--------------F-RN- 68%, 77%    S18038
Prd Dm         Q--C----SAS---------ER-Q---IYT---L-Q-TN-----I----S----RL--QH 65%, 78%    P06601
Chx10 mu       K--H--I--SY--E---K--NEA-----YA--ML-MKTE-P-D-I--------------- 65%, 77%    L34808
Ceh-10 Ce      K--H--I---Y-I----K--QDS----IYA--VL-GKTE-Q-D-I-------------T- 65%, 80%    U19995
gsb-p Dm       Q--S-----AE--EA-----SR-Q----YT---L-QTTA-----I----S----RL--HS 65%, 75%    P09083
bsh4 Dm         --S-----AE--EA--G--SR-Q----YT---L-QTTA ----I----S----RL--HS 63%, 73%    M14941
Otd  Dm        Q--E-----RA---V--AL-G--R---I-----V-LKIN-P-S------K-----C-QQL 63%, 72%    X58983
Otx1 mu        Q--E-----RS---V--AL-A--R---I-----V-LKIN-P-S------K-----C-QQQ 63%, 72%    X68883
gsb-d Dm       Q--S----SND-I-A---I-AR-Q----YT---L-QSTG----------S----RL--QL 62%, 72%    P09082
mab18 Ce       LQ-N--S---V-IES--KE-ER-------A--RL-QKIQ-P---I----S-------RE- 60%, 78%    U29145
Pax6 zf        LQ-N--S---E-IEA--KE-ER-------A--RL-AKID-P---I----S-------RE- 60%, 78%    P26630
prop-1 mu      R--H----NPA--EQ--S--GRNQ---IWA--GL-QDTG-S---I----------Q--Q- 58%, 68%    U77946
Pax-B Hl       M--V----SL--RRA--D--E--P---AEQ----SIQCD-P-P------S-K---L-RQD 57%, 67%    U96194
gsc-mu         K--H--I--DE--EA--NL-QE-K----GT--QL-RK---R-EK-E---K-------RQK 57%, 65%    Q02591
Hesx1/rpx mu   G--P--A---N-VEV--NV-RVNC--GIDI--DL-QKLN-E-D-I-I--------MKRSR 47%, 65%    X80040
Antp Dm        RK-G-Q-Y-RY-TL---KE-HFNR-LTRRR-I---HALC---RQIKI------M--K-EN 43%, 57%    P02833

Prd-SPECIFIC   Q...........LD...RA..RTH.PDIYT.E....KT....AR.Q...S...A......
RESIDUES       1.......10........20........30........40........50........60
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C

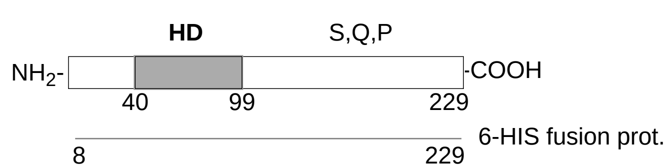

Fig. 1. (A) cDNA (uppercase) and genomic (lowercase) sequences of the *prdl-a* gene identified in *Hydra vulgaris*. The homeodomain is underlined. Numbers on the left refer to nucleotides, on the right to amino acids. (B) Alignment of the *Hydra vulgaris* (Hv) prdl-a HD sequence with vertebrate (mu, murine; rt, rat; zf, zebrafish) *Drosophila* (Dm), *C. elegans* (Ce), sea urchin (su), *Schistosoma mansoni* (Sm) or *Hydra littoralis* (Hl) related HDs. The conserved intron between Q46 and V47 is indicated by an arrow. Percentages on the right correspond to identity (first number) and similarity rates when conservative substitutions are considered. Accession numbers are listed on the right. Residues specific to *paired*-type and *paired*-like HDs are shown at the bottom; those underlined were noted by Schneitz et al. (1993). (C) Diagram of the prdl-a protein structure. S,Q,P: Ser, Gln, Pro-rich C-terminal domain. HD, homeodomain. Numbers indicate amino acids.

a was expressed in the most apical part of the hydra head, the hypostome, in ectodermal cells surrounding the mouth opening, and around the root of the tentacles (Fig. 2A). A 6His-prdl-a fusion protein (Fig. 1C) was produced in *E. coli* and used to raise a polyclonal antiserum. The prdl-a protein distribution confirmed previous findings and showed nuclear staining in ectodermal cells of the hypostome, with a maximal density around the mouth opening, and along the tentacles (Fig. 2B,C). In *Hv*, no prdl-a expressing cells were detected below the level of the tentacle insertion ring, nor in the body column or the foot region. The prdl-a antibody was further assayed on *Podocorynae carnea*, a marine hydrozoan species. We detected *prdl-a* expressing cells in the most apical part of feeding polyps, as well as in early stage polyps that will develop as medusae, suggesting that prdl-a has a similar function in other hydrozoans (Fig. 2D, arrows). Finally, we analysed the cell-type specificity of *prdl-a* expression in hydra adult polyps by northern blot analysis using RNA extracted from elutriated cells (Greber et al., 1992). This analysis documented *prdl-a* expression in fractions containing nerve cells and their precursors and *prdl-b* expression in fractions containing nematoblasts (Fig. 2F). These results were confirmed by in situ hybridization on dissociated hydra; *prdl-a* was expressed in a few small interstitial cells and in a subset of nerve cells (Fig. 2E), while *prdl-b* was detected in groups of synchronously dividing nematoblasts located in the body column (Fig. 2G,H and not shown). We thus concluded that, in adult polyps, *prdl-a* and *prdl-b* were both expressed in cells deriving from the interstitial cell lineage, *prdl-a* being restricted to a subset of the apical nerve cell lineage.

prdl-a expression during apical regeneration

Northern blot analysis of mRNA prepared at various time points during apical and basal regeneration showed that *prdl-b* expression did not display any modulation while *prdl-a* transcripts, which were barely detectable in lower halves shortly after cutting, progressively reappeared during apical regeneration, between 3 and 9 hours after mid-gastric section, to reach a steady state level after 12 hours (Fig. 3A, right). In upper halves undergoing basal regeneration (Fig. 3A, left), no significant modulation was observed when compared to actin mRNA levels. In situ hybridization performed on whole-mount regenerating hydra showed modulation of *prdl-a* expression in those lower halves undergoing apical regeneration (Fig. 3B-G). While not observed 1 hour after cutting, *prdl-a* transcripts were detected in endodermal cells of the regenerating tip 6 hours after cutting, i.e. 30 hours before any apical structure was visible (Fig. 3B, white arrows). This signal reached its maximum after 12 hours and then progressively declined (Fig. 3C,D). Several hours before tentacle buds appeared, about 24 hours post-bisection, *prdl-a* expression was progressively reestablished in apical ectodermal cells, first on a broad domain covering the regenerating tip (Fig. 3E-F), then restricted to the head region above the tentacle insertion ring as in the adult polyps (Fig. 3G). Finally, *prdl-a* expression was not detected in upper halves where the stump was regenerating a foot (Fig. 3B,C); however, in these upper halves, which contained a fully formed adult head, a transient decrease in adult-type *prdl-a* expression was noted (Fig. 3C).

This biphasic modulation of *prdl-a* transcripts during regeneration was confirmed by immunohistochemistry (not shown). Endodermal *prdl-a* expressing cells were detected in regenerating tips from 10 hours after mid-gastric section. Several hours before tentacle buds emerged (28 hours post-cutting), at a stage where prdl-a positive endodermal cells were still visible, a few apical *prdl-a* positive ectodermal cells appeared at the apex of regenerating tips. 4 hours later, these cells were numerous and became restricted to the tentacular and supra-tentacular zones as soon as tentacle buds emerged.

prdl-a expression during budding

The budding process, which initiates in the lower part of the

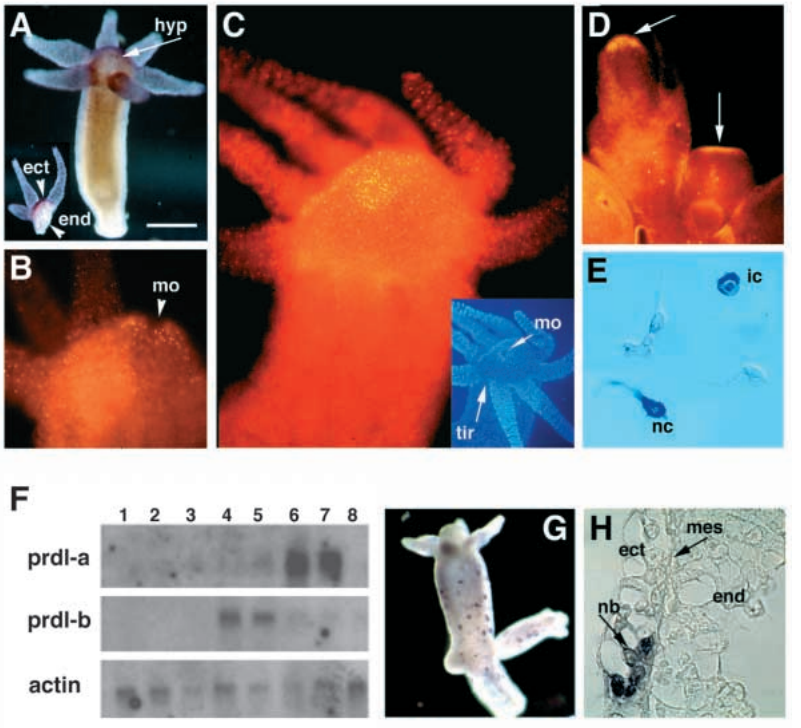

Fig. 2. *Prdl-a* (A-E) and *prdl-b* (G,H) expression in hydrozoan polyps. (A) Whole-mount in situ hybridization on *Hv* adult polyps with *prdl-a* antisense riboprobe; insert on left shows a sectioned head. (B-D) Prdl-a immunohistochemistry analysis in *Hv* adult polyps (B,C) and *Podocorynae carnea* polyps (D). Prdl-a positive cells were not detected with the pre-immune serum or when prdl-a protein was added to the immune serum (not shown). Mouth opening (mo) and tentacle insertion ring (tir) are better seen with Dapi staining (C, inset). (E) In situ hybridization performed on dissociated hydra; ic, small interstitial cell; nc, nerve cell. (F) Northern blot analysis of mRNAs from elutriated cell fractions: lane 1, endodermal epithelial cells; lane 2, endodermal and ectodermal epithelial cells; lane 3, ectodermal epithelial cells; lane 4, nematoblast cells; lane 5, nematoblasts and interstitial cells; lane 6, interstitial and nerve cells; lane 7, nerve cells; lane 8, total mRNA. (G) Whole-mount in situ hybridization on *Hv* adult polyps with *prdl-b* antisense riboprobe. (H) Ectodermal *prdl-b* expressing cells in longitudinal section after paraffin-embedding. ect, ectoderm; end, endoderm; hyp, hypostome; mes, mesoglea; nb, nematoblasts. Bar, 0.8 mm.

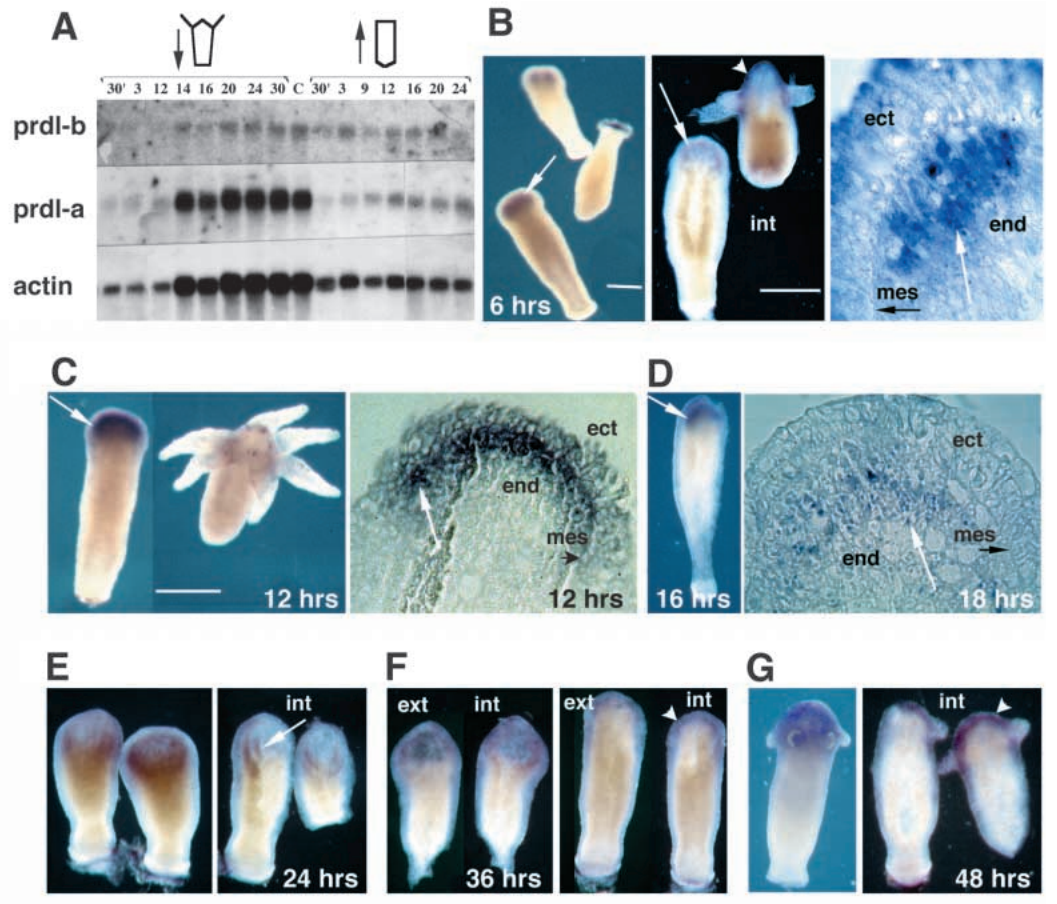

Fig. 3. *Prdl-a* expression during hydra regeneration. (A) Northern blot showing the relative amounts of *prdl-a*, *prdl-b* and *actin* transcripts in regenerating *Hydra vulgaris (Hv)*. The right part, which corresponds to head regeneration, shows a specific increase in level of *prdl-a* transcripts between 3 and 9 hours (time indicated at top). C, uncut control animals. (B-G) Spatial pattern of *prdl-a* expression analysed by whole-mount mRNA in situ hybridization. Endodermal expression, white arrows; ectodermal expression, arrowheads. (B-D, right panels) Histological sections after *prdl-a* whole-mount in situ hybridization. From 6 to 18 hours (B-D), expression was exclusively endodermal; from 24 hours on (E, F), expression was observed in both layers; the ectodermal expression became progressively restablished while the endodermal expression was disappearing. At 48 hours (G), expression was exclusively ectodermal. Black arrows (in C and D) point to the mesoglea (mes); ect, ectoderm; end, endoderm; int, internal ext, external views after longitudinal section. Bars, 0.8 mm.

body column, follows 10 different stages to produce a mature bud ready to detach from the parent (Otto and Campbell, 1977). The whole process requires about 70 hours and the first tentacle rudiments appear 24 hours after a bud is detected on the parental polyps (stage 2). Early on, at stage 2 (peak stage), a front of endodermal *prdl-a* expressing cells was visible while no expression was noted in the sus-jacent ectoderm (Fig. 4A). From stage 3 (mound stage) onwards, ectodermal expression became predominant all over the budding zone whereas the number of endodermal cells expressing *prdl-a* was reduced. In addition, these latter cells were no longer organized as a front adjacent to the mesoglea but, instead, were dispersed amongst endodermal cells (Fig. 4B). At stage 7, when tentacle rudiments emerged, a strong ectodermal expression domain restricted to the most apical part of the bud, the hypostome and the roots of the tentacles was noticed, while the most distal part of the tentacle rudiments were negative (Fig. 4A). Immunohistochemistry analysis confirmed these observations by detecting *prdl-a* expressing cells in ectoderm as early as stage 3 and showing an expression domain that was mostly apical (Fig. 4C). Thus a similar biphasic mode of expression was observed for *prdl-a* during budding and regeneration, although with slightly different kinetics; primary endodermal expression appeared to be more transient during budding.

prdl-a expression during concomitant apical regeneration and budding

Apical regeneration and budding both lead to apical differentiation and are supposed to follow similar morphogenetic cascades. When they take place concomittantly, they are known to be mutually inhibitory (Müller, 1995). We therefore examined *prdl-a* expression in this context in eight different cases (Fig. 4D). The precise timing of budding (Otto and Campbell, 1977) allowed us to evaluate, for each case, the extent of temporal overlap between budding and regeneration, as well as the time when budding was initiated during regeneration. In three cases, we observed a significant delay of *prdl-a* expression in the regenerating stump of budding animals when compared to that observed in budless regenerating animals. In a 12 hours-regenerating/late stage 4 budding hydra (case 1), *prdl-a* expression was very weak in the stump, which was similar to a much earlier stage, but comparable to control levels in the bud. In three distinct 16 hours-regenerating hydra displaying buds of different stage (cases 2-4), only that harboring the oldest bud showed a complete lack of *prdl-a* expression in the stump. Finally, a 32 hours-regenerating/stage 7 budding hydra (case 7) displayed an appropriate ectodermal prdl-a expression in the bud, but none in the regenerating stump. Instead of the characteristic evagination observed at that stage, this stump exhibited a round apical shape, suggesting a delayed development. In these three cases, budding was initiated at the time or just before hydra were bisected. These cases of delayed *prdl-a* expression in the regenerating stump, but not in the bud, illustrate the correlation between an arrest in regeneration, due to concomitant budding, and the delay in *prdl-a* expression. Accordingly, in cases where buds started to

develop slightly later, on young apical-regenerating hydra, *prdl-a* expression was slightly or not affected in the stump (cases 2, 3 and 5). Finally, when budding occurred much later, no clear alteration of apical development and *prdl-a* expression was noted (cases 6 and 8). These results suggest that during an early and limited period of time, *prdl-a* expression in the endoderm was dependent on signals common to budding and regeneration, which are likely to be required for apical differentiation and to be tightly regulated. Whenever budding occurred out of this time window, no competition was observed. Interestingly, hydra harboring double buds, which are quite common in a daily fed culture, displayed an appropriate pattern of *prdl-a* expression in each of the buds. Nevertheless, we never observed twins in double-budding hydra; the second bud usually appeared when the first one reached stage 5 or 6, 15 or 25 hours after the first bud went through stage 2. According to these comparisons, we would expect the time window important for induction of *prdl-a* expression to be located between 1 and 6 hours after bisection, or during stage 2 for budding. If bisection is performed on a hydra initiating budding, then *prdl-a* expression will be deficient in the stump and regeneration will be delayed.

Up-regulation of prdl-a in multiheaded mutant

As the multiheaded mutant of *Chlorohydra viridissima* (*Cv mh*) displays stronger head organizing activity, as indicated by the high number of tentacles and heads that are produced all along its body column (Fig. 5A), we next analysed *prdl-a* expression in this mutant. While the *prdl-a* expression patterns were similar in two different hydra species, *Hv* and *Chlorohydra viridissima (Cv wt*, Fig. 5B), an extended pattern of *prdl-a* expression was clearly detected in the *mh* mutant. Most animals exhibited *prdl-a* expressing cells in the ectoderm of the upper gastric region, below the insertion ring of the tentacles (Fig. 5C). As expected, western analysis showed a higher level of prdl-a protein in whole cell and nuclear extracts of the *mh* mutant when compared to that of budless *Cv wt* or *Hv* (Fig. 5D). Due to the abundancy of chloroplasts in *Cv* endodermal cells, detection of prdl-a positive endodermal cells during regeneration was not reliable. Finally, we investigated the conservation of prdl-a DNA-binding activity. In contrast to Antp-type homeoproteins, *paired*-type and *paired*-like

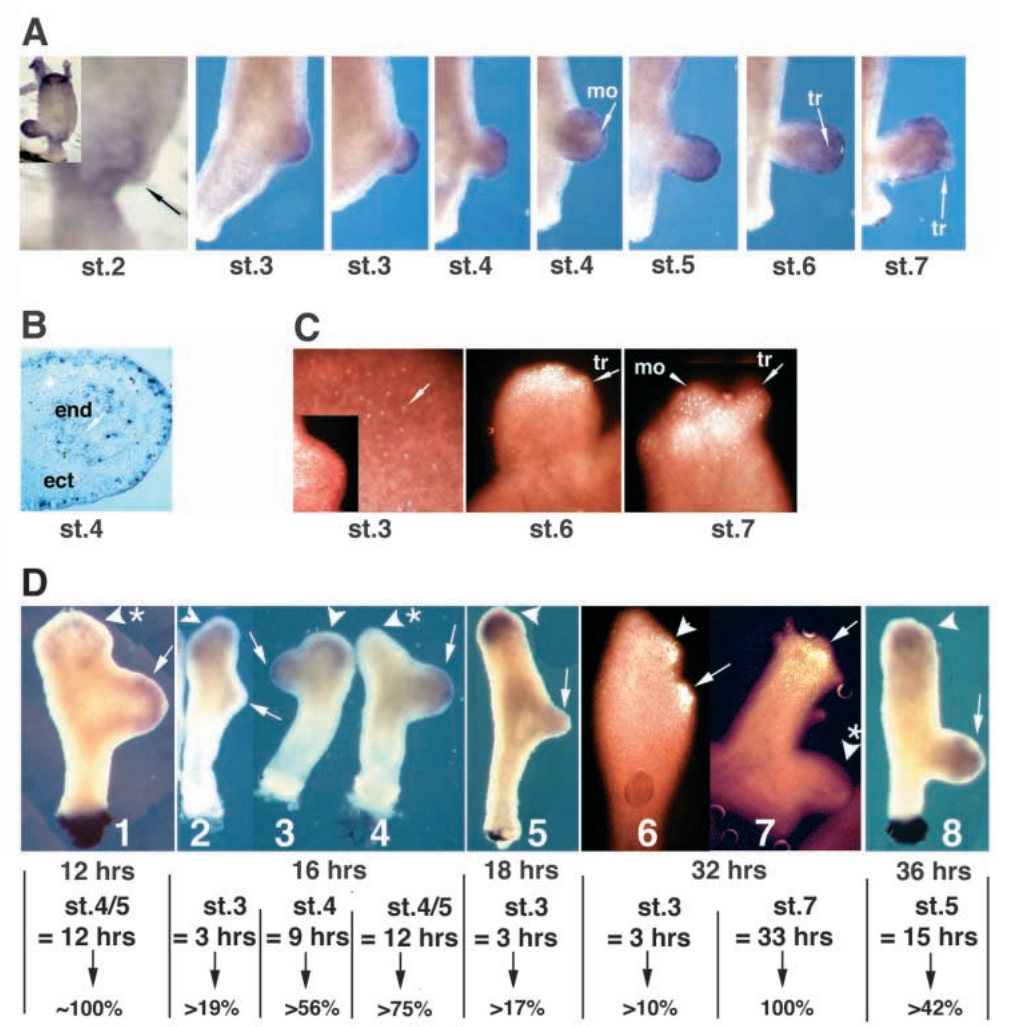

Fig. 4. (A-C) *Prdl-a* expression during budding from stage 2, when prdl-a expressing cells were detected only in the endoderm (black arrow), to stage 7 when tentacle rudiments (tr) have emerged. From stage 4, the future mouth opening (mo) became visible. (B) In histological sections after whole-mount in situ hybridization, *prdl-a* positive cells were detected in the ectoderm (ect) and dispersed in the endoderm (end) of a stage 4 bud. (C) Antiprdl-a immunohistochemistry: at stage 3, the first positive cells were detected; at stages 6 and 7, prdl-a positive cells were restricted to the future head region. (D) Influence of budding on *prdl-a* expression during apical regeneration. Arrowheads indicate apical-regenerating stumps; arrows point to buds. In each case, we have indicated the regeneration time since mid-gastric section (12 hours in case 1; 16 hours in cases 2-4; 18 hours in case 5; 32 hours in case 6 and 7 and 36 hours in case 8) and stage of the bud with the corresponding developmental time (according to Otto and Campbell, 1977). From these two time values, we deduced in each case the overlap between budding and regeneration (budding time over regeneration time expressed as a percentage). Cases where *prdl-a* expression was strongly affected in the stump are indicated with an asterisk. In cases 5 and 8, the longer distance between the stump and the bud probably diminish the competition effect. Cases 6 and 7 were examined at the protein level; note that the level of prdl-a expression in the regenerating stump was drastically affected when overlap between budding and regeneration was complete (case 7).

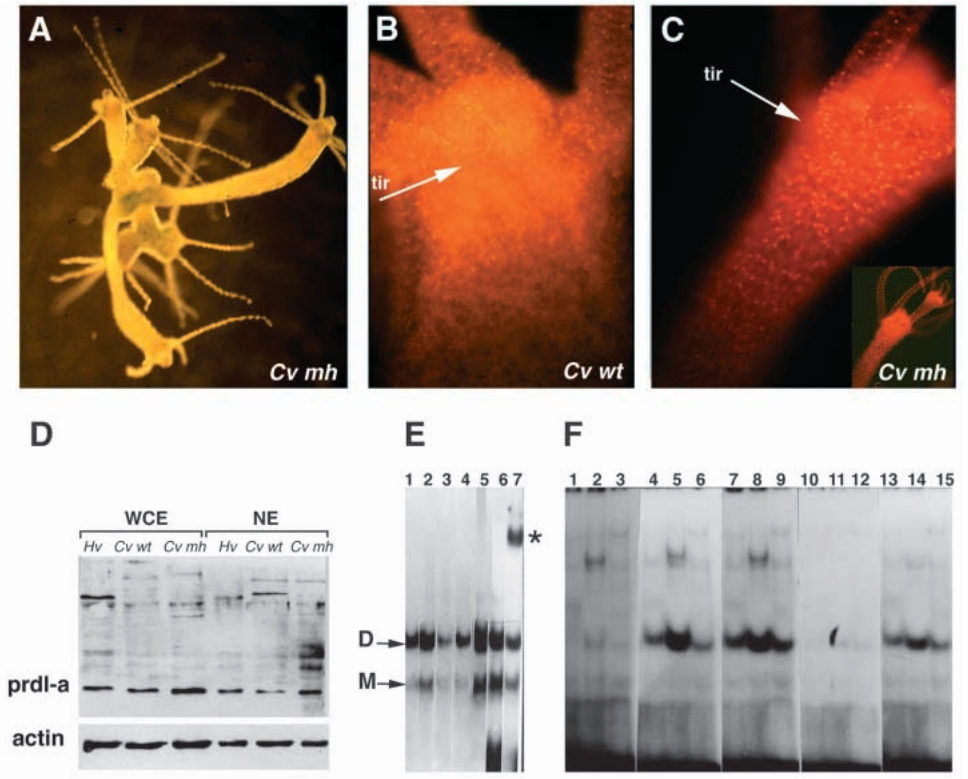

Fig. 5. Overexpression of *prdl-a* in the *Chlorohydra viridissima* multiheaded mutant (*Cv mh*). (A) Phenotype of *Cv mh*: one single animal forms multiple heads. (B,C) Prdl-a immunohistochemistry performed on wild type (*Cv wt*, B) and *Cv mh* (C) adult polyps; tir, tentacle insertion ring; 40× enlargement of region shown in inset. (D) Western analysis showing higher levels of prdl-a protein in either nuclear (NE) or whole cell (WCE) extracts from *Cv mh* when compared to that of either *Cv wt* or *Hv*. (E) DNA-binding activity of the *E. coli* produced prdl-a protein (10 ng) onto the palindromic P3 site (Wilson et al., 1993); competitor oligonucleotides were added in lane 2 (10× wt), 3 (100× wt) and 4 (100× mutant); preimmune serum in lane 6 and immune serum in lane 7; asterisk indicates the supershift; M, monomer; D, dimer. (F) DNA-binding activity of NE (10 μg) from *Hv* (lanes 1, 4, 7, 10, 13), *Cv mh* (lanes 2, 5, 8, 11, 14) or *Cv wt* (lanes 3, 6, 9, 12, 15), tested on the half-palindromic P1 (lanes 1-3) and palindromic P3 (lanes 4-15) motifs in the absence or presence of competitor oligonucleotides (lanes 7-9, 20× wt; lanes 10-12, 200× wt; lanes 13-15, 200× mutant).

homeoproteins bind DNA also as dimers: *paired*-like HDs specifically bind to a TAATPyNPuATTA palindromic motif with a 3 bp spacing (P3 site; see Wilson et al., 1993). When tested in band-shift assays, the prdl-a protein, produced either in *E. coli* as a 6HIS-fusion protein, or in reticulocyte lysate as a full-length *prdl-a* cDNA product, strongly bound the P3 sequence, generating two specific retarded bands, probably corresponding to monomer- and dimer-binding complexes, and these were supershifted upon addition of the prdl-a antiserum (Fig. 5E and not shown). These results showed that hydra *paired*-like homeoproteins could cooperatively bind as dimers to the DNA motif, which is usually bound by related triploblastic proteins. As expected, nuclear extracts prepared from the *mh* mutant displayed a stronger P3-binding activity when compared to that of *Hv* or *Cv wt* (Fig. 5F), confirming the higher level of *prdl-a* expression in *mh* mutant.

DISCUSSION

Paired-like genes in diploblastic animals

We report the isolation of two hydra *paired*-like homeogenes, *prdl-a* and *prdl-b*, which encode homeodomains highly similar to the aristaless-related (al, Arx) HDs. The degree of conservation, reaching 85% of identity, was unusually high when compared with the different cnidarian HD sequences reported so far: 74% identity for the msx homolog; 72% for the Hox-related cnox2 and 71% for the NK-2 homolog (Schummer et al., 1992; Grens et al., 1996). Conservation at the nucleotide level was lower, as expected from the codon usage (Galliot and Schummer, 1993). Surprisingly, we noted a similar rate of conservation between *Pax6*-type, *paired*-like and *prdl-a* homeoboxes. This apparent inconsistency between nucleotide and amino acid relatedness might reflect the fact that *paired*-type *(PAX)* and *paired*-like genes probably evolved from a common ancestor (Noll, 1993) and may thus have poorly diverged in cnidaria, at least within their HDs. Our observation of the conservation of an intron at position 46 is the second report of the identity between intron locations within DNA-binding domains from diploblastic to triploblastic species (Galliot et al., 1995). This conservation may be linked to the transcriptional efficiency of regulatory genes (Brinster et al., 1988). Thus *prdl-a* might exemplify ancestral forms of the *paired*-like class of homeogenes.

prdl-a is a marker for adult apical ectodermal nerve cells and their response to apical signals

In adult hydra, morphogenesis can be considered as a steady-state level of a permanent process maintained through the inductive influences of two organizing centers, apical and basal, with the prevalence of the apical over the basal. Upon standard conditions, nerve cells play a key role in this process as they produce signaling molecules, while epithelial cells are the direct effectors of morphogenesis (Sugiyama and Wanek, 1993; Galliot, 1997). Migratory processes, initiated in the body column, are important in the hydra apical region as a large number of cells migrate towards the tentacles whereas only few migrate into the inner hypostome to become a stationary population (Dübel, 1989). In adult polyps, *prdl-a* expression was restricted to a limited number of ectodermal cells, mostly nerve cells or their precursors, located in the most apical part of the head region, the hypostome, and in tentacle roots. Therefore, our results suggest that *prdl-a* might be involved in the maintenance of apical differentiation in adult polyps.

Alternatively, *prdl-a* may have a regulatory function in the feeding and/or light response as sensory nerve cells located in the hypostome are involved in these behaviours (Passano and McCullough, 1964; Grimmelikhuijzen and Westfall, 1995).

In the *mh* mutant, the *prdl-a* expression domain extended below the ring of tentacles, at a position where positive cells were not observed in wild-type animals. Since cells migrate from the body column to the extremities they probably originated in the body column, rather than in the hypostome. During their migration towards the head region, nerve cells and/or their precursors may thus start to express *prdl-a* at a lower position along the body axis than in wild-type animals, possibly because they sensed inducing signals more efficiently. The multiheaded mutant was reported to contain higher levels of both the neuropeptide head activator (HA) (Schaller et al., 1977) and its receptor (Neubauer et al., 1991). Therefore, *prdl-a* may be a direct or indirect target for apical signals such as HA.

prdl-a expression in endoderm during apical morphogenesis

Different morphogenetic contexts of head organizing activity correlated with two different *prdl-a* expression patterns: in the adult polyps, where maintenance was supposed to predominate, an apical nerve cell-restricted ectodermal expression was observed, whereas during budding and regeneration, at the time when induction was maximal, an apical transient endodermal expression was scored well before the adult pattern was reestablished. This similar biphasic modulation of *prdl-a* expression during budding and regeneration supports the view that molecular mechanisms leading to apical morphogenesis are similar in these two contexts and that endodermal cells are involved in inductive interactions. Grafting experiments have shown that the head-organizing activity (or apical-forming potential) could be assessed by measuring the ability of regenerating tips to induce a secondary head in the host (MacWilliams, 1983). After several hours of post-cutting inhibition, head-organizing activity was progressively reestablished at the regenerating tip, reaching a plateau level about 9 hours after mid-gastric section. Two different components were distinguished: on the one hand, a labile activity decaying rapidly after 12 hours, which was confined to the presumptive apical region at the apex of regenerating tip; on the other hand, a rather stable activity, still present after 48 hours, which was distributed as a gradient from the regenerating tip and required the determination of novel nerve cells (MacWilliams, 1983). Transient endodermal expression of *prdl-a* during apical regeneration was observed at a time when, and at a place where, head-organizing activity reached its maximal value. This suggests that *prdl-a* plays a role in differentiation of hydra apical structures by participating in the labile component of head-organizing activity.

Evolutionary aspect of *prdl-a* function

In cnidaria, the region surrounding the mouth opening is involved in food detection and ingestion, and thus named the head. In hydra, nerve cell density is maximal in the head region with, in some species, a nerve ring at the base of the tentacle insertion ring (see Grimmelikhuijzen and Westfall, 1995). Contraction-burst potentials, a process that is altered upon light exposure, originate in the hypostome and are conducted throughout the body column (Passano and McCullough, 1964). Thus, the head is the place where a high level of cellular and morphological organisation correlates with complex behaviours. In triploblastic species, food detection, ingestion and partial processing are also located in the head region, which contains sense organs, complex neural structures and, in vertebrates, the distribution of respiratory gases (see Gans and Northcutt, 1983). Most of the vertebrate head has supposedly arisen de novo, rather than by modification of a preexisting structure (Thorogood, 1993). Whether ancestral elements defining a ‘minimal head region’ might be present in less complex species, including diploblastic species, remains, however, an open question. In vertebrates, regulatory genes have been isolated that are parts of a molecular head-organizing activity. For example, *twist*, a basic helix-loop-helix transcription factor, regulates differentiation and behaviour of head mesenchymal cells in mice (Chen and Behringer, 1995). The *Lim-1* gene, a LIM-class homeobox gene, is required for formation of an early organized node and anterior axial mesoderm (Shawlot and Behringer, 1995), whereas the *Cart1* gene is required for the proliferation of forebrain mesenchyme cells: *Cart1*-deficient mice displayed strong anomalies of neural tube closure consecutive to head mesenchyme defects (Zhao et al., 1996). The early and transient pattern of *prdl-a* expression during regeneration suggests a function for *prdl-a* in the differentiation of the most apical part of the animal. It is thus tempting to speculate that this morphogenetic role is reminiscent of that observed and/or supposed for *Arx* in vertebrate brain development (Miura et al., 1997) and *Cart1* in mouse head formation (Zhao et al., 1996). It was recently proposed that the anterior primitive endoderm, rather than the prechordal plate mesoderm, induces head development in vertebrate embryo (see Bouwmeester and Leyns, 1997). In mouse, the patterning of the rostral neuroectoderm requires expression of a *paired*-like gene (*Hesx1*), which shows a successive endodermal to ectodermal expression (Thomas and Beddington, 1996). Hence, despite fundamental differences in vertebrates, insects, nematodes and cnidarian head development, the intriguing hypothesis whereby ancestral molecular mechanisms driving anterior/apical organizer activity to differentiate a rudimentary head might have been conserved throughout animal evolution and recruited to participate in the elaboration of a more complex head, has to be considered. This ancestral anterior/apical organizer activity might be shared by all members of the animal kingdom and involve members of the *paired*-like gene family.

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