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Abstract
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 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 concomitant 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 [...]
**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 concomitant 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; Aerné 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 aristaleal (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 NaPO₄, pH 7.4, 1 mM CaCl₂, 0.1 mM KCl, 0.1 mM MgCl₂ 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 in 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 10⁶ 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 GCGAGGAACTTGTGTCGGGT (forward) and GTACTGCACTGGTTCGGTTACAG (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 transferred 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 (10⁶ cpn/ml). Washes were performed in 2x 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 antisera 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 antisera 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 GATCGAGGGAATTCCCTTAGC, 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 aristaleal 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

1  CANATTTTACGAAACATTTGAAAGGAGGCAAGCAGCTAAGGCTTTGTTAGG  MTLG  5
73  TATGCTGCAATGTTGAAACTCGGCGTATGTCAGGAAATAAAGAACTGAGGGGC  29
145  AAAAGAGAAGATGATCTTACGAGAAGAAGAGGCAAGCAGCTAAGGCTTTGTTAGG  NQRSFGKRNERRRTFTTFQFLD  53
217  TGACATGAAAAGACATTTGGATATTCGTGACGATGAAACTCGGCGTATGTCAGGAAATAAAGAACTGAGGGGC  77
289  TGACATGAAAAGACATTTGGATATTCGTGACGATGAAACTCGGCGTATGTCAGGAAATAAAGAACTGAGGGGC  85

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; zl, zebrasfish) 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.

B

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C

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Head development in cnidarian 1639
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 adult 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 about 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...
Head development in cnidarian 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 concomitantly, 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

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**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 reestablished 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.
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 multiheded mutant**

As the multiheded 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
homeoproteins bind DNA also as dimers: paired-like HDs specifically bind to a TAATPyNPuATTATAT 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.

**Fig. 5.** Overexpression of prdl-a in the Chlorohydra viridissima multieathed 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; 40x 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 (10x wt), 3 (100x wt) and 4 (100x 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, 20x wt; lanes 10-12, 200x wt; lanes 13-15, 200x 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 homebox 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 consequent 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 (Hexx1), 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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**REFERENCES**


Schummer, M., Scheurlen, I., Schaller, C. and Galliot, B. (1992). HOM/HOX homeobox genes are present in hydra (Chlorohydra viridissima) and are differentially expressed during regeneration. EMBO J. 11, 1815-23.


