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Pax–Six–Eya–Dach network during amphioxus development: Conservation *in vitro* but context specificity *in vivo*

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Abstract

The *Drosophila* retinal determination gene network occurs in animals generally as a Pax–Six–Eyes absent–Dachshund network (PSEDN). For amphioxus, we describe the complete network of nine PSEDN genes, four of which–*AmphiSix1/2*, *AmphiSix4/5*, *AmphSix3/6*, and *AmphiEya*–are characterized here for the first time. For amphioxus, *in vitro* interactions among the genes and proteins of the network resemble those of other animals, except for the absence of Dach–Eya binding. Amphioxus PSEDN genes are expressed in highly stage- and tissue-specific patterns (sometimes conspicuously correlated with the local intensity of cell proliferation) in the gastrular organizer, notochord, somites, anterior central nervous system, peripheral nervous system, pharyngeal endoderm, and the likely homolog of the vertebrate adenohypophysis. In this last tissue, the anterior region expresses all three amphioxus *Six* genes and is a zone of active cell proliferation, while the posterior region expresses only *AmphiPax6* and is non-proliferative. In summary, the topologies of animal PSEDNs, although considerably more variable than originally proposed, are conserved enough to be recognizable among species and among developing tissues; this conservation may reflect indispensable involvement of PSEDNs during the critically important early phases of embryology (e.g. in the control of mitosis, apoptosis, and cell/tissue motility).

Keywords: Cephalochordata; Lancelet; Somitogenesis; Neurogenesis; Placodes; Pituitary

Introduction

The Retinal Determination Gene Network (RDGN) of *Drosophila* plays a major part in specifying eye development. The genes constituting the network (*twin of eyeless*, *eyeless*, *sine oculis*, *eyes absent*, and *dachshund*) are interrelated by reciprocal feedback loops and encode nuclear proteins that can form multi-molecular complexes to control target gene transcription (Silver and Rebay, 2005; Friedrich, 2006). Animals other than *Drosophila* also have recognizable RDGNs (Duncan et al., 1997; Bebenek et al., 2004; Colosimo et al., 2004; Kozmik, 2005; Mazet et al., 2005). For example, vertebrate RDGNs, which are involved in such human pathologies as branchio–oto–renal syndrome, comprise over a score of genes belonging to the following four families: *Pax* (corresponding to *twin of eyeless* and *eyeless*), *Six* (corresponding to *sine oculis*), *Eyes absent* (*Eya*, unaltered terminology), and *Dachshund* (*Dach*, unaltered terminology). To accommodate animals in general, Kawakami et al. (2000) replace the *Drosophila*-specific term "RDGN" with the more widely applicable term "PSEDN" (Pax–Six–Eya–Dach Network), a convention that we will follow in the present paper. During vertebrate development, PSEDN genes play key developmental roles not only in the eyes, but also in such structures as muscles, endocrine glands, placodes, and pharyngeal pouches (Hanson, 2001; Silver and Rebay, 2005; Rebay et al., 2005). Recent work (reviewed by Donner and Maas, 2004) shows that PSEDN

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configurations may differ considerably from one tissue to the next, although there is more uniformity when a given tissue (excepting muscles) is compared among vertebrate classes. Some of these within-tissue similarities in PSEDNs are even found when vertebrates are compared with their closest invertebrate chordate relatives, the tunicates (Bassham and Postelthwait, 2005; Mazet et al., 2005).

The present paper extends the study of PSEDNs to amphioxus (*Branchiostoma floridae*), now thought to represent the most basal group of living chordates (Blair and Hedges, 2005; Philippe et al., 2005; Bourlat et al., 2006; Delsuc et al., 2006) and one of the best available proxies for the protochordate ancestor of the vertebrates (Schubert et al., 2006). The amphioxus PSEDN comprises nine genes—four characterized here for the first time (namely *AmphiSix1/2*, *AmphiSix3/6*, *AmphiSix4/5*, and *AmphiEya*), plus five characterized previously, namely *AmphiPax1/9* (Holland et al., 1995), *AmphiPax2/5/8* (Kozmik et al., 1999), *AmphiPax3/7* (Holland et al., 1999), *AmphiPax6* (Glardon et al., 1998), and *AmphiDach* (Candiani et al., 2003). In comparison to vertebrates, amphioxus has less than half as many PSEDN genes. Moreover, for amphioxus, it is likely that no more of these genes will be found, since no additional ones are present in the recently completed *B. floridae* genome sequence (draft version 1.0). Here we provide comprehensive expression profiles for all the amphioxus PSEDN genes at four developmental stages. Furthermore, because several of these genes are involved in cell cycle control, we compare our expression data with tissue-specific cell proliferation as determined by bromodeoxyuridine (BrdU) labeling. Finally, as a first step toward a mechanistic understanding of the PSEDN in amphioxus, we analyze interactions between proteins encoded by key genes of the network as well as protein–DNA interactions and Six-mediated nuclear translocation of Eya. Considered as a whole, the amphioxus data indicate that the patterns of association among PSEDN components are not as stereotyped as initial comparisons between *Drosophila* and vertebrates reasonably seemed to indicate (Relaix and Buckingham, 1999). Even so, animal PSEDNs have tended to persist in variable, but still recognizable, constellations during evolution, possibly because of their indispensable involvement in such processes as cell cycling, apoptosis, and cell/tissue motility during the early stages of development.

Materials and methods

Animal culture, isolation of cDNA sequences, and phylogenetic analysis

Ripe males and females of the Florida amphioxus (*B. floridae*) were collected in Tampa Bay, Florida. Spawning was stimulated electrically, and the embryos and larvae were raised according to Holland and Holland (1993). A cDNA library in λZap II (Stratagene, La Jolla, CA) from mixed-stage (8 h to 18 h) embryos served as a template in PCR with degenerate primers specific for conserved regions of *Eya* (corresponding to amino acids WDLDET and MRKLAF) and *Six* genes (corresponding to amino acids PRTIWDG and NWFKNRR). The sub-cloned PCR fragments were sequenced, and this information was used to design the following gene-specific primers: *AmphiEya* (3′RACE, gtacctacaattttgcaacagacgg; 5′RACE, ccgtctgttgcaaaattgtaggtac), *AmphiSix1/2* (3′RACE, ggtgtgttacgggagtggtacgct; 5′RACE, gggaagggtacgggttgtgagcgta), *AmphiSix4/5* (3′RACE, agaccgtctactgcttcaaggaga; 5′RACE, ttctcgtctggagtcgggtaccg), *AmphiSix3/6* (3′RACE, tgttacgagaatggtatctacaagacc; 5′RACE, cttttgcgggtttgggtaagggtctt).

For phylogenetic analysis, we aligned sequences and generated neighbor-joining trees with bootstrap with ClustalX and the GeneDoc program. To construct a Six tree, we used the following organisms (with accession numbers): *Aurelia aurita*, cnidarian, Six1/2 (AY652604); *Podocoryne carnea*, cnidarian, Six1/2 (AY542527), Six3/6 (AY542528); *Cladonema radiatum*, cnidarian, Six1/2 (AY542529), Six3/6 (AY542530), Six4/5 (AY542531); *Drosophila melanogaster* sine oculis (NM_057385), optix (Q95RW8), myotonix (AAF63760); *Ciona intestinalis*, tunicate Six1/2 (AK112392), Six3/6 (BAE06688), Six4/5 (AK116814); *Platynereis dumerilii*, annelid Six2 (CAC86663); *Danio rerio*, zebrafish, Six1 (NM_207095), Six2 (NM_057385), Six3 (NM_131362), Six4 (NM_131720); *Xenopus laevis*, frog Six1 (AF279254), Six2 (AF108810), Six3 (AF183571), XOptx2 (AAD47356), Six6 (BC042277); *Homo sapiens*, human SIX1 (AF323497), SIX2 (AF332198), SIX3 (NM_005413), SIX4 (AB024685), SIX5 (NM_175873), SIX6 (AF141651), *B. floridae*, amphioxus AmphiSix1/2 (EF195742), AmphiSix4/5 (EF195741), AmphiSix3/6 (EF195743); *Girardia tigrina*, flatworm, sine occulis (AJ251660); *Gallus gallus*, chicken Optx2 (AF050131), Six3 (Y15106). To construct an Eyes absent tree, we used the following organisms (with accession numbers): *D. melanogaster* eya (CG9554); *Dugesia japonica*, flatworm, Eya (AJ557023); *B. floridae*, amphioxus AmphiEya (EF195740); *Strongylocentrotus purpuratus*, sea urchin Eya (XP_789024); *Oikopleura dioica*, tunicate Eya (AAZ23131); *C. intestinalis*, tunicate Eya (BW252124); *Xenopus tropicalis* frog Eya1 (ENSXETP00000034849), Eya2 (ENSXETP00000007517), Eya3 (ENSXETP00000028181), Eya4 (ENSXETT00000000214); *G. gallus*, chicken Eya1 (ENSGALP00000025135), Eya2 (ENSGALP00000007168, NP_990246), Eya3 (ENSGALP00000001125), Eya 4 (ENSGALP00000022622); *Mus musculus*, mouse Eya1 (NP_034294), Eya2 (NP_034295), Eya3 (NP_0997592), Eya4 (NP_034297); *H. sapiens* human EYA1 (ENSP00000342626, NP_742056), EYA2 (ENSP00000342173, NP_005235), EYA3 (ENSP00000342919, NP-001981), EYA4 (ENSP00000351322, NP_004091).

In situ hybridization; BrdU labeling

Antisense probes were prepared against 3′ clones of *AmphiSix1/2* and *AmphiSix3/6*, mixed 3′ and 5′ clones of *AmphiSix4/5* and *AmphiSix3/6*, and a 3′ clone of *AmphiEya*. *In situ* hybridization and histological sectioning were according to Holland et al. (1996). In addition, the developmental expression of *AmphiPax1/9* is amended (see Supplemental Figures A–D) to include the somitic mesoderm expression domain not noticed by Holland et al. (1995). To study regional differences in cell proliferation intensity, embryos and larvae were pulse-labeled with BrdU according to Holland and Holland (2006). We photographed 25 different 36-h larvae (examples in Supplemental Figures E–H) and superimposed the photographs to accentuate the patterns of the BrdU-labeled nuclei in the cerebral vesicle and preoral pit.

GST pull-down analysis of protein–protein interactions

For pull-down assays, either the N-terminal region (amino acids 1–115) or the C-terminal region (amino acids 338–637) of AmphiEya was fused to glutathione *S*-transferase (GST) by cloning the respective nucleotide sequences of *AmphiEya* into pGEX-6P-1 vector using oligonucleotides 270E/270Eco and 270F/270G, respectively. Plasmids encoding GST plus the N-terminus of AmphiEya and those encoding GST plus the C-terminus (which included the entire Eyes absent domain) were transformed in *E. coli* strain BL21. Expression of fusion proteins was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM for 2 h. The bacteria were harvested by centrifugation and resuspended in 5 ml of NTEN buffer (20 mM Tris pH 8, 100 mM NaCl, 1 mM EDTA, and 0.5% NP40) at 4 °C for 20 min. After sonication and centrifugation to remove cell debris, the supernatant was incubated with 200 μl of glutathione–sepharose beads (BD Bioscience, Franklin Lakes, NJ) at 4 °C for 1 h. After three washes in 5 ml of binding buffer (20 mM Tris pH 8, 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 20% glycerol, and 0.1% NP40), the levels of GST fusion proteins bound to the beads were checked by SDS–PAGE stained with Coomassie blue. Normalized amounts of the bead-