Flexibly deployed *Pax* genes in eye development at the early evolution of animals demonstrated by studies on a hydrozoan jellyfish

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Pax transcription factors are involved in a variety of developmental processes in bilaterians, including eye development, a role typically assigned to Pax-6. Although no true *Pax-6* gene has been found in nonbilateral animals, some jellyfish have eyes with complex structures. In the cubozoan jellyfish *Tripedalia*, *Pax-B*, an ortholog of vertebrate *Pax-2/5/8*, had been proposed as a regulator of eye development. Here we have isolated three *Pax* genes (*Pax-A*, *Pax-B*, and *Pax-E*) from *Cladonema radiatum*, a hydrozoan jellyfish with elaborate eyes. *Cladonema Pax-A* is strongly expressed in the retina, whereas *Pax-B* and *Pax-E* are highly expressed in the manubrium, the feeding and reproductive organ. Misexpression of *Cladonema Pax-A* induces ectopic eyes in *Drosophila* imaginal discs, whereas *Pax-B* and *Pax-E* do not. Furthermore, *Cladonema* Pax-A paired domain protein directly binds to the 5′ upstream region of eye-specific *Cladonema* opsin genes, whereas Pax-B does not. Our data suggest that *Pax-A*, but not *Pax-B* or *Pax-E*, is involved in eye development and/or maintenance in *Cladonema*. Phylogenetic analysis indicates that *Pax-6*, *Pax-B*, and *Pax-A* belong to different *Pax* subfamilies, which diverged at the latest before the Cnidaria–Bilateria separation. We argue that our data, showing the involvement of *Pax* genes in hydrozoan eye development as in bilaterians, supports the monophyletic evolutionary origin of all animal eyes. We then propose that during the early evolution of animals, distinct classes of *Pax* genes, which may have played redundant roles at that time, were flexibly deployed for eye development in different animal lineages.

biodiversity | *Cladonema radiatum* | Cnidaria | evo-devo | gene duplication

The *Pax* gene family encodes transcription factors involved in a variety of developmental processes in metazoans (1–3). It can be subdivided into five subfamilies—*Pax-4/6*, *Pax-2/5/8*, *Pax-1/9*, *Pax-3/7*, and *pox neuro* (*poxn*)—on the basis of structural comparison and phylogenetic relationship (4). These subfamilies diverged from each other at the latest before the separation of cnidarians and bilaterians (4, 5).

It is widely accepted that *Pax-6*, a member of *Pax-4/6* subfamily, is one of the most significant components of the gene network that controls eye development in many bilaterians (e.g., refs. 6, 7); mutations in *Pax-6* genes cause severe eye defects both in mammals and in *Drosophila* (8, 9), and *Pax-6* genes cloned from diverse bilaterians can ectopically initiate eye development both in *Drosophila* and in *Xenopus* when they are misexpressed (6, 10, 11).

Cnidaria are the earliest branching animal phylum containing species with multicellular eyes, which sometimes show complex structures such as lens, iris, pigmented layer, and photosensitive layer (12). Among the five classes of the phylum Cnidaria (Anthozoa, Hydrozoa, Cubozoa, Scyphozoa, and recently recognized Staurozoa), four of them (Hydrozoa, Cubozoa, Scyphozoa, and Staurozoa) include eye-bearing species (12). However, no bona fide *Pax-6* has been identified in cnidarians to date.

Kozmik et al. (13) have proposed that *Pax-B*, a member of the *Pax-2/5/8* subfamily, is responsible for eye development in *Tripedalia cystophora*, a cubozoan jellyfish. *Tripedalia Pax-B* is expressed in the rhopalia, which are batteries of sensory organs including eyes. This gene is also able to transactivate the promoters of a *Tripedalia* crystallin gene and a *Drosophila* rhodopsin gene in cell culture, and it induces ectopic eyes in *Drosophila* when misexpressed in imaginal discs. These data suggest that *Pax-B* is responsible for eye development in *Tripedalia*. The authors have further proposed that *Pax-6* diverged from *Pax-B* (*Pax-2/5/8*) by gene duplication in the bilaterian lineage after the separation from cnidarians and was independently recruited for controlling eye development in the bilaterian lineage (13, 14). Accordingly, they have hypothesized that cnidarian and bilaterian eyes arose independently.

Besides the class Cubozoa, the class Hydrozoa includes several species with complex eyes (12). Sun et al. (15) have investigated *Cladonema californicum*, a hydrozoan jellyfish bearing eyes. However, they have found only a *Pax-B* gene, whose function has not been well studied in *Cladonema*.

In this study, we have isolated and characterized three *Pax* genes from *Cladonema radiatum*, which possesses eyes with elaborate structures including lens, pigmented cell layer, and photosensitive cell layer (16, 17) (Fig. 1*A*). Our data suggest that *Pax-A*, a member of the *poxn* subfamily, rather than *Pax-B*, plays a major role in the *Cladonema* eye. We argue that our results support a hypothesis that all of the animal eyes have a single evolutionary origin (6). We propose that, for development and/or maintenance of eyes, distinct lineages of animals flexibly selected different classes (corresponding to subfamilies) of *Pax* genes, the ancestors of which may have had redundant roles in the common ancestor of cnidarians and bilaterians.

Results

Identification of *Pax* Genes from Hydrozoan Jellyfish and Marine Sponge. By performing degenerate PCR with multiple primer combinations, we obtained three cDNAs encoding Pax proteins from *C. radiatum*. Two of the cloned *Pax* genes show high sequence similarities and identical domain structures (Fig. 1*B*) to previously identified cnidarian *Pax* genes, *Pax-A* and *Pax-B*; *C. radiatum* Pax-A and Pax-B (designated CrPax-A and CrPax-B) show 100% and 83% amino acid

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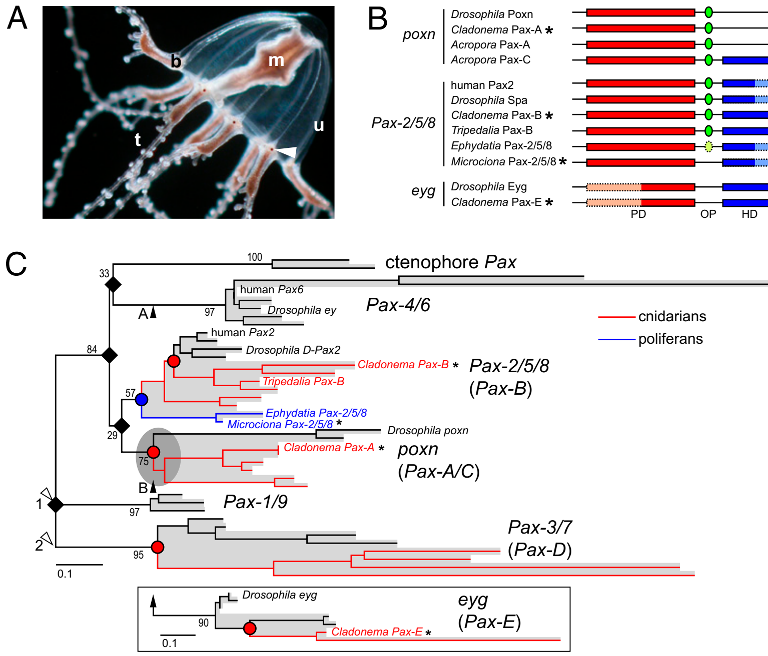

Fig. 1. *Pax* genes cloned from *Cladonema* and *Microciona*, and molecular phylogenetic tree of the *Pax* family. (*A*) Medusa of *C. radiatum*. Arrowhead indicates an eye. b, tentacle bulb; m, manubrium; t, tentacle; u, umbrella. Photo courtesy of Claudia List. (*B*) Structures of Pax proteins. Those cloned in this study are marked by asterisks. Red box, green ellipse, and blue box represent the PD, octapeptide (OP), and HD, respectively. Octapeptide-like motifs of the *poxn* subfamily are found at amino acid positions 360–367 for *Drosophila* Poxn, 435–442 for *Cladonema* Pax-A, 324–331 for *Acropora* Pax-A, and 153–160 for *Acropora* Pax-C. Symbols with dashed line and pale color indicate highly divergent sequences. (*C*) The ML tree inferred from a comparison of the whole PD amino acid sequences. See Fig. S1 for details. Two possible root positions are indicated according to previous publications (4, 5, 22) (white arrowheads). The *eyg* subtree derived from an additional ML analysis (Fig. S2*B*), which is performed on the basis of comparison of the latter halves (RED subdomains) of the PD sequences, is shown in a box. Black arrowheads indicate two possible ML positions of the *eyg* subtree suggested by further ML analyses based either on a comparison of the RED subdomain sequences (arrowhead A; Fig. S3*A*) or on that of the RED subdomain plus the whole HD sequences (arrowhead B; Fig. S3*B*). Because complete HDs are present only in *Pax-C* among the members of the *poxn* subfamily, *Pax-A* and *poxn* were excluded from the latter analysis. The position of the *eyg* subtree is therefore ambiguously shown at the root of *poxn* subtree (gray ellipse). Subtrees corresponding to distinct subfamilies are shaded gray and the subfamily names are shown on the right, with the names of cnidarian members in parentheses. Cnidarian sequences and poriferan sequences are shown in red and blue, respectively. Red and blue circles indicate the cnidarians–bilaterians and the poriferans–eumetazoans splits, respectively. Filled rhombi indicate the gene duplications that gave rise to the subfamilies. The extended local bootstrap probability is shown at each branch that separates subfamilies.

identity in the paired domain (PD) to *Hydra magnipapillata* Pax-A and Pax-B, respectively. The third gene (*CrPax-E*) does not show a particularly close relationship to any of the cnidarian *Pax* genes identified thus far. Interestingly, an identical domain structure is shared by CrPax-E and *Drosophila* Eyegone (Eyg), both of them having a highly divergent N-terminal subdomain (called PAI) of PD, and a complete homeodomain (HD), but lacking an octapeptide sequence (Fig. 1*B*).

To gain further insights into the diversity of *Pax* genes in basal metazoans, we investigated several additional organisms. Among metazoans, poriferans (sponges) show the simplest body plans, lacking nervous system and true organs. Although some sponge larvae show photosensitive responses (18, 19), they seem to use different cell- and molecular-level mechanisms of photoreception from those of eumetazoans (18–21). All of the *Pax* genes identified so far from various species of demosponges are of the *Pax-2/5/8* type. Only one *Pax* cDNA that we could obtain from the marine sponge *Microciona prolifera* by degenerate PCR is closely related to the *Pax-2/5/8* of the freshwater sponge *Ephydatia fluviatilis* (22) (92% amino acid identity in the PD). The only *Pax* gene found in the complete genome sequence of the marine sponge *Amphimedon queenslandica* (named *AmqPaxB*) (23), and another *Pax* gene recently found in another marine sponge, *Chalinula loosanoffi* (24), also belong to the *Pax-2/5/8* subfamily. From the genome sequence of the choanoflagellate *Monosiga brevicollis*, a protist thought to be the closest relative to metazoans (25), no *Pax* gene was found.

Phylogenetic Tree Analyses of the *Pax* Gene Family. We inferred a phylogenetic tree based on a comparison of the whole PD sequences by the maximum likelihood (ML) method (Fig. 1*C*). The phylogenetic tree provides supports to the previous classification of the *Pax* genes into five subfamilies: *Pax-4/6*, *Pax-2/5/8*, *Pax-1/9*, *Pax-3/7*, and *pox neuro* (*poxn*) (4, 5). The ctenophore *Pax* genes form an independent cluster, being away from the other metazoan *Pax* subfamilies. It remains unclear whether they represent a novel subfamily or it is simply an artifact caused by the high evolutionary rate (4).

In Fig. 1*C*, *CrPax-A* and *CrPax-B* are classified into the *poxn* and *Pax-2/5/8* subfamilies, respectively. The classification of *CrPax-E* was carried out separately because its PD is incomplete. The identical domain structure shared by CrPax-E and *Drosophila* Eyg suggests their close evolutionary relationship. Searches of the genome sequences of *Anopheles*, two species of nematodes, and *Hydra* revealed in each organism the presence of a *Pax* gene that lacks a PAI subdomain or has a highly divergent one, as happens in *CrPax-E* and *Drosophila eyg*. Phylogenetic analyses using the sequences of the second half of PD (RED subdomain) provides a strong support to the independent clustering of all those *Pax* genes that have the truncated PDs (Fig. S2; Bayesian posterior probability of 1.0 and extended local bootstrap probability of 90%). We therefore propose that *CrPax-E* and *eyg* compose a novel subfamily (*eyg* subfamily) together with the other *Pax* genes bearing the degenerated PDs. To determine the branching position of the *eyg* subfamily, we exhaustively evaluated all of the possible branching positions of the *eyg* subtree on the ML tree in Fig. 1*C* by log-likelihood values. These analyses using (*i*) only the RED subdomains and (*ii*) the RED subdomains plus the whole HDs indicated two different branching positions (Fig. 1*C*; arrowheads A and B, respectively) as most likely, although none of them excluded the other possibilities statistically significantly (Fig. S3).

Consistent with previous reports (4, 26), our phylogenetic tree analysis strongly suggests that the majority of gene duplications (subfamily-generating duplications; indicated by black rhombi in Fig. 1*C*) that gave rise to different *Pax* subfamilies occurred before the separation of cnidarians and bilaterians (Fig. 1*C*, red circles), no matter where the tree root is situated (white arrowheads) (4, 5, 22). The affiliation of the sponge *Pax* genes to the *Pax-2/5/8* subfamily (4, 22) suggests an even earlier occurrence (before the separation of poriferans and eumetazoans) of these duplications (4, 22). It is thus likely that at the latest the common ancestor of cnidarians and bilaterians once possessed a gene belonging to the *Pax-4/6* subfamily, which was subsequently lost or has not yet been found in the cnidarian lineage.

***Cladonema Pax-A* Is Expressed in the Eye, Whereas *Pax-B* Is Expressed in the Oocytes.** In the cubozoan jellyfish *T. cystophora*, *Pax-B* is highly expressed in the eyes (13). This supports the hypothesis that *Pax-B* is involved in eye development in this species. To identify a *Pax* gene that is potentially involved in eye development in the hydrozoan jellyfish *C. radiatum*, we performed expression analyses of the *Pax* genes by real-time quantitative RT-PCR (Fig. 2 *A–C*). The data unexpectedly showed that *CrPax-B* is mainly expressed in the manubrium, the feeding and reproductive organ, but not at the bases of the tentacles (tentacle bulbs) where the eyes develop. *CrPax-E* shows a similar expression profile to *CrPax-B*. Instead, *CrPax-A* turned out to be highly expressed in the tentacle bulbs.

The expression pattern of *CrPax-A* (Fig. 2 *D–G*), analyzed by whole-mount in situ hybridization, is consistent with the result of real-time RT-PCR analysis. *CrPax-A* is expressed both in the ocellus and in the peripheral tissue of the tentacle bulb (Fig. 2 *D* and *E*). Cryosections clearly show that the staining is localized in the retina (Fig. 2*F*) and in the ectodermal tissue of the tentacle bulb (tentacle bulb ectoderm; TBE) (Fig. 2*G*), where numerous differentiating nematoblasts (developing stinging cells) (27) are observed (Fig. 2*G*, *Inset*).

CrPax-B transcripts were detected by in situ hybridization in the aboral region of the manubrium, where gonads develop (Fig. 2*H*). In a young medusa with no apparent gonads, the expression was undetectable (Fig. 2*I*). The *CrPax-B* expression was observed in the cytoplasm of oocytes at various stages of development (Fig. 2*J*). We further quantified the *CrPax-B* expression levels in 10 medusae at two different growth stages: just-detached and full-grown (see *Materials and Methods* for definitions). The expression of *CrPax-B* in the full-grown medusae with gonads was 3.9–77 times higher than that in the just-detached medusae that lack gonads (Fig. 2*K*). These data indicate that *CrPax-B* is active in the maturing oocytes.

***Cladonema Pax-A* Induces Ectopic Eyes in *Drosophila*, Whereas *Pax-B* and *Pax-E* Do Not.** *Tripedalia Pax-B* can ectopically initiate eye development in *Drosophila* when it is misexpressed in imaginal discs by activating the intrinsic gene network that directs compound eye development (13). We tested the ability of *Cladonema Pax* genes to induce ectopic eyes in *Drosophila* by using the yeast Gal4/UAS system (28). The targeted expression of *CrPax-A* in imaginal discs using the dpp^{blink}-Gal4 driver induced ectopic eyes, but only in a limited portion of the screened flies. However, the additional use of UAS-Gal4, which increases Gal4 expression, resulted in ectopic eye inductions on more than 50% of the screened flies (Fig. 3*A*). Most of the induced eyes were observed in the notum, which develops from the wing imaginal disk. The induced eyes clearly displayed ommatidia when analyzed by SEM (Fig. 3*B*). In contrast to *CrPax-A*, *CrPax-B* and *CrPax-E* did not induce ectopic eyes either with the dpp^{blink}-Gal4 driver alone or with the combination of that driver and the UAS-Gal4 transgene.

***Cladonema Pax-A* Can Activate the *Drosophila* Eye Determination Gene Network in the Absence of *ey*.** Initiation of eye development in *Drosophila* requires expression of a set of genes that compose a transcriptional regulatory network, sometimes referred to as the retinal determination gene network (RDGN) (7). This network

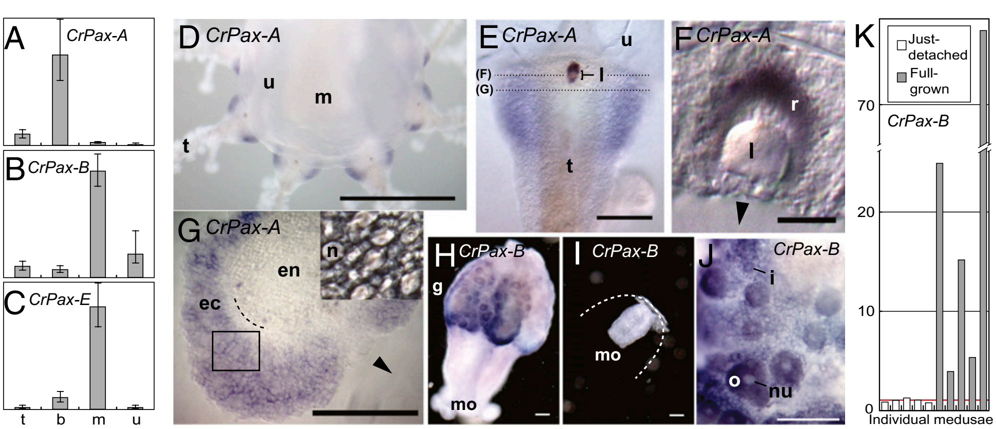

Fig. 2. Expression of *Cladonema Pax* genes. *Cladonema* medusae were dissected into four body parts, tentacles (t), tentacle bulbs with eyes (b), manubrium (m), and umbrella (u) (Fig. 1*A*) and expression levels of *CrPax-A* (*A*), *CrPax-B* (*B*), and *CrPax-E* (*C*) were quantified by real-time RT-PCR analysis. The expression level in each body part relative to the whole body (1.0) was calculated and subsequently normalized to the expression level of elongation factor 1α (EF1α). The quantifications were performed three times on different cDNAs generated independently, and geometric means were calculated. The *y* axes are arbitrary. Error bars represent SDs. (*D–J*) Whole-mount in situ hybridization analyses with anti-sense RNA probes for *CrPax-A* (*D–G*) and *CrPax-B* (*H–J*). Cross-sections of the eye (*F*) and the whole tentacle (*G*) of the *CrPaxA* probe-hybridized jellyfish are shown. The cutting planes are indicated by the dashed lines in *E*. Note that the natural eye pigment disappears during the hybridization procedure. (*G*) A phase contrast microscopic picture of the ectoderm tissue, which corresponds to the box, is shown in the *Inset*. Dashed line indicates the border between endoderm and ectoderm. Arrowheads indicate the frontal side of a tentacle. (*H* and *I*) Excised manubriums. The original position of the umbrella is indicated by a dashed line in *I*. ec, ectoderm; en, endoderm; g, gonads; i, immature oocyte; l, lens; m, manubrium; mo, mouth; n, nematoblast; nu, nucleus; o, mature oocyte; r, retina; t, tentacle; u, umbrella. (Scale bars, 500 μm in D, 100 μm in *E* and *G–I*, 10 μm in *F*.) (*K*) *CrPax-B* expression levels quantified independently for five just-detached and five full-grown medusae by real-time RT-PCR. Their expression levels relative to the average (red line) of the just-detached medusae are presented. Data normalized to EF1α.

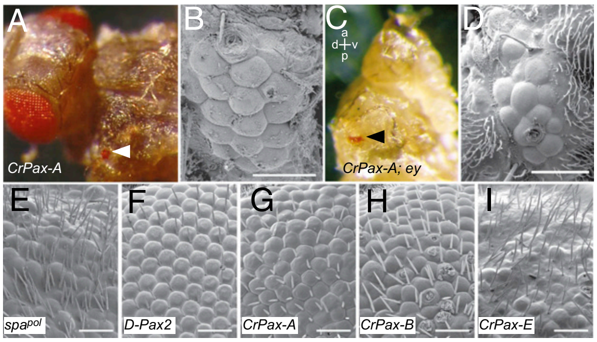

Fig. 3. Ectopic eye induction in *Drosophila* and rescue of the *spa*^{pol} mutant phenotype by *Cladonema Pax* genes. (*A*) *CrPax-A* was expressed under the control of *dpp*^{blink}-Gal4 driver with UAS-Gal4. Arrowhead indicates the induced eye. (*B*) SEM picture of the induced eye. (*C*) Misexpression of *CrPax-A* under the control of *dpp*^{blink}-Gal4 driver in a homozygous *ey* null mutant (*ey*$^{J5.71}$) background induced ectopic eyes (arrowhead). The anteroposterior (a-p) and dorsoventral (d-v) axes are shown. Note that the natural compound eye is absent. The genotype of *ey* mutant was confirmed by the absence of the second exon of *ey* (30) by PCR. (*D*) SEM picture of the induced eye. (*E*) Eye phenotype of the *spa*^{pol} homozygous fly. (*F–I*) Rescue experiments of the *spa*^{pol} mutant phenotype. UAS-*D-Pax2* (*F*; positive control), UAS-*CrPax-A* (*G*), UAS-*CrPax-B* (*H*), and UAS-*CrPax-E* (*I*) transgenic lines were crossed with the *spa*-Gal4 driver line in a *spa*^{pol} homozygous background. (Scale bars, 30 μm.)

contains a positive feedback transcriptional loop comprising *eyeless* (*ey*; *Drosophila Pax-6*), *sine oculis*, *eyes absent*, and *dachshund* (7, 29). Initially, this transcriptional loop was thought to be ignited only by the activation of *ey* by *twin of eyeless* (*toy*; another *Pax-6* gene) (29). However, it has been shown that *toy* alone is able to initiate the transcriptional loop in the absence of *ey* by directly activating another loop member, *sine oculis* (30). To examine how the jellyfish *Pax* gene activates the *Drosophila* RDGN, we tested whether *CrPax-A* can still induce ectopic eyes in the absence of *ey*. Indeed, the expression of *CrPax-A*, driven by the *dpp*^{blink} enhancer, induced ectopic eyes in an *ey* null mutant (*ey*$^{J5.71}$) background (Fig. 3 *C* and *D*). Our data indicate that the *CrPax-A* initiates the *Drosophila* RDGN by activating the components of the transcriptional loop, even in the absence of *ey*, as *toy* does.

Both *Cladonema Pax-A* and *Pax-B* Can Substitute for *Pax-2* in the *Drosophila* Eye. In the *Drosophila spa*^{pol} mutant (Fig. 3*E*), the expression of *D-Pax2,* a member of the *Pax-2/5/8* subfamily, in cone and primary pigment cells is abolished, resulting in a severely disturbed development of ommatidial cells (31). We used this mutant to test whether the *Cladonema Pax* genes can substitute for the *D-Pax2* functions in ommatidial cells, by performing rescue experiments. Interestingly, not only *CrPax-B*, an ortholog of *D-Pax2*, but also *CrPax-A* significantly rescued the *spa*^{pol} eye phenotype when they were expressed under the control of the cone- and pigment cell–specific enhancer (*spa* enhancer) (31, 32) of *D-Pax2*. *CrPax-E*, however, did not rescue the phenotype. SEM pictures clearly show that the hexagonal shape of each ommatidium and the regular arrangement of interommatidial bristles were largely recovered both by the expression of *CrPax-A* and by that of *CrPax-B* in the developing eye (Fig. 3 *F–I*).

CrPax-A PD Directly Binds to the Upstream Regions of Eye-Specific Opsin Genes. Previous publications had proposed that *Drosophila* Ey directly regulates the expression of rhodopsin genes through its HD (33, 34). Likewise, *Tripedalia* Pax-B can transactivate a *lacZ* reporter gene under the control of a *Drosophila* rhodopsin promoter, presumably through its HD (13). *Cladonema Pax-A*, however, lacks an HD. We therefore tested the possibility that CrPax-A can still directly bind the promoter regions of *Cladonema* eye-specific opsin genes, which had been characterized in our previous study (17), in the absence of an HD. We screened ≈1 kb of 5′ promoter fragments by performing EMSA with PD proteins using sets of probes that cover the fragments. In two examined opsin genes (CropG1 and CropN1), one and two CrPax-A–specific PD binding sites (red vertical lines in Fig. 4*A*) were identified, respectively (Fig. 4*B*). No or very faint binding was detected when the putative binding sites were mutated (Fig. 4*B*). The sequences of the identified CrPax-A PD binding sites moderately match the chordate Pax-6 PD consensus binding sequence (35) (Fig. S4). In contrast, CrPax-B PD showed little, if any, binding to any of the tested probes (Fig. 4*B*; only the three probes that showed the CrPax-A binding are shown). These results are consistent with the notion that *Pax*-A, but not *Pax-B*, is involved in eye development and/or maintenance in *Cladonema*.

Discussion

Functional Diversity of *Cladonema Pax* Genes. In this study, we have characterized three *Pax* genes (*CrPax-A*, *CrPax-B*, and *CrPax-E*) from *C. radiatum*, a hydrozoan jellyfish. From the expression analyses, we assume that *CrPax-A* is involved in eye development and/or maintenance, whereas *CrPax-B* is required for the oocyte maturation process. Although *CrPax-E* is predominantly expressed in the manubrium, attempts to detect its transcripts by in situ hybridization were not successful, probably owing to its low expression level. Interestingly, the dramatic gene up-regulation observed for *CrPax-B* upon gonad formation (Fig. 2*K*) was not observed for *CrPax-E*, suggesting that they exert different functions in the manubrium.

The expression of *CrPax-A* in the TBE, in addition to the retina, suggests its involvement in nematogenesis because the TBE is the specialized site for the tentacular nematocyte differentiation in

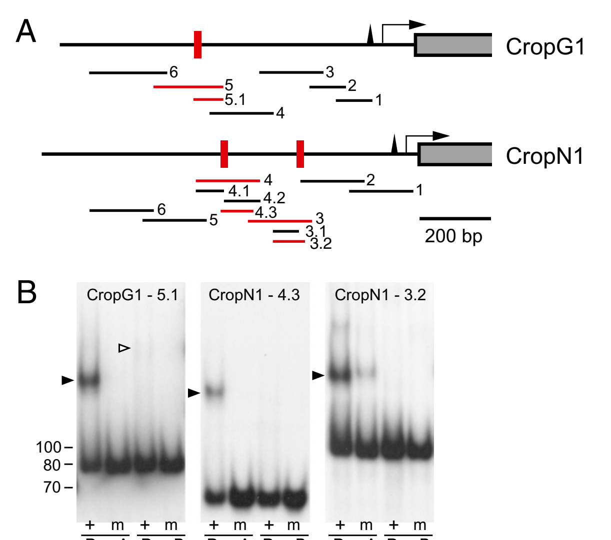

Fig. 4. CrPax-A PD binding sites in the upstream region of eye-specific *Cladonema* opsins. (*A*) Schematic drawing showing the positions of the probes generated for EMSA. Probes to which CrPax-A PD bound are shown in red. Red vertical bars indicate the positions of the CrPax-A binding sites, which were precisely identified by the use of mutated probes. Black triangles, arrows, and gray boxes represent TATA boxes, transcription starting sites, and protein coding regions, respectively. (*B*) EMSA for the three positive probes: probe 5.1 of CropG1, and probe 4.3 and 3.2 of CropN1. +, wild-type probes; m, mutated probe. The size (bp) of the probe is shown at left. Arrowheads indicate the band shifts caused by the binding of proteins. Note that the bindings of CrPax-B PD are very faint or undetectable (white arrowhead), even though the same amount of the proteins as CrPax-A PD were used. CrPax-B PD was separately proven to be active by the use of a control probe carrying D-Pax2 binding sites (*Materials and Methods*).

hydrozoan jellyfish (27, 36, 37). Like bilaterian *Pax* genes (2, 3, 38), *CrPax*-A seems to be involved in multiple aspects of developmental processes.

Involvement of *Pax-A* in *Cladonema* Eye Development and/or Maintenance. By targeted expression experiments, we have shown that *CrPax-A* is able to ectopically initiate eye development in *Drosophila*, whereas *CrPax-B* and *CrPax-E* are not. This agrees with the results of our expression analyses, which show that only *CrPax-A* is highly expressed in the eye (Fig. 2).

The *Drosophila ey* gene includes an eye-specific enhancer that contains Pax-6 protein binding sites (29). It is possible that an introduced *Pax* gene simply augments the endogenous Ey protein level, which then induces the ectopic eyes. By showing the ability of *CrPax-A* to induce ectopic eyes in *Drosophila* in an *ey* null mutant, however, we have demonstrated that the induction of ectopic eyes by *CrPax-A* is not solely the effect of this jellyfish *Pax* gene-mediated induction of the *Drosophila* endogenous *ey*. It seems that, even in the absence of *Drosophila* Ey protein, the RDGN components expression is directly or indirectly induced by the CrPax-A protein.

The ability of the *Cladonema* Pax-A PD to bind to the 5′ upstream regions of two eye-specific opsin genes raises the possibility that CrPax-A directly regulates the expression of opsin genes during eye development and/or maintenance. In contrast, CrPax-B PD is incapable of binding to the same sequences. These data are consistent with the expression analyses and the ectopic eye induction experiments. The direct interaction of Pax proteins with opsin promoters might have contributed to the evolution of ancestral animal eyes (13). Intercalation of other transcription factors into this simple regulatory cascade may explain how the complex gene network regulating eye development evolved (39). It should be noted, however, that DNA–protein interactions demonstrated by EMSA do not always reflect the same interactions in vivo. Studies on other species of jellyfish bearing eyes should allow testing of our hypothesis.

Flexible Choice of Distinct *Pax* Genes for Eye Development in Different Animal Lineages. *Pax-6* has been characterized as one of the central components of the gene network that controls eye development in most of the bilaterians studied to date (6). In *Tripedalia*, a cubozoan jellyfish that seems to lack a bona fide *Pax-6*, *Pax-B* has been implicated in eye development (13). We have now shown that *Pax-A*, rather than *Pax-B*, seems to be involved in eye development and/or maintenance in *Cladonema*, a hydrozoan jellyfish. *Pax-6*, *Pax-B,* and *Pax-A* belong to different *Pax* subfamilies, which diverged from each other by gene duplication at the early stage of animal evolution, at the latest before the separation of cnidarians and bilaterians. It is thus very likely that for eye development and/or maintenance, three distinct animal lineages use three distantly related *Pax* genes, which were generated by gene duplication before the three animal lineages separated from each other.

It can be argued that these data provide evidence in favor of three independent origins of animal eyes during evolution (13). If it is the case, however, completely different sets of transcription factors could have been recruited for eye development in different animal lineages. The observation that the three animal lineages use genes that belong to the same gene family (i.e., *Pax* family) in their eyes rather supports the hypothesis of the monophyletic origin of all animal eyes (6). This hypothesis is further supported by the functional conservation of the *Six* family genes that are involved in eye development and/or regeneration both in *Cladonema* and in several bilaterians studied to date (40–44). In addition, we recently cloned the *Cladonema* homolog of *eyes absent*, one of the main components of *Drosophila* RDGN (45), and detected its expression in the eye. Recent publications revealed that not only the transcription factor network controlling eye development but also its potential downstream targets that in-

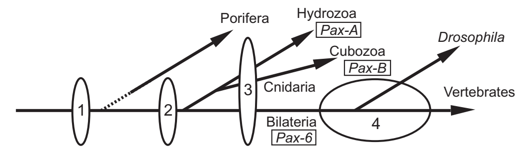

Fig. 5. Evolution of *Pax* genes deployed for animal eye development. 1: Gene duplications that gave rise to distinct *Pax* classes (corresponding to subfamilies) occurred. 2: The ancestral animal eye evolved and different classes of *Pax* genes were redundantly recruited for eye development. 3: In each of three different animal lineages, a specific *Pax* gene was selected for the eye development. 4: *Pax* genes responsible for eye development were altered in some bilaterians. See text for detailed description.

deed constitute the eye, such as genes involved in photoreception, phototransduction, and pigmentation, are also well conserved between cnidarians and bilaterians (17, 46). The common ancestry of cnidarian eyes and bilaterian ones seems to be the most reasonable interpretation of these data.

In our present model, gene duplications that gave rise to distinct subfamilies occurred most likely before the separation of poriferans and eumetazoans (1 in Fig. 5), as suggested by the statistical tests in refs. 4 and 22. We cannot, however, completely eliminate the possibility that some (or all) of these duplications postdate the poriferans–eumetazoans split (dashed line in Fig. 5) (4) until more sponge *Pax* genes that do not belong to the *Pax-2/5/8* subfamily are found. When the ancestral animal eye evolved in the common ancestor of cnidarians and bilaterians, *Pax* genes may have been recruited as components of the gene network responsible for eye development (2 in Fig. 5). We assume that, at this stage, several classes (corresponding to subfamilies) of *Pax* genes were redundantly involved in this network. After the divergence of bilaterians and cnidarians on one hand, and hydrozoans and cubozoans on the other hand, the three distinct animal lineages selected different classes of *Pax* genes for the roles in eye development and/or maintenance (3 in Fig. 5). Such molecular-level opportunism is often observed in evolution (e.g., lens crystallins) (47). Interestingly, formation of some bilaterian eyes seems to be *Pax-6* independent (ref. 14 for review). This suggests that the gene network directing eye development can be anomalously modified, making *Pax-6* dispensable for eye development in some bilaterian lineages (4 in Fig. 5).

Our model predicts that genes from different *Pax* subfamilies may still retain to some extent the ability to perform each other's function redundantly. The ability of *Cladonema Pax-A* (*poxn* subfamily), *Pax-B* (*Pax-2/5/8* subfamily), and *Drosophila ey* and *toy* (*Pax-4/6* subfamily) to rescue the *D-Pax2* (*Pax-2/5/8* subfamily) mutant spa^{pol} in the *Drosophila* eye is in agreement with this prediction (Fig. 3 *G* and *H* and ref. 13). Similarly, misexpressed *D-Pax2* induces eyes in the imaginal discs, as *ey* and *toy* do (13). Also at the molecular level, chordate Pax-6 and Pax-2 PDs recognize almost identical sequences, even though they show clear differences in their affinities to certain DNA sequences (35).

In summary, our study uncovers the diversity of the *Pax* genes used for development and/or maintenance of animal eyes. We propose that in the ancestral animal eye, which is likely to have evolved in the common ancestor of cnidarians and bilaterians, different classes (corresponding to the present subfamilies) of *Pax* genes were redundantly recruited and may then have been flexibly selected in distinct animal lineages for their roles in eye development.

Materials and Methods

Detailed descriptions of animal culture, fly strains, and all of the technical information regarding the gene cloning and sequencing, real-time PCR, molecular phylogenetic tree analysis, in silico search for *Pax* genes, in situ hybridization, protein expression, and EMSA are found in the *SI Materials and Methods*.

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