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[REVIEW]

Acquisition of Retinoic Acid Signaling Pathway and Innovation of the Chordate Body Plan

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ABSTRACT—Retinoic acid (RA) regulates many of the chordate-specific and vertebrate-specific characters. These include the anteroposterior pattern of the dorsally located central nervous system, pharynx with gill slits, neural crest cells, limb morphogenesis and anteroposteriorly organized vertebrae. The necessity of endogenous RA and the RA receptor (RAR) has been demonstrated by mutant analyses, vitamin A-deficient animals and various other methods. Since RAR has been identified only in chordates, the acquisition of the RAR-mediated RA signaling pathway is thought to be an important event for the innovation of the chordate body plan. RA-synthesizing aldehyde dehydrogenases and RA-degrading enzymes also seem to be chordate-specific. The expression pattern of these genes in ascidian embryos is similar to that in vertebrate embryos. These results suggest that the RA signaling cascade, with various regulators and modifiers, had been already well established in the common chordate ancestor. RA also regulates morphogenesis during the asexual reproduction of ascidians, suggesting that RA may also have played a part in producing diversity within the chordate groups.

Key words: chordate, body plan, retinoic acid receptor, retinoic acid metabolism, evolution

INTRODUCTION

The origin of chordates, as well as that of vertebrates, has long attracted many scientists in various fields of biology (for review see Gee, 1996; Hall, 1998). Therefore, we would like to avoid repeating well-conceived theories and debates here. We would like to concentrate our discussion on the possibility that the acquisition of the metabolic and signaling pathways of retinoic acid (RA) have played important roles in the evolution of the chordate and vertebrate body plans and divergence within the phylum Chordata.

It is widely accepted that the innovation of a new body plan can be achieved by gene duplication (Ohno, 1970). Gene duplication allows one of the derivatives to change to an extent that may otherwise be fatal (Holland *et al*., 1994). This may finally result in the elaboration of a gene with novel function and novel expression pattern (Holland *et al*., 1994). This seems quite applicable to vertebrate evolution, where two rounds of genome-wide gene duplication are believed to have occurred (Sidow, 1996). Attention has been concentrated on the duplication of the *Hox* gene cluster (Ruddle *et al*., 1994; Holland and Garcia-Fernàndez, 1996). Indeed, the duplication of *Hox* genes produced a complicated vertebrate body plan. However, the vertebrate *Hox* genes are functionally interchangeable with *Drosophila* counterparts (Malicki *et al*., 1990; Lutz *et al*., 1996). The genes upstream and/or downstream to the Hox function, as well as Hox-independent developmental programs, should also be considered for seeking “difference” between vertebrates and invertebrates, as carefully pointed out by Holland and Garcia-Fernàndez (1996). In addition, cephalochordate amphioxus and urochordate ascidians belong to the phylum Chordata, together with vertebrates (Kowalevsky, 1866, 1867; Garstang, 1928; Katz, 1983). They share many characters with vertebrates, even though their genomes possess a single *Hox* gene cluster (Garcia-Fernàndez and Holland, 1994; Dehal *et al*., 2002). The genes important for the innovation of the basic chordate-specific body plan may therefore have to be sought among those commonly found in all chordate species but not in non-chordate species. The draft genome sequence of the ascidian *Ciona intestinalis* revealed that about one-sixth (ca. 2570) of the *Ciona* genes possess homologs only within chordates, suggesting that these genes arose in the common ancestor of chordates (Dehal *et al*., 2002). A gene encoding the RA receptor (RAR) belongs

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to this category (Dehal *et al*., 2002). RA regulates proliferation, differentiation, morphogenesis and pattern formation in a wide variety of tissues, organs and cell lines in vertebrates (De Luca, 1991; Conlon, 1995; Ross *et al*., 2000). Similar effects have been observed exclusively in chordates as discussed below, suggesting that the acquisition of the RAR function was a driving force for innovation of the chordate body plan. In the following sections we show how RA and RAR, and RA-metabolizing enzymes, are involved in the cell differentiation and morphogenesis of key chordate-specific cell types. Then, we discuss the possibility that a urochordate-specific life, with asexual reproduction, was elaborated by the acquisition of the RA signaling pathway.

RAR-mediated RA signaling pathway is acquired in the chordate ancestor

RA signaling is mediated not only by RAR but also mediated by retinoid X receptor (RXR) (Mangelsdorf *et al*., 1990). RXR is a heterodimeric partner that can bind various nuclear receptors including RAR (Mangelsdorf and Evans, 1995). RAR, but not RXR, can bind all-*trans* RA and 9-*cis* RA when it forms a heterodimer with RXR (Kurokawa *et al*., 1994). By contrast, RXR can bind 9-*cis* RA (Heyman *et al*., 1992), when it dimerizes with RXR or a few other nuclear receptors (NURR1 and LXR) (for review see Leblanc and Stunnenberg, 1995). The RXR-encoding genes have been cloned from a wide variety of animals. In *Drosophila* an RXR homolog, Ultraspiracle (USP; Oro *et al*., 1990), does not bind RA but forms a heterodimer with the ecdysone receptor (Oro *et al*., 1990; Yao *et al*., 1992). Although there is no evidence suggesting endogenous RA function in *Drosophila*, the possibility cannot be excluded that 9-*cis* RA regulates some biological functions in other invertebrates.

In contrast, RAR-encoding genes have been identified exclusively in chordates. Vertebrates possess three RAR-encoding genes (Fig. 1; for review see Mangelsdorf and Evans, 1992). The RAR/RXR heterodimer binds to the specific DNA sequences, called RA response element (RARE), and mediates RA signaling (Umesono *et al*., 1991). The unliganded form of the RAR/RXR can still bind to RARE and functions as a transcriptional repressor through protein-protein interaction with nuclear receptor co-repressors SMRT and N-CoR (Chen and Evans, 1995; Hörlein *et al*., 1995). Ascidians (Hisata *et al*., 1998; Devine *et al*., 2002; Nagatomo *et al*., 2003) and amphioxus (Escriva *et al*., 2002) possess a single RAR-encoding gene (Fig. 1). Sequence-specific DNA-binding, heterodimerization with RXR, and RA-dependent transcriptional activation of reporter genes were experimentally demonstrated for these protochordate RARs (Kamimura *et al*., 2000; Escriva *et al*., 2002). No RAR has been reported in any non-chordate species. The well-characterized genomes of *Caenorhabditis elegans* and *Drosophila melanogaster* do not contain any gene similar to RAR (The *C. elegans* Sequencing Consortium 1998; Adams *et al*., 2000).

RA-mediated malformations have been reported in a number of non-chordates, including sponges (Imsiecke *et al*., 1994), cnidarians (Müller, 1984), molluscs (Créton *et al*., 1993), crustaceans (Hopkins and Durica, 1995), and echinoderms (Sciarrino and Matranga, 1995). However, RA-induced phenotypes can hardly be interpreted by analogy from those of chordates, suggesting that the RA-mediated developmental programs, if any, are not homologous to those in chordates. Phenotypes induced by exogenous RA are similar among the chordate species as described in the following sections. The protochordate RARs are expressed during embryogenesis in a stage- and tissue-specific manner (Escriva *et al*., 2002; Nagatomo *et al*., 2003), suggesting specific roles in the embryo. An RAR-mediated RA signaling pathway seems to have been established in the chordate ancestor.

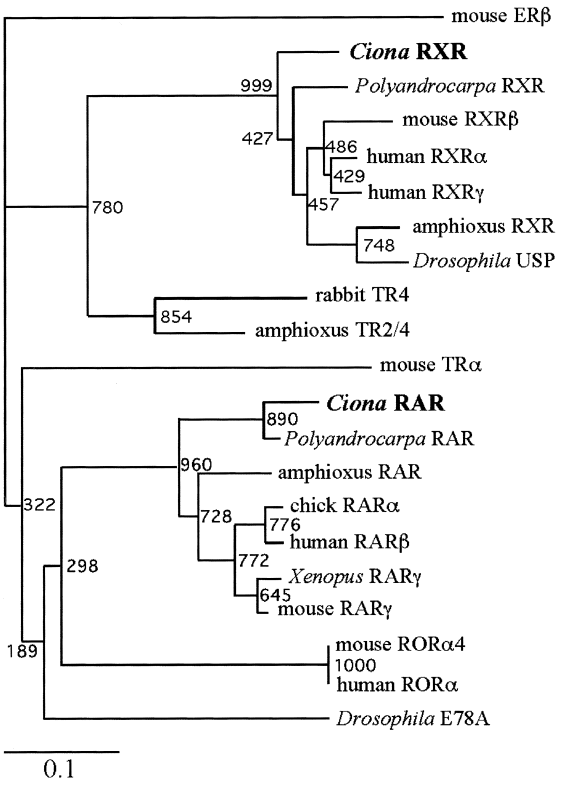

Fig. 1. A phylogenetic tree of nuclear receptors constructed by the neighbor-joining method (Saitou and Nei, 1987) using the amino acid sequences of the DNA-binding domains. The RARs from two ascidian species (C*iona intestinalis* and *Polyandrocarpa misakiensis*) form a sister group of the vertebrate RARs. Similarly, the RXRs from ascidians form a cluster with other RXR homologs. The divergence within this group does not seem to be accurate, probably because the sequences of the DNA-binding domain of RXRs are extremely highly conserved. Among *Drosophila* proteins, the orphan receptor E78A shows the highest similarity to RARs. Note that rabbit TR4 and amphioxus TR2/4 belong to a group of "testis-specific receptors (TR)" and are different from mouse TRα (thyroid hormone receptor α). ER, estrogen receptor; ROR, retinoid-related orphan receptor.

RA signaling is important for the expression of chordate-specific characteristics

The central nervous system (CNS) in chordates is formed from the folding of the dorsally located neural plate and is subdivided along the anteroposterior axis (Gee, 1996). The anteroposteriorly organized, but ventrally located CNS is observed in the annelids and arthropods, and was once regarded to be homologous to the chordate CNS (Dohrn, 1875; see also Nübler-Jung and Arendt, 1994). However, the homology between chordate dorsal CNS and protostome ventral CNS is rather unsolid (Lacalli, 1995; Peterson, 1995). The homology between hemichordate dorsal "neurocord" and the chordate neural tube is not widely accepted either (Brusca and Brusca, 1990). It seems therefore likely that the organization of the dorsal, hollow CNS has been acquired during the evolution of the common "chordate" ancestor (Garstang, 1928).

RA causes the homeotic transformation of the identity of rhombomeres in the hindbrain by affecting the expression pattern of *Hox* genes along the anteroposterior axis of the neural tube in vertebrates (Morriss-Kay *et al*., 1991; Papalopulu *et al*., 1991). A dominant negative form of RARβ interferes with the normal rhombomere patterning and reduces RA-induced teratogenesis (van der Wees *et al*., 1998). Similarly, another type of the dominant negative RAR eliminates *HoxD1* expression and affects the pattern formation in the hindbrain (Kolm *et al*., 1997). Since the dominant negative RAR inhibits RAR-mediated but not RXR-mediated signaling, the RAR/RXR heterodimer was revealed to play a key role in the normal hindbrain patterning (Kolm *et al*., 1997; van der Wees *et al*., 1998). The necessity of endogenous RA was also demonstrated by the defect of the hindbrain patterning in vitamin A-deficient quail (Gale *et al*., 1999). Koide *et al*. (2001) showed that the repressor activity of unliganded RAR is required for correct differentiation of the forebrain. This also suggests complicated but important endogenous roles of RAR for the chordate body plan.

In amphioxus and ascidians, the region-specific expression pattern of many developmental regulatory genes along the anteroposterior axis of the central nervous system is similar to that in vertebrates (Wada and Satoh, 2001). Although there is no obvious indication of the metameric regionalization in the putative hindbrain region, the expression pattern of *Hox* genes is also similar to that in vertebrates (Katsuyama *et al*., 1995; Gionti *et al*., 1998; Locascio *et al*., 1999; Wada *et al*., 1999; Nagatomo and Fujiwara, 2003). RA affects the *Hox* gene expression pattern in amphioxus (Holland and Holland, 1996) and ascidians (Katsuyama *et al*., 1995; Nagatomo and Fujiwara, 2003), although the malformation of the anterior neural tissues in ascidians can hardly be regarded as homeotic transformation (Fig. 2; Denucé 1991; Nagatomo *et al*., 2003).

The pharynx, with gill slits and the endostyle, is another chordate-specific characteristic (Young, 1981). The protochordate endostyle is thought to be homologous to the vertebrate thyroid gland (Gee, 1996; Ogasawara *et al*., 1999). The hemichordate pharynx possesses gill slits but no endostyle (Gee, 1996). RA causes the loss of pharynx in RA-treated lamprey (Kuratani *et al*., 1998) and mammalian (White *et al*., 1998) embryos. RA is involved in the transcriptional regulation of *Pax1* and *Pax9* genes in the pharyngeal endoderm in mice (Wendling *et al*., 2000). Targeted mutagenesis of RARs resulted in the loss of the second and third pharyngeal arches (Lohnes *et al*., 1994). Abnormalities are also obvious in the thymus and thyroid gland (Mendelsohn *et al*., 1994). The reduction of the posterior pharyngeal endoderm in vitamin A-deficient quails provides additional support for endogenous RA requirement for pharyngeal patterning (Quinlan *et al*., 2002).

In ascidians the development of pharyngeal basket starts after metamorphosis. RA treatment during postlarval development leads to reduced expression of *otx* gene and eventual loss of the pharyngeal basket (Hinman and Degnan, 1998, 2000). In amphioxus, the size of the pharynx is reduced and gill slits do not form in RA-treated embryos (Holland and Holland, 1996). In contrast, the pharynx is expanded in embryos treated with an RA antagonist, BMS009 (Escriva *et al*., 2002). In this case, RA affects the expression of a *Pax1/9* homolog (Holland and Holland, 1996; Escriva *et al*., 2002). The pharynx of ascidians and hemichordates also expresses *Pax1/9* homolog (Ogasawara *et al*., 1999). These results suggest that RA regulates the pharyngeal morphogenesis through a similar genetic cascade in all the chordate groups.

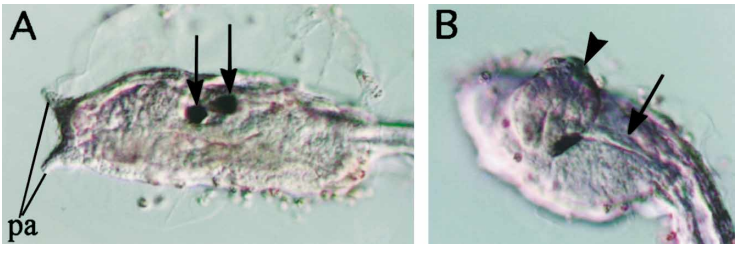

Fig. 2. RA-induced phenotype in the central nervous system of ascidians. The photographs were obtained by Tomoko Ishibashi of our laboratory. (A) Head of a normal *Ciona intestinalis* larva, the anterior is to the left, and the dorsal is up. The adhesive papillae (pa) and the brain vesicle with two sensory pigment cells (arrows) are differentiated. (B) Head of an RA-treated larva, dorsal view. The anterior neural tube failed to close and was exposed to the dorsal surface (arrow), and the presumptive brain cells form a cell mass outside the body (arrowhead).

RA signaling is also important for the expression of vertebrate-specific characteristics

Many important vertebrate-specific characteristics are found in the complicated structure of the head, and thus vertebrates (and hagfish) are called, "craniates" (Gee, 1996). The neural crest cells give rise to the cranial nerves, cephalic skeletal components and many other tissues comprising the head structure (Gans and Northcutt, 1983). Since most of these and other vertebrate-specific cell types derive from the neural crest cells, their evolution is thought to be an extremely important event in vertebrate evolution (Hall, 2000). The neural crest cells come only from the posterior neural plate (the hindbrain and spinal cord regions), suggesting that posteriorization of the neural plate, according to "the activation/transformation model" (Nieuwkoop and Albers, 1990), is involved in the neural crest development (Villanueva *et al*., 2002). The neural crest differentiation depends, at least partly, on the induction from the paraxial mesoderm (Bonstein *et al*., 1998). This and the fact that the posterior paraxial mesoderm is the site of retinoic acid synthesis (discussed below, see Fig. 4) suggest that RA could be one of the candidate posteriorization factors. In fact, RA treatment and a constitutively active form of RAR can induce the neural crest in the presumptive forebrain region (Villanueva *et al*., 2002). The presumptive neural crest cells die by apoptosis in vitamin A-deficient quails (Maden *et al*., 1998). Wada (2001) proposed that the dorsal midline epidermis in ascidians and amphioxus is the origin of the neural crest and thus the neural crest cell population itself is not an innovation of vertebrates. An enhancer element of the amphioxus *Hox-1* gene (*AmphiHox-1*) can be activated in the vertebrate neural crest, although *AmphiHox-1* expression is restricted to the neural tube in amphioxus embryos (Manzanares *et al*., 2000). Since *Hox-1* expression in the neural crest depends on RA (Manzanares *et al*., 2000), the acquisition of the RA signaling pathway could have conferred the neural crest cell properties on the dorsal midline epidermis of the vertebrate ancestor. Wada (2001) is skeptical to this idea because an *AmphiHox-3* enhancer containing an RARE was not expressed in the neural crest cells (Manzanares *et al*., 2000). However, requirement of a few additional changes does not necessarily deny the importance of the acquisition of RA-responsiveness for the neural crest evolution. A large part of the amphioxus nerve cord is thought to be homologous to the vertebrate brain (Holland *et al*., 1992). The *Hox-3* and *Hox-5* homologs of ascidians are expressed only at the late stages of development (Gionti *et al*., 1998; Locascio *et al*., 1999). These observations suggest that the expression and function of the posterior (5') *Hox* genes were largely modified after the divergence of protochordate and vertebrate groups.

The limbs are also vertebrate-specific, and missing in protochordates (Gee, 1996). RA affects the proximodistal axis in the regenerating amphibian limbs (Niazi and Saxena, 1978; Maden, 1982; Thoms and Stocum, 1984). The effect of RA on the anteroposterior pattern formation of developing chick wing bud was remarkable (Tickle *et al*., 1982). The organizing role of endogenous RA in the limb has long been expected, but also doubted. Although RA-induced ectopic digit formation follows the upregulation of RARβ expression, the endogenous organizing region (called ZPA) does not express RARβ (Noji *et al*., 1991). RA thus came to be regarded as a notorious example of the chemical that only mimics the action of endogenous factors. ZPA expresses *Sonic hedgehog (Shh)* gene that organizes the anteroposterior pattern in the limb bud (Riddle *et al*., 1993). The question then moved to how the *Shh* expression was activated in the posterior region of the limb bud (Johnson and Tabin, 1997). It seems that the anteroposterior organization of the limb bud is a sophisticated modification of the pre-existing pattern in the lateral plate mesoderm (Cohn *et al*., 1997) Lu *et al*. (1997) demonstrated that RAR antagonists block the formation of ZPA. In addition, the expression domain of *Hoxb-8*, a direct RA target gene, correlates with the domain of polarizing activity in the lateral plate mesoderm (Lu *et al*., 1997). Inhibition of the RA synthesis (Stratford *et al*., 1996) and the RAR/RXR function (Helms *et al*., 1996) in the lateral plate mesoderm disturbed limb formation. These results suggest that RA truly acts as an endogenous factor for ZPA induction and limb formation.

The name "vertebrates" comes from the vertebral column. RA induces homeotic transformations of the vertebrae along the anteroposterior axis (Kessel and Gruss, 1991). This phenotype is derived from altered pattern of *Hox* expression (Kessel and Gruss, 1991; Kessel, 1992).

It should be noted that all these vertebrate-specific characters are affected by targeted mutagenesis of at least two of three RAR subtypes (Lohnes *et al*., 1994). The phenotypes of RAR double mutants are similar to the fetal vitamin A-deficient syndrome (Wilson *et al*., 1953). This suggests that endogenous RAR plays important roles in the expression of these characteristics.

RA-synthesizing enzyme and RA-degrading enzyme are also chordate-specific

Is RA synthesized only in the chordates? In vertebrates, a major RA-synthesizing enzyme in the embryo is the aldehyde dehydrogenase encoded by *Raldh2* gene (Zhao *et al*., 1996). *Raldh2* is expressed in the posterior mesoderm in the pre-somite stage vertebrate embryos (Niederreither *et al*., 1997; Swindell *et al*., 1999). Targeted disruption of Raldh2 gene causes early embryonic lethality (Mic *et al*., 2002). Conditional rescue of the *Raldh2* null mutant mice by providing RA within maternal food revealed that the forelimb formation requires normal RA synthesis (Niederreither *et al*., 2002b). These results suggest an essential role of RA in vertebrate embryogenesis. Although many experiments have been carried out in which exogenous RA was applied to various invertebrate species as described above, little attention has been paid to the RA-synthesizing activity in invertebrates.

Retinal, of various isomers, is stored in the unfertilized

egg of the ascidian *Halocynthia roretzi* (Irie *et al*., 2003). Recently, we identified a candidate *Raldh2* homolog (*Ci-Raldh2*) from the ascidian *Ciona intestinalis* (Fig. 3A; Fujiwara *et al*., 2002; Nagatomo and Fujiwara, 2003). The amino acid sequence deduced from the *Ci-Raldh2* cDNA shows the highest similarity to human RALDH2. However, low bootstrap values suggest that the branching pattern of a few related retinaldehyde dehydrogenases {RALDH3 (Grün *et al*., 2000; Niederreither *et al*., 2003), mitochondrial ALDH2 (Chen *et al*., 1994) and RALDH2} is uncertain (Fig. 3A). The expression of *Ci-Raldh2* was restricted in the anterior paraxial mesoderm (a few muscle cells on both sides of the notochord) (Fig. 4A, C; Nagatomo and Fujiwara, 2003). Although it is still under dispute, the ascidian muscle cells are thought to correspond to the segmented somites in vertebrates (Crowther and Whittaker, 1994). If so, the expression pattern of *Ci-Raldh2* is similar to that of vertebrate *Raldh2* in the somites (Fig. 4A-D; Niederreither *et al*., 1997). The role of 11-*cis*-retinal in vision is thought to be evolutionarily ancient and retinal-metabolizing enzymes were isolated from invertebrates (for review see Duester, 2000). There is no evidence suggesting endogenous roles for all-*trans* RA and 9-*cis* RA in non-chordate invertebrates. Furthermore, no *Raldh2* homolog has been reported in any non-chordate species. However, it is difficult to draw conclusions from sequence comparison. The sequence of aldehyde dehydrogenases is extremely highly conserved from bacteria, fungi and plants to animals, and we can hardly predict the substrate from the sequence.

Vertebrates possess cytochrome P450 enzymes (CYP26) that catalyze the reaction from RA to 4-oxo- and 4-hydroxy-RA (White *et al*., 1997, 2000). *Cyp26* is expressed in the presumptive forebrain/midbrain region of the gastrulating embryo, while *Raldh2* is expressed in the presomitic and lateral plate mesoderm at the same stage (Fig. 4B; Swindell *et al*., 1999). This complementary expression pattern suggests that CYP26 is limiting the range of RA action (Fig. 4A–D). The region where RA is deduced to be active coincides with the spatial expression pattern of an RARE-containing reporter gene (Fig. 4E; Rossant *et al*., 1991). *Cyp26* null mutant mice exhibited the posterior transformations of cervical vertebrae and abnormal rhombomere pat-

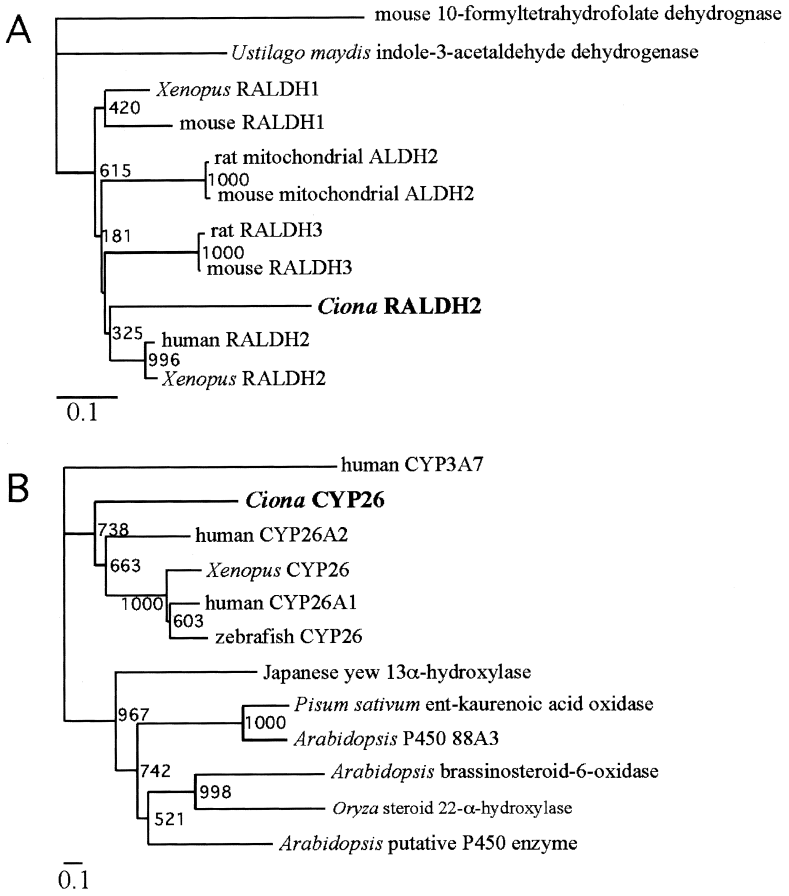

Fig. 3. (A) A phylogenetic tree of aldehyde dehydrogenases constructed by the neighbor-joining method (Saitou and Nei, 1987). Vertebrates possess three RALDH groups (RALDH1-3). The *Ciona* RALDH2 shows the highest similarity to RALDH2. Indole-3-acetaldehyde dehydrogenase of the fungus *Ustilago maydis* and 10-formyltetrahydrofolate dehydrogenase showed relatively high similarity to a group of retinaldehyde dehydrogenases. (B) A phylogenetic tree of cytochrome P450 enzymes constructed by the neighbor-joining method. The *Ciona* CYP26 forms a cluster with vertebrate CYP26 enzymes. Among various cytochrome P450 enzymes, the *Ciona* CYP26 showed relatively high similarity to the plant enzymes.

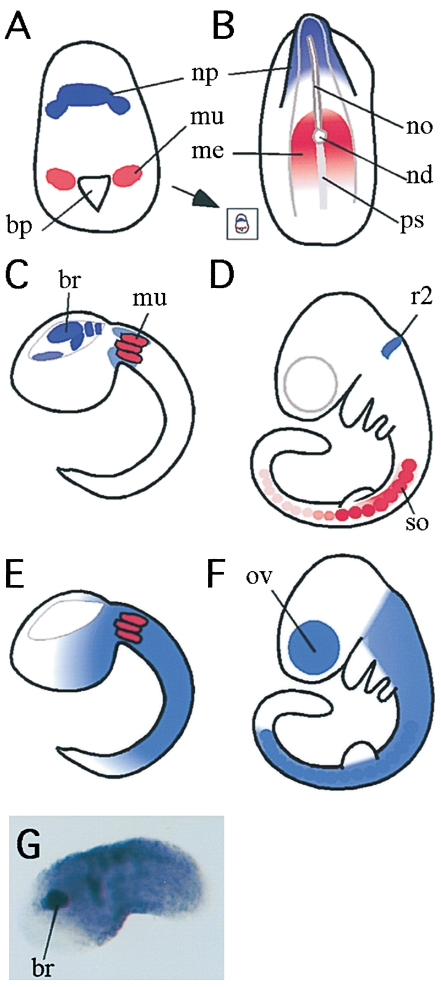

Fig. 4. (A–D) Schematic representation of the expression pattern of *Raldh2* (red) and *Cyp26* (blue) in gastrulae and tailbud embryos of ascidians and vertebrates. bp, blastopore; br, brain; me, paraxial and lateral plate mesoderm; mu, muscle; nd, node; no, notochord; np, neural plate; ps, primitive streak; r2, the second rhombomere segment; so, somites. (A) Ascidian gastrula, dorsal view. (B) Vertebrate gastrula, dorsal view. The ascidian gastrula is shown in a box in a roughly equal magnification. (C) Ascidian tailbud embryo, lateral view. Three anterior-most muscle cells express *Raldh2*. (D) Vertebrate somite-stage embryo, lateral view. (E) The region where endogenous RA functions, speculated from the expression pattern of a *Ciona* homolog of *Meis*, of which expression is activated by RA (G). (F) The expression pattern of the RARE-containing reporter gene that depicts the region where RA is acting (Rossant *et al*., 1991). The expression of the reporter gene in the optic vesicle (ov) is thought to be activated by RA synthesis catalyzed by RALDH3. (G) The expression pattern of a *Ciona Meis* homolog. The strong expression in the brain (br) is thought to be activated by an RA-independent mechanism, since *Cyp26* is strongly expressed in this region. The photograph was obtained by Kan-ichiro Nagatomo (in preparation).

terning (Abu-Abed *et al*., 2001). This phenotype resembles that observed in RA-treated embryos, suggesting the important endogenous roles for CYP26 in regulating RA action during development (Abu-Abed *et al*., 2001).

We identified a *Cyp26* homolog in ascidians (Fig. 3B; Fujiwara *et al*., 2002; Nagatomo and Fujiwara, 2003). This gene is expressed in the anterior neural plate of the gastrula and neurula, and in the brain of the tailbud embryos (Fig. 4A, C; Nagatomo and Fujiwara, 2003). This expression domain does not overlap that of *Raldh2* and is similar to that of vertebrate *Cyp26* (Fig. 4A-D; Nagatomo and Fujiwara, 2003). The expression of *Cyp26* in ascidian embryos is dramatically upregulated by exogenous RA (Nagatomo and Fujiwara, 2003), as is the case in vertebrates (White *et al*., 1997). The enzymatic activity of these ascidian homologs remains to be demonstrated biochemically.

Variation of the repertoire of RA-target genes in some chordate species

If the acquisition of the RAR gene is a key event during the chordate evolution, the history of the RAR's recruiting target genes depicts at least a part of chordate morphological evolution. RAR regulates developmental programs mainly through transcriptional regulation of *Hox* genes (Simeone *et al*., 1990; Gould *et al*., 1994; Manzanares *et al*., 2000). A few developmental regulatory genes are also known as RA target genes. These include RARβ (Mendelsohn *et al*., 1991) and *Meis2* (Oulad-Abdelghani *et al*., 1997). The systematic analyses of RA target genes, based on cDNA arrays, were performed using embryonic stem cells (Kelly and Rizzino, 2000) and embryonal carcinoma cells (Freemantle *et al*., 2001) but not in embryos of any animal species.

Recently we identified a number of RA target genes in the ascidian embryos by means of a cDNA microarray-based screening (Ishibashi *et al*., 2003). The strong activation of *Hox-1* and *Cyp26* was similar to that in vertebrates (Nagatomo and Fujiwara, 2003; Ishibashi *et al*., 2003). As previously shown by *in situ* hybridization analysis (Nagatomo *et al*., 2003), RAR expression was not activated by RA (Ishibashi *et al*., 2003). This is in marked contrast to the RA-inducibility of the RAR genes in amphioxus (Escriva *et al*., 2002) and vertebrates (Mendelsohn *et al*., 1991). Many candidate target genes seemed to be involved in the neuronal functions, suggesting that not only morphogenesis but also cellular differentiation of the nervous system are regulated by RA. However, RA-treated larvae can respond to natural metamorphosis inducers even though they lack the adhesive papilla that is responsible for the chemosensory response (Hinman and Degnan, 1998). This suggests that RA does not cause homeotic transformation of the anterior cells into completely different cell types in ascidians.

RA-mediated transdifferentiation in the budding ascidians

RA also plays an important role in the asexual reproduction of the ascidians, which is a characteristic acquired only in the urochordate lineage of chordate evolution. In the ascidian *Polyandrocarpa misakiensis*, the gut, pharynx, endostyle, neural gland and many other tissues are formed through the pluripotent transdifferentiation of the atrial epithelium (Fujiwara and Kawamura, 1992; Kawamura and

Fujiwara, 1994). Exogenous RA induces ectopic transdifferentiation, resulting in a completely duplicated body axis (Hara *et al*., 1992). Localized activation of aldehyde dehydrogenase suggests that endogenous RA acts as a regulator of transdifferentiation (Kawamura *et al*., 1993). Genes encoding RAR and RXR are expressed in the developing bud (Hisata *et al*., 1998; Kamimura *et al*., 2000). Functional analysis revealed that they bind specific DNA sequence as a heterodimer and activate reporter gene expression depending on RA (Kamimura *et al*., 2000). A differential display screening identified a few target genes, including a serine protease with a complex modular structure (Ohashi *et al*., 1999). This serine protease, named TRAMP, is a candidate transdifferentiation factor, since recombinant TRAMP protein can stimulate proliferation and probably dedifferentiation of the cell line derived from the atrial epithelium (Ohashi *et al*., 1999).

About a half of the ascidian species can proliferate asexually by budding. Various modes of budding have been described (for review see Nakauchi, 1982). The origin of the newly formed tissues is the atrial epithelium in Styelidae and Botryllidae, the stolonial septum in Perophoridae, and the epicardial epithelium in Polycitoridae, Polyclinidae and some other families (Nakauchi, 1982). Since the common chordate ancestor was thought to be a free-swimming tadpole-like animal (Wada, 1998), the ability of asexual reproduction seems to have been independently acquired several times during the ascidian evolution (Wada *et al*., 1992). This suggests that small genetic changes can cause the conversion of solitary to colonial lifestyle. Despite the extreme diversity in the type of multipotent formative tissues, the early buds of most species look similar to one another. In the developing bud, the gut and pharynx are usually the first organs to be formed (Nakauchi, 1982 and references therein). The molecular mechanisms underlying the bud development may be roughly common to all the budding ascidians. RA affects morphogenesis of the pharyngeal endoderm in all chordate groups, as described above. And *Hox* genes determine the anteroposterior pattern of the digestive tract in vertebrate embryos (Sakiyama *et al*., 2001). Considering these facts, the ability of asexual reproduction may have been acquired by re-activation of RA-driven developmental programs in some adult cell type. Since the asexual reproduction includes reconstruction of the whole body from a piece of tissues, the process can be thought as regeneration (Kawamura and Fujiwara, 2000). RA affects amphibian limb regeneration as described above. Inhibition of RA synthesis disturbs regeneration, suggesting that endogenous RA plays an important role in the amphibian limb regeneration (Maden, 1998). Although RA is also involved in the regeneration of a few other tissues (for review see Maden and Hind, 2003), we have little evidence to connect, in general, the ability of regeneration to the ability of re-activation of RA signaling pathway.

Conclusions and perspectives

The RAR-mediated RA signaling pathway is involved in the formation of most chordate-specific and vertebrate-specific characteristics. The necessity of endogenous RA functions has been demonstrated for each case, using dominant negative RARs, RAR mutants and vitamin A-deficient animals. RA-synthesizing and RA-degrading enzymes also seem to be chordate-specific. Considering this, the basic framework of the RA signaling cascade, target genes and metabolizing enzymes that modify RA functions has already been established in the common chordate ancestor. How were the complicated network and cascade established? How were RAR expression and function modified during the evolution of vertebrates? Thinking about the origin, it is important to obtain the information about the sister groups, hemichordates and echinoderms. In addition to the functional analysis of the genes involved in the RA signaling, screening and comparative analysis of homologous genes in hemichordates and echinoderms will be of great interest.

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