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Developmental role of endogenous retinoids in the determination of morphallactic field in budding tunicates

Kazuo Kawamura*, Kenji Hara and Shigeki Fujiwara

Department of Biology, Faculty of Science, Kochi University, Akebono-cho, Kochi 780, Japan

*Author for correspondence

SUMMARY

We have extracted retinoids from the budding tunicate *Polyandrocarpa misakiensis* and, using HPLC, identified some major peaks as *cis*-retinal, all-*trans*-retinal and all-*trans*-retinoic acid, of which *cis*-retinal was most abundant (~2 μM). In developing buds, the amount of *cis*-retinal was about one-fifth that of the adult animals. In those buds, aldehyde dehydrogenase, which could metabolize retinal in vitro, was expressed in epithelial cells and then in mesenchymal cells at the proximal extremity, that is, the future developmental field of the bud. Exogenous retinoic acid comparable to the endogenous level could induce an additional field at the distal end of the bud, resulting in a double monster. The induction always accompanied an ectopic expression of aldehyde dehydrogenase. The results of this work suggest that retinoic acid or related molecule(s) act as an endogenous trigger of morphallactic development of *Polyandrocarpa* buds.

Key words: tunicate, budding, morphallaxis, retinoic acid, aldehyde dehydrogenase, *Polyandrocarpa*

INTRODUCTION

Asexual reproduction of the tunicate *Polyandrocarpa misakiensis* is equivalent to morphallactic regeneration, a complete animal arising from a piece of the parental tissues (Kawamura and Nakauchi, 1986). Buds form as evaginations of the parental body wall consisting of epidermis and atrial epithelium (Fig. 1A). Together the epithelia form a double-walled vesicle elongated proximodistally. The atrial epithelial cells are multipotent and play a key role in bud morphogenesis (Kawamura and Nakauchi, 1984, 1991). Most tissues and organs arise as a folding or outgrowth of the epithelium at the proximal region (Fig. 1B).

It is noteworthy that the multipotent cells of *Polyandrocarpa* are not undifferentiated (Fujiwara and Kawamura, 1992). They are quiescent in growing buds (Kawamura and Nakauchi, 1991), but enter the cell division cycle only after buds are isolated from the parent (Kawamura and Nakauchi, 1986). Then, organogenesis proceeds through a transdifferentiation-like process (Fujiwara and Kawamura, 1992). We would like to know how mechanical stimulus, such as incision, triggers the activation of quiescent multipotent cells. Because of the structural simplicity, *Polyandrocarpa* buds may be useful for our general understanding of regeneration-inducing signal(s) expressed following incision.

Retinoic acid, a retinoid with a carboxyl group, exerts a wide spectrum of biological effects on vertebrate embryos. It promotes specific gene expression and cell differentiation of embryonic stem cells and embryonic carcinoma cells (Colberg-Poley et al., 1985; Deschamps et al., 1987). It has potent teratogenic effects on the neural epithelium (Durston et al., 1989) and mesodermal tissues such as heart (Taylor et al., 1981). Some investigators assume that retinoic acid acts as an endogenous morphogen for axial pattern formation (Maden, 1982; Tickle et al., 1985). For example, retinoic acid can mimic the action of polarizing region in avian limb buds (Tickle et al., 1982) and is distributed differently along the limb anteroposterior axis (Thaller and Eichele, 1987). Other workers (Noji et al., 1991) suggest that it is not a morphogen but a determinant for the polarizing region. Nevertheless, retinoic acid appears to provide a spatiotemporal cue for the expression of retinoic acid receptor genes and homeobox genes in the developing limb bud (for review, Tabin, 1991). The floor plate of the neural tube, which is a local source of retinoic acid, is able to exert a polarizing activity (Wagner et al., 1990). Retinoic acid also affects the regeneration of urodele limbs (e.g., Thoms and Stocum, 1984), but it is somewhat obscure if retinoic acid is naturally involved in limb regeneration.

Recently, we have shown that retinoic acid can induce the secondary bud axis of *Polyandrocarpa misakiensis* (Hara et al., 1992). In this work, a single bead soaked with retinoic acid was grafted through a pinhole into a mesenchymal space at the distal extremity (non-morphogenetic domain) of the bud. The value of this work was that complete morphogenetic events took place at the implantation

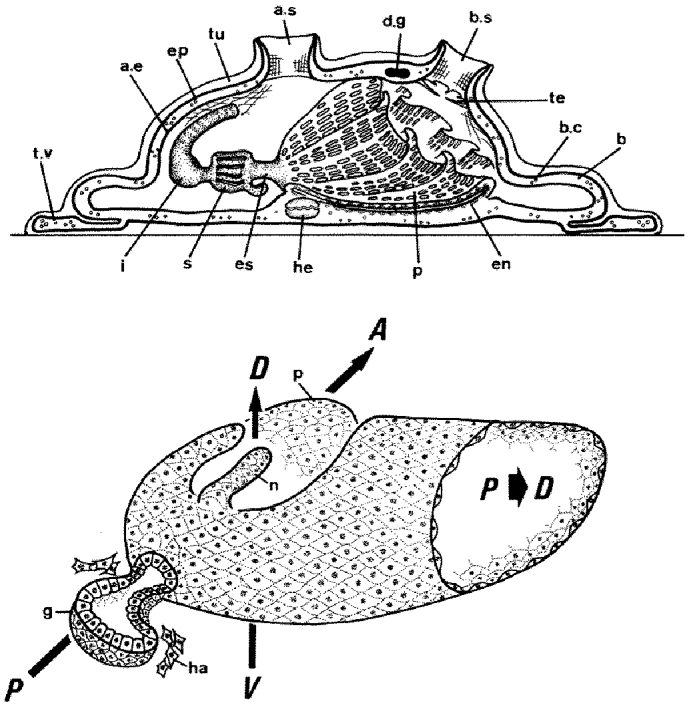

Fig. 1. Diagrams of the budding tunicate, *Polyandrocarpa misakiensis*. (A) Lateral view of an adult animal with protruding buds. The anteroposterior organization of the animal is represented mainly by the pharynx and digestive tract. The body wall consists of the epidermis and atrial epithelium, between which mesenchymal blood cells exist. These components grow out and form the palleal bud. (B) Developing bud of the earliest stage, from which the epidermis is omitted. Major organ rudiments are derived from the multipotent, inner (atrial) epithelial vesicle elongated proximodistally (P-D). The pharynx and gut represent the anteroposterior (A-P) axis and the neural complex represents the dorsoventral (D-V) axial component. Mesenchymal stem cells, haemoblasts, take part in these morphogenetic events (Kawamura et al., 1991). It should be noted that these events normally occur at the proximal extremity of the bud after the bud is isolated from its parent. a.e, atrial epithelium; a.s, atrial siphon; b, bud; b.c, blood cell; b.s, branchial siphon; d.g, dorsal ganglion; en, endostyle; ep, epidermis; es, esophagus; g, gut rudiment; ha, haemoblast; he, heart; i, intestine; n, neural complex; p, pharynx; s, stomach; te, tentacle; tu, tunic; t.v. tunic vessel.

site without drastic surgical incision. The results suggest that retinoic acid may be an endogenous signalling molecule to promote bud development.

In the present study, the working hypothesis mentioned above was considered. (1) We examined endogenous retinoids from *Polyandrocarpa* adult animals and developing buds. (2) We studied histochemically the spatiotemporal expression of aldehyde dehydrogenase in developing buds and also examined biochemically if the enzyme contained the activity of retinoic acid synthase. (3) We investigated whether exogenous retinoic acid could affect the in vivo expression of the enzyme. (4) We treated buds with amounts of retinoids comparable to endogenous levels and quantified their effects on the developmental fields of *Polyandrocarpa* buds. The results of this study are discussed in relation to the manner by which endogenous retinoids might play a role in the morphallactic development of tunicate buds.

MATERIALS AND METHODS

Animals

Polyandrocarpa misakiensis was used (for taxonomic description, see Watanabe and Tokioka, 1972). Bud formation and bud development of this animal have been described elsewhere (Kawamura and Nakauchi, 1984, 1986, 1991). Animals were attached to glass slides and reared in a culture box floating in Uranouchi Inlet near the Usa Marine Biological Institute, Kochi University.

Extraction of endogenous retinoids

After removal from the glass slide, animals were frozen in liquid nitrogen and stored at −80°C. About 10 g of the samples were thawed and washed briefly with 7.5 mM phosphate buffer (pH 7.0). They were homogenized on an ice bath in the same buffer containing 10% methanol and 0.2% tocopherol (anti-oxidation agent). The same volume of the organic solvents consisting of ethyl acetate and petroleum ethel (1:1) was added to the homogenate. The organic phases were extracted twice after vigorous shaking and centrifugation. They were evaporated at room temperature and resuspended in 300 μl dimethyl sulfoxide. The precipitate formed was removed by the column guird filter (440-84, Millipore Ltd).

High performance liquid chromatography

HPLC was performed with a Jasco's liquid chromatographic system consisting of an 801-SC system controller, an 880-02 gradient unit, an 880-PU pump and an 875-UV detector (Japan Spectroscopic Co. Ltd). Cosmosil $5C_{18}$ column (4.6×150 mm) (Nakarai Tesque Co. Ltd) was used for the reverse phase HPLC. According to Thaller and Eichele (1987), the column was eluted with acetonitrile/methanol/1% acetic acid (6:2:2) at a flow rate of 1.2 ml/minute.

For the normal phase HPLC, cosmosil $5NH_2$ (Nakarai Tesque Co. Ltd) was used. The solvent systems consisted of solutions A (chloroform/methanol (9:1)) and B (chloroform/methanol/10% acetic acid (9:1:0.1)). The column was eluted first with the solution A for 10 minutes and then with the solution B at a flow rate of 2.0 ml/minute. This normal phase HPLC was performed to isolate all the isoforms of retinoic acid from other retinoids.

Identification and quantification of endogenous retinoids

Seven kinds of standard retinoids were purchased from Sigma (all-*trans*-retinol, R7632; 13-*cis*-retinol, R6132; all-*trans*-retinoic acid, R2625; 13-*cis*-retinoic acid, R3255; all-*trans*-retinal, R2500; 9-*cis*-retinal, R5754; 13-*cis*-retinal, R6256). These retinoids (0.6 ng-1.2 mg) were applied to the column to examine their retention times and their peak areas. The peak areas were used to draw the calibration curve. The curve was linear in the range of 10 ng and 1.2 mg. A portion of standard retinoids was extracted with the organic solvents (ethyl acetate/petroleum ethel, 1:1), evaporated and applied to the HPLC column in the same manner as the endogenous retinoids. The peak area was compared with that obtained by the immediate analysis in order to calculate recovery ratios of respective retinoids.

Retinoids were also quantified with the aid of a spectrophotometer (Ubest-35, Japan Spectroscopic Co. Ltd). They were dissolved in the solution B mentioned above and were scanned to estimate the maximal wave length of absorption spectrum and the

peak height. The calibration curve of standard retinoic acid was linear, at least, in the range of 0.1 μg/ml and 2 μg/ml.

Whole-mount histochemistry of aldehyde dehydrogenase

Growing buds were allowed to enter the developmental phase by extirpating them from the parental animal (Kawamura and Nakauchi, 1986). They were fixed at various developmental time periods in Zamboni's fixative (Zamboni and DeMartino, 1967) for 10 minutes at 4˚C, followed by acetone for 10 minutes at −30˚C. They were washed with 60 mM phosphate buffer (pH 7.2) and incubated for 120 minutes in the color development solution for aldehyde dehydrogenase (McCaffery et al., 1991). In brief, the solution contained 10 mg nicotinamide adenine dinucleotide (or nicotinamide adenine dinucleotide phosphate), 1 mg phenazine methosulfate and 10 mg nitro blue tetrazolium in 10 ml of 60 mM phosphate buffer (pH 7.2). Either 14 mM propionaldehyde or 20 mM benzaldehyde was used as the substrate. As a control, some specimens were incubated in the color development solution lacking the substrate. Reactions were stopped by dehydrating the buds in graded ethanol. Buds were cleared by placing them in methyl salicylate.

Some of colored samples were embedded in JB-4 plastic medium (Polysciences, Inc.), as described previously (Kawamura et al., 1991). They were sectioned with glass knives at 2 μm and mounted serially on coverslips.

Isolation and characterization of *Polyandrocarpa* aldehyde dehydrogenase

As the enzyme can be induced in both processes of budding and regeneration of *P. misakiensis* (this paper and unpublished observation of Kawamura et al.), we have extracted the enzyme from regenerating animals one or two days after cutting into anterior and posterior halves. These animals (about 20 g) were homogenized in 7.5 mM phosphate buffer (pH 7.0) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation (12000 ***g***, 15 minutes), the supernatant was dialyzed against 0.1 M ammonium acetate containing 0.5 mM EDTA and passed through a gel filtration column (Ultrogel AcA44; 3×100 cm) equilibrated with the same buffer. Each fraction was tested for aldehyde dehydrogenase activity, using the dot-blot apparatus. In brief, 200 μl solution was loaded on each hole of the apparatus and blotted to nitrocellulose membrane. The membrane was washed with 60 mM phosphate buffer (pH 7.2) and stained with the color development solution mentioned above.

Positive fractions were collected, concentrated and dialyzed against 10 mM phosphate buffer (pH 8.0). They were applied to a column (16×40 mm) of DEAE Toyopearl 650M (Tosoh Co. Ltd), equilibrated with the same buffer. The column was eluted overnight with a linear gradient of 0-0.5 M NaCl in the same buffer. The enzyme activity was monitored, as mentioned above.

Polyandrocarapa aldehyde dehydrogenase was tested for RA synthase activity. The enzyme fractions were dialized against distilled water and concentrated to about 40 μg proteins/ml. The amount of proteins was determined by the method of Lowry et al. (1951). The enzyme solution (0.1 ml) was added to 0.9 ml of 60 mM phosphate buffer (pH 7.2) containing 1 mg NAD and 0.1 mg PMF. As substrates, either 1.2 μg all-*trans*-retinal or 13-*cis*-retinal was added. The reaction was carried out in the dark for 120 minutes at room temperature. It was stopped by the addition of the same volume of ethyl acetate/petroleum ethel (1:1). The organic phases were extracted and analyzed by HPLC, as mentioned above.

Bioassays

Growing buds were truncated at both proximal and distal extremities. They were removed from the substratum, put on the new glass slide and placed in a moist chamber for 10 minutes, so that they would adhere firmly to the substratum (Kawamura and Watanabe, 1983).

The glass slide containing 5-6 buds was incubated for 3-24 hours in 0-0.6 μg/ml retinoids diluted with filtered sea water, and then transferred to natural sea water. In order to estimate body patterns formed, 6-day-old buds were fixed in Zamboni's fixative, washed with phosphate-buffered salt solution (pH 7.2) and stained with borax carmine for 10 minutes. The specimens were dehydrated and cleared, as above.

RESULTS

Endogenous retinoids of *Polyandrocarpa*

In our preparation procedure of HPLC samples, recovery ratios of standard retinoids were 53% to 67% of the original amounts. The lower limit of quantification in our HPLC system was about 0.5 ng (retinoic acid) and 5 ng (retinol and retinal) at UV 350 nm. But, in practice, more than 10 ng was the reliable amount. Fig. 2A shows the reverse phase HPLC elution profile of *Polyandrocarpa* extracts taken from adult animals. The retention time of peak 6 was consistent with that of all-*trans*-retinoic acid (Fig. 2C). The extracts were applied to the column again, but all-*trans*-retinoic acid (10 ng), retinol (50 ng) and retinal (50 ng) were added to them as internal standards (Fig. 2B). The signal of peak 6 was augmented specifically. The shoulder of peak 7, showing the same retention time as all-*trans*-retinal, became also higher than the original one (cf., Fig. 2A). In contrast, the signal indicating all-*trans*-retinol could not be detected at all. The *Polyandrocarapa* extracts were also monitored at UV 280 nm. Peak 6 and neighboring peaks decreased remarkably in height (Fig. 2D), consistent with the absorption spectrum of retinoids.

In order to confirm the above-mentioned results and identify other peaks, extraction of retinoids was repeated for *Polyandrocarpa* adult animals. Fig. 3A shows that the elution profile is reproducible. In addition to all-*trans*-isoforms of retinoids, three kinds of 13-*cis*-retinoids were used as internal standards. (Fig. 3B). The peak of all-*trans*-retinoic acid was completely consistent with peak 6, and that of 13-*cis*-retinal was consistent with peak 7 (Fig. 3B). The shoulder of peak 7 was considered as all-*trans*-retinal. From the calibration curve, all-*trans*-retinoic acid was estimated at 5-17 ng per 1 g samples, all-*trans*-retinal at 10-26 ng and *cis*-retinal at about 300 ng.

Next, retinoids were extracted from developing buds and analyzed by HPLC (Fig. 3C). Cis-retinal (peak 7) decreased remarkably in amount (60 ng/g). Instead, peak 3, neighboring the peak of 13-*cis*-retinoic acid (cf., Fig. 3B), increased in height. The retention time of peak 4′ overlapped with all-*trans*-retinol (Fig. 3D) and 13-*cis*-retinol (Fig. 3E), but was not completely consistent with either of them. We have not yet determined whether or not peak 4′ was identical to peak 4 from adult animals. The amount of all-*trans*-retinoic acid was below the calibration curve.

Although there is a possibility that the peak 3 is an isoform of *cis*-retinoic acid, we could not determine its accurate chemical structure. Instead, we attempted to quantify

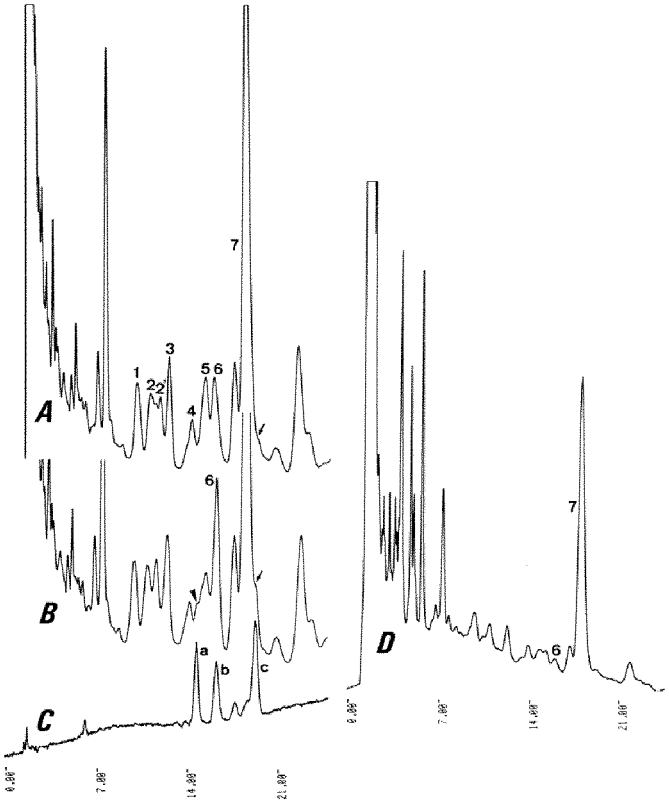

Fig. 2. Reverse phase HPLC analyses of endogenous retinoids extracted from adult animals of *P. misakiensis*, monitored at UV 350 nm except D. (A) Elution profile of organic phase of the extracts. Peaks showing the retention time from 10 to 20 minutes were numbered serially. Arrow shows the shoulder of peak 7. (B) The extracts were re-chromatographed together with internal standards, all-*trans*-retinol (50 ng), all-*trans*-retinoic acid (10 ng) and all-*trans*-retinal (50 ng). Both peak 6 and the shoulder (arrow) increased in height. Arrowhead shows the peak of all-*trans*-retinol that could not be detected from the original extracts. (C) Elution profile of standard retinoids, all-*trans*-retinol (a), all-*trans*-retinoic acid (b) and all-*trans*-retinal (c). (D) Elution profile of the extracts, monitored at UV 280 nm. Note that numbered peaks decreased remarkably in height.

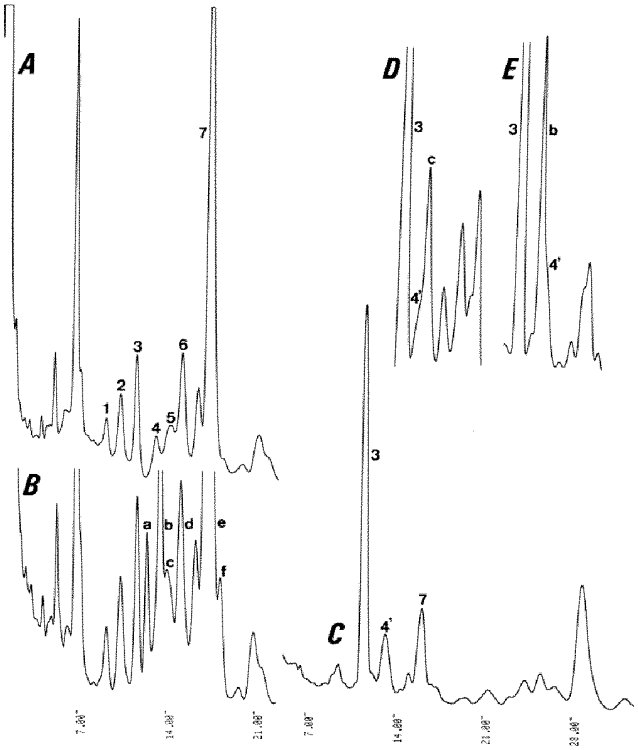

Fig. 3. Further analyses of endogenous retinoids of *Polyandrocarpa*, monitored at UV 350 nm. (A) The organic phase was extracted again from adult animals. Peaks numbered serially were eluted in a reproducible manner, although peak 2′ disappeared from this chromatogram. (B) Six kinds of internal standards were added to the extracts: a, 13-*cis*-retinoic acid; b, 13-*cis*-retinol; c, all-*trans*-retinol; d, all-*trans*-retinoic acid; e, 13-*cis*-retinal; f, all-*trans*-retinal. Peak 7 was completely consistent with that of 13-*cis*-retinal. (C) One- or two-day developing buds were used as source of endogenous retinoids. The elution profile was characterized by the decrease in height of peak 7 and alternative increase of peak 3. It is unknown if peak 4′ is identical with peak 4 from adult animals. (D) Bud extracts were coeluted together with several internal standards. Peak 4′ overlapped with all-*trans*-retinol (c) but not identical with it. (E) Similar chromatography, but 13-*cis*-retinol (b) was added to the extract.

the total amount of isoforms of retinoic acid from asexually developing *Polyandrocarpa* colony. In the normal phase HPLC, both standard retinol and retinal were eluted in the void volume (chloroform/methanol, 9:1) (Fig. 4A), whereas retinoic acid was bound to NH_2 group of the immobile phase (Fig. 4B). The bound retinoic acid could be eluted easily by the addition of 0.1% acetic acid (Fig. 4B arrow). Using this method, endogenous retinoids with carboxyl group were separated from other retinoids. Three major peaks were detected in the mobile phase containing acetic acid (Fig. 4C). The last one was eluted at the same retention time as standard retinoic acid. It showed the maximum of absorption spectrum at UV 355 nm and 370 nm (Fig. 4D), the former consistent with the λmax of standard retinoic acid in chloroform/methanol/acetic acid. From the height of peaks, the total amount of endogenous retinoic acid was estimated at 0.09 μg per 1 g samples. Taking the recovery ratio of 60% into consideration, we could correct the value to 0.15 μg/g.

Developmental expression of aldehyde dehydrogenase

Retinoic acid can be produced from retinal, or vitamin A aldehyde, via oxidation of its aldehyde group. Therefore, it is expected that, in *P. misakiensis*, retinoic acid-producing cells, if present, would show enzyme activity of aldehyde dehydrogenase. This was examined histochemically.

Growing buds did not show any signs of aldehyde dehydrogenase activity (Fig. 5A). Five hours after isolation from the parent, the bud epidermis expressed enzyme activity at the proximal end (Fig. 5B arrow). At 12 hours, the signal spread over the epidermis of the morphogenesis domain (Fig. 5C). At 24 hours of bud development, it was also observable in the inner multipotent epithelium (Fig. 5D arrow). This specimen was truncated at both proximal and distal ends and allowed to develop. In the distal, non-morphogenetic area (Fig. 5D arrowhead) neither epidermis nor multipotent epithelium showed enzyme activity.

Fig. 5E shows a histological section of the morphogen-

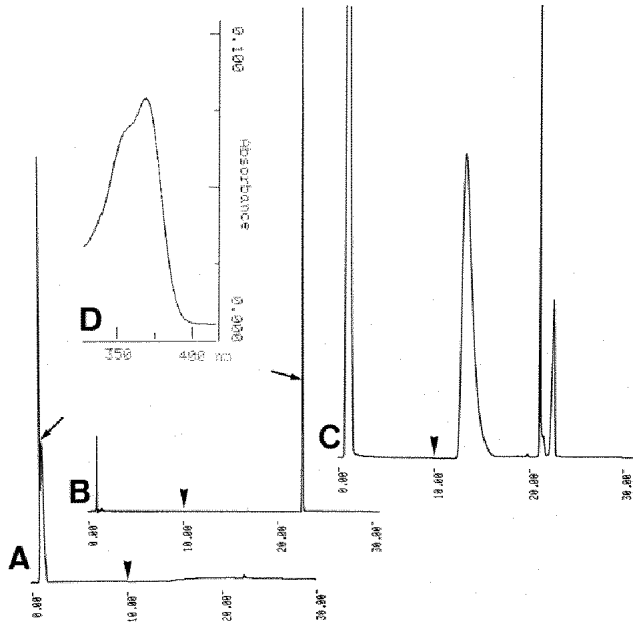

Fig. 4. Isolation and quantification of all isoforms of endogenous retinoic acid from budding *Polyandrocarpa* colonies. (A,B) Elution profiles of standard retinoids at UV 350 nm in the normal phase HPLC. The column was eluted with the solution A (chloroform/methanol, 9:1) for 10 minutes, and then (arrowhead) the mobile phase was changed to the solution B (chloroform/methanol/10% acetic acid, 9:1:0.1). (A) Both 13-*cis*-retinol and retinal (1.2 μg) were eluted in the void volume (arrow). (B) 13-*cis*-retinoic acid (1.2 μg) was eluted about 12 minutes (arrow) after changing to the solution B. (C) Elution profile of the organic phase from asexually developing *Polyandrocarpa* colonies. Three major peaks were eluted in the solution B. The retention time of the last one was consistent with that of standard retinoic acid. (D) Absorption spectrum of the last peak. It showed λmax at UV 355 nm and 370 nm. The total amount of endogenous retinoic acid was estimated from the peak height at 355 nm. For this purpose, the calibration curve of standard retinoic acid (λmax, about UV 355 nm in the solution B) was used.

esis domain of 24-hour developing buds. The epidermis was usually stained over the whole area of cell bodies, but in some cases only the basal surface was stained. In the mesenchymal space, or haemocoel, most of mesenchymal cells were stained. Large amoeboid cells, or leucocytes (after Wright's nomenclature, 1981), with a diameter of about 10 μm, were most prominent. In the multipotent epithelium, the signal was usually restricted to the apical surface. In the following 24 hours, the epithelium would evaginate to form the gut rudiment (Kawamura and Nakauchi, 1986). Fig. 5F shows the distal area of the same section as Fig. 5E. The enzyme activity could not be detected at all.

From this developmental stage onward, the expression pattern of enzyme activity was not changed dramatically and the signal became weakened gradually (not shown). The negative control, in which the substrate was subtracted from the color development solution, did not show any signals (Fig. 5G).

Retinoic acid synthase activity of aldehyde dehydrogenase

We have found that aldehyde dehydrogenase is also induced in the process of regeneration of adult animals in *P. misakiensis* (Kawamura et al., unpublished data). These regenerating animals, which could be collected easily, were used as the source of the enzyme and *Polyandrocarpa* aldehyde dehydrogenase was tested for retinoic acid synthase activity.

Fig. 6A shows the result of gel filtration chromatography. Fig. 6B shows a typical example of enzyme assay. Peaks of the enzyme activity always appeared just behind the void volume. The enzyme fractions were collected, concentrated and passed through the column of anion exchange chromatography (Fig. 6C). They were eluted soon after the onset of NaCl gradient.

Polyandrocarpa aldehyde dehydrogenase (about 4 μg proteins) was allowed to react in vitro with 1.2 μg all-*trans*-retinal or 13-*cis*-retinal for 2 hours at room temperature. The reaction products were extracted with organic solvents and analyzed by HPLC. In the absence of the enzyme, about 70% of the original amount of retinal was recovered in the organic phase (Fig. 7E). However, in the presence of the enzyme, only 35% was recovered (average of four experiments) (Fig. 7F). The difference was statistically significant. The results suggested that a large amount of the aldehyde group of retinal had been oxidized by the enzyme. It should be noted, however, that the amount of product (all-*trans*-retinoic acid or 13-*cis*-retinoic acid) was 4-10 ng at most (compare Fig. 7B,D with Fig. 7A,C). This value was inconsistent with the decrease in amount of the substrate.

The enzyme treatment was repeated, but 20 ng 13-*cis*-retinal was reacted with the enzyme for 30 minutes. About 10 ng of the substrate and 1 ng of 13-*cis*-retinoic acid were recovered (Fig. 7G). The discrepancy between the missing substrate and the product to be synthesized was decreased to some extent, but we could not determine improved experimental conditions.

The effect of retinoids on *Polyandrocarpa* bud development

If buds were treated with 0.3-0.6 μg/ml retinoic acid for 3-6 hours, aldehyde dehydrogenase was often induced ectopically at the distal extremity (Fig. 8A arrow). This induction of enzyme was strengthened remarkably by treating buds with 0.6 μg/ml retinoic acid for 24 hours (Fig. 8B). Enzyme activity was spread over the whole area of the epidermis, inner multipotent epithelium and mesenchymal cells. The mesenchymal space became expanded widely anywhere along the proximodistal axis of the bud. These characteristics were normally restricted to the proximal end of bud (cf., Fig. 5D-F).

In normally developing whole-mount buds, the pharynx and gut that represent the anteroposterior organization became visible within 6 days after bud isolation from the parent (Fig. 8C). Most of malformed animals induced by retinoids were double monsters, of which the secondary bud axis induced distally had a complete anteroposterior organization (Fig. 8D arrow). Table 1 shows that retinoic acid gives the highest induction ratio of pattern duplication. When 0.6 μg/ml retinoic acid was applied to buds, an adequate incubation time was 3-4 hours. If lower doses were used, longer incubation time periods were needed. The

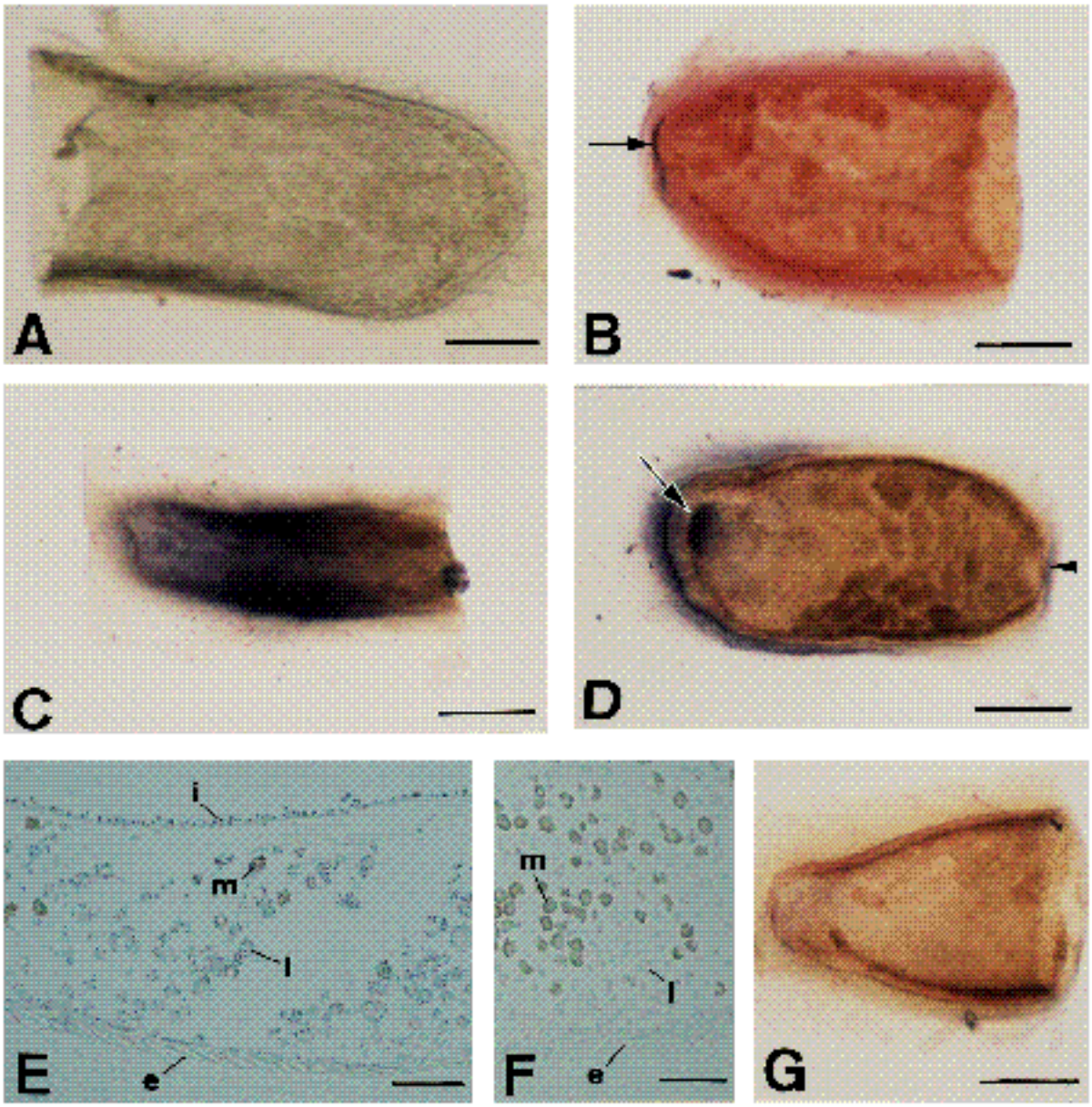

Fig. 5. Whole-mount histochemistry of aldehyde dehydrogenase activity in developing *Polyandrocarpa* buds. In each bud, the proximal end is at the left. Propionaldehyde (A,E,F) or benzaldehyde (B,C,D,G) was used as substrate. (A) Growing bud before morphogenesis. Bar, 0.5 mm. (B) 5 hours after isolation from the parent. The distal half was extirpated from the bud after fixation. The signal appeared in the epidermis at the proximal end (arrow). Bar, 0.5 mm. (C) Developing bud, 12 hours later. The distal half was removed from the specimen. Bar, 0.5 mm. (D) Developing bud, 24 hours later. The distal end (arrowhead) was cut off at the onset of bud development to examine the effect of injury on enzyme expression. Arrow shows the multipotent epithelium at the morphogenesis domain with the enzyme activity. Bar, 0.5 mm. (E) Section of a 24-hour bud, proximal end. Signals were observed in the epidermis (e), multipotent inner epithelium (i) and mesenchymal cells such as leucocytes (l) and morula cells (m). Bar, 50 μm. (F) The distal area of the same section as E. No signals were observable. Bar, 50 μm. (G) Control, 24-hour bud. The substrate was subtracted from the color development solution. Bar, 0.5 mm.

induction ratio of double monsters was about 50% when buds were treated with 0.1 μg/ml 13-*cis*-retinoic acid for 24 hours. Longer treatment of buds with high dose of retinoic acid (cf., Fig. 8B) resulted in the failure of development of anteroposterior organization in the majority of cases (Table 1). Uniposterior-like animals had a single, enormously large gut-like structure (Fig. 8E), and biposterior-like animals had an additional gut-like diverticulum (Fig. 8F).

Retinal showed a secondary axis-inducing activity, but the activity was significantly lower than retinoic acid (Table 1). Retinol, which could not be detected from either adults or buds of *P. misakiensis*, had no apparent effect on bud development as far as we tested in the present work.

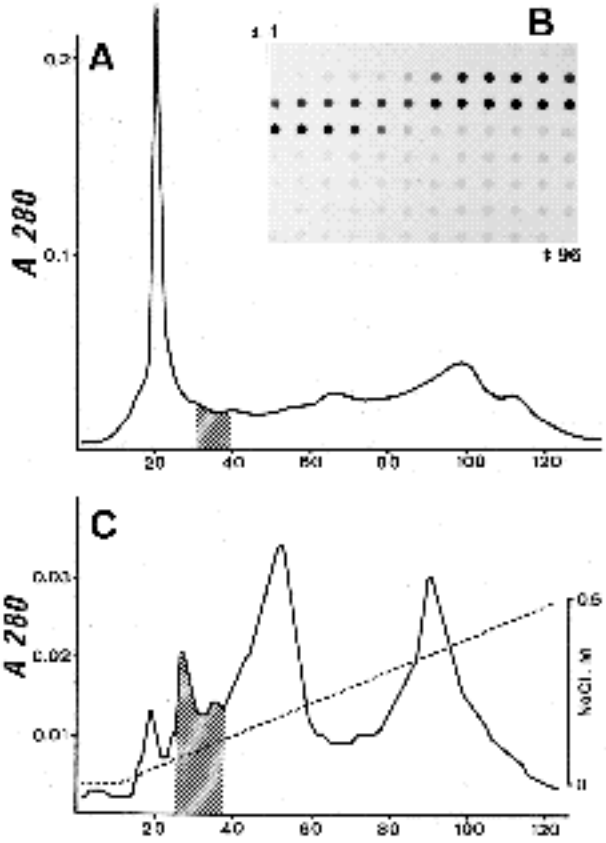

Fig. 6. Isolation and partial purification of *Polyandrocarpa* aldehyde dehydrogenase. (A) Gel filtration chromatography of crude extracts from regenerating animals. The enzyme fractions (hatched) eluted after the void volume. (B) Dot-blot analysis of the enzyme activity. Coloration of circles from peaks 20 to 29 was due to pigments that eluted in the void volume. (C) Anion exchange chromatography. The enzyme fractions (hatched) eluted under low concentrations of NaCl.

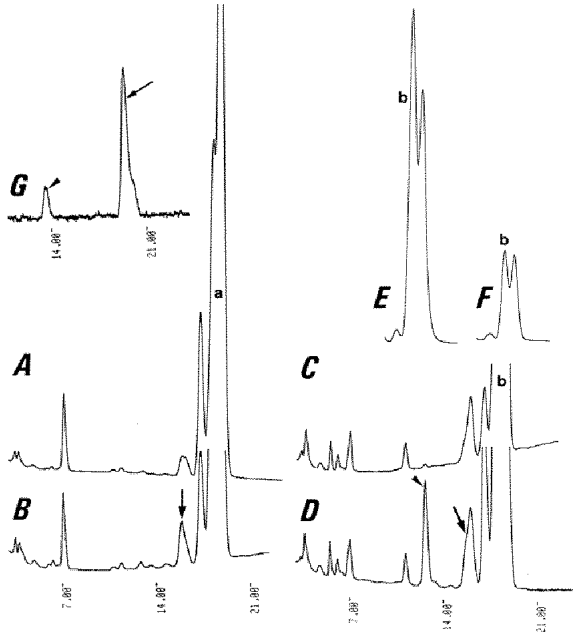

Fig. 7. Retinal-metabolizing activity of *Polyandrocarpa* aldehyde dehydrogenase. (A,B) All-*trans*-retinal (a) was used as the substrate. (C-F) 13-*cis*-retinal (b) was used. Retinal (1.2 μg) was incubated for 2 hours in the reaction solution in the absence (A,C,E) or presence (B,D,F) of the enzyme. Arrows and arrowhead show all-*trans*-retinoic acid and 13-*cis*-retinoic acid, respectively. (E,F) Comparison of remaining amounts of the substrate. Note the significant decrease in amount in the presence of the enzyme (F). (G) Smaller amount of 13-*cis*-retinal (20 ng) was treated with the enzyme for only 30 minutes. About 10 ng of retinal (arrow) and 1 ng of 13-*cis*-retinoic acid (arrowhead) were recovered. The discrepancy between the loss of the substrate and the increase in amount of the product became small.

DISCUSSION

We have described here the amounts of endogenous retinoids in adult animals and buds, the spatiotemporal distribution of aldehyde dehydrogenase activity, its substrate-specificity for retinal (vitamin A aldehyde) and biological effects of retinoids on body patterning in the budding tunicate, *Polyandrocarpa misakiensis*. On the basis of these findings, we discuss the possibility that retinoids act as endogenous signalling molecules to trigger the onset of morphallactic bud development.

Identification of endogenous retinoids and their dynamics during budding

We detected about 300 ng/g *cis*-retinal (1.25-2 μM), 10-26 ng/g all-*trans*-retinal (50-100 nM) and 5-17 ng/g all-*trans*-retinoic acid (20-60 nM) in adult animals of *P. misakiensis*. It should be noted that these values are underestimations as adult animals are hollow and devoid of cells and tissues owing to the branchial and atrial cavities (see Fig. 1). Unfortunately, we could not determine the kind of isoform(s) of endogenous *cis*-retinal, as the peak of 13-*cis*-retinal used as standard overlapped completely with that of 9-*cis*-retinal in our HPLC condition (our unpublished data). The presence of enormous amounts of *cis*-retinal in *Polyandrocarpa* contrasts with the case of embryonic fields in vertebrates. Chick limb buds seem not to contain a substantial amount of retinal, but they contain about 600 nM all-*trans*-retinol (Thaller and Eichele, 1987). Likewise, axolotl spinal cords possess all-*trans*-retinol but not retinal (Hunter et al., 1991).

In *P. misakiensis*, developing buds contained about one fifth the amount of *cis*-retinal as adult animals. We assume that this decrease in amount may have come partly from the metabolism of *cis*-retinal during bud development. Some unpublished observations support this idea. First, immunohistochemistry using anti-retinoid monoclonal antibody and HPLC analysis of fractionated cell populations showed that the epidermis and mesenchymal cells were major sources of endogenous retinoids. These cell populations are shared by both adult animals and buds (Fig. 1). This observation does not support the idea that in adult animals *cis*-retinal is abundant because it is included in differentiated tissues and organs that are absent in developing buds. Second, *cis*-retinal also decreased remarkably in amount in the process of regeneration of adult animals (Kawamura et al., unpublished data). As shown in Fig. 7F,

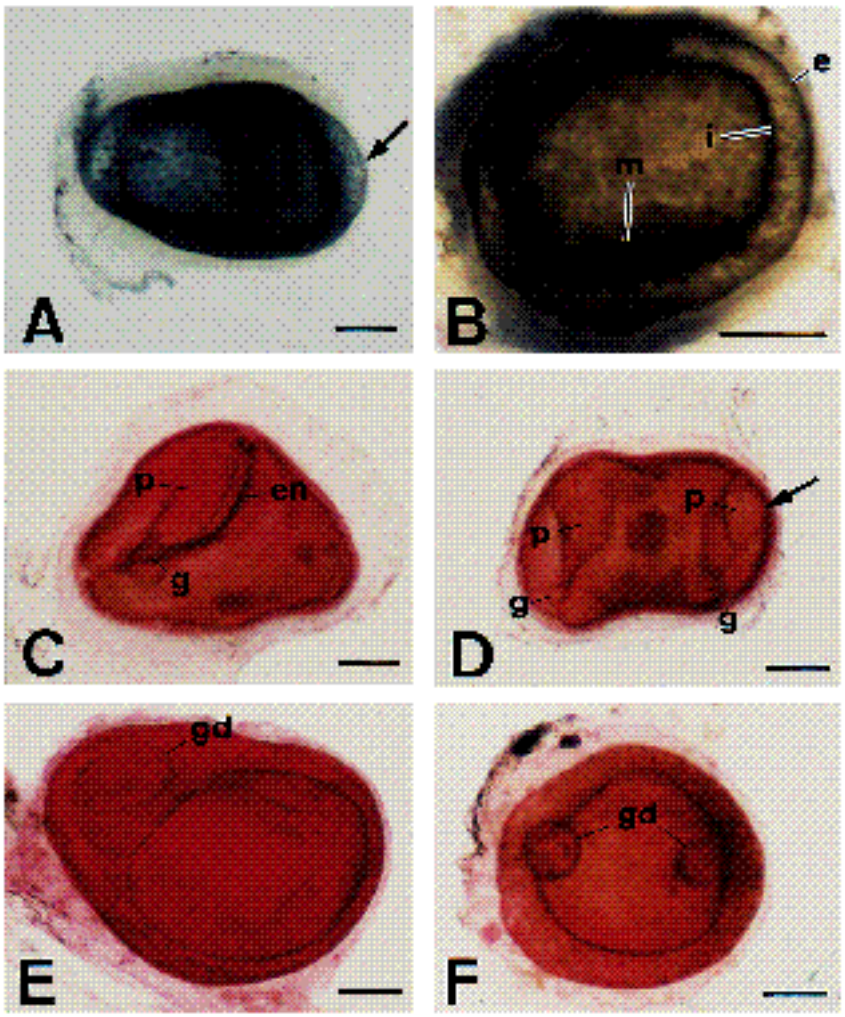

Fig. 8. Abnormalities of bud development caused by retinoic acid. In each bud the proximal end is at the left. (A,B) Propionaldehyde staining. (A) Ectopic expression of aldehyde dehydrogenase at the distal extremity (arrow). Growing buds were truncated at both extremities, treated with 0.3 μg/ml 13-*cis*-retinoic acid for 6 hours, and then allowed to develop for one day in normal sea water. (B) Aldehyde dehydrogenase of 1-day-old bud treated continuously with 0.6 μg/ml all-*trans*-retinoic acid. The enzyme activity could be seen anywhere in the whole-mount bud. (C) Six-day-old normal bud. The anteroposterior organization has been well established. (D) Double malformation derived from a bud treated as in A. In the distal, new developmental field (arrow), the complete anteroposterior organization was established. (E,F) Hypomorphosis derived from buds treated as in B. In both uniposterior-like (E) and biposterior-like (F) animals, anterior structures failed to develop. e, epidermis; en, endostyle; g, gut; gd, gut-like diverticulum; i, inner epithelium; m, mesenchymal cell; p, pharynx. Bars, 0.5 mm.

aldehyde dehydrogenase extracted from regenerating animals caused a decrease in amount of exogenous 13-*cis*-retinal. The metabolism of *cis*-retinal may be characteristic of regenerative events including budding in *P. misakiensis*.

The amount of all-*trans*-retinoic acid extracted from developing buds was unexpectedly small, which may be evidence against a role of all-*trans*-retinoic acid in bud development. The alternative possibility is that, during bud development, retinoic acid might be bound to high affinity receptors (Takase et al., 1986; Giguere et al., 1987) and, after fulfilling biological functions, it might be metabolized further.

In contrast, peak 3 increased in height during budding. This was also the case in regeneration (our unpublished data). Unfortunately, the peak could not be identified accurately, although our unpublished data suggested that it might be an isoform of *cis*-retinoic acid. Instead, we estimated the total amount of isoforms of retinoic acid in asexually developing *Polyandrocarpa* colonies. This quantification is important, as different isoforms of retinoic acid were similarly effective in inducing the secondary bud axis (Table 1). Using the normal phase HPLC and spectrophotometry, endogenous retinoic acid (strictly speaking, retinoids with carboxyl group) was estimated at about 150 ng/g (450 nM), a value more than three times as much as that of adult animals (our preliminary data). Both endogenous and exogenous retinoic acids at this concentration exhibited morphallaxis-inducing activity in *Polyandrocarpa* buds (Table 1 and Kawamura et al., unpublished data).

Does aldehyde dehydrogenase reflect the localization of retinoic acid synthase activity?

Many different isoforms of aldehyde dehydrogenase have been isolated from the mouse liver (Manthey et al., 1990) and from the retina (McCaffery et al., 1991). Some of them oxidize a broad range of aldehydes and others are relatively substrate-specific. Both isoforms 2 and 7 of aldehyde dehydrogenase show retinoic acid synthase activity and are characterized by basic isoelectric points (Lee et al., 1990). The former prefers an aliphatic substrate such as propionaldehyde and the latter an aromatic, or cyclic substrate such as benzaldehyde. The enzyme of *P. misakiensis*, both in buds and regenerates, showed similar affinity for each substrate.

In *P. misakiensis*, enzyme activity was always localized at the morphogenesis domain, usually the proximal end of developing buds. When buds were pulse-treated with retinoic acid, the enzyme was often induced ectopically at

Table 1. Effects of retinoids on bud development of *Polyandrocarpa misakiensis*

Reagents	Conc. (μg/ml)	Incubation period (hours)	No. of cases	Body patterns formed (%)				
				Normal	Double monster	Unipost.-like	Bipost.-like	Not developed
		1	38	81.6	7.9	10.5	0	0
	0.6	6	50	22.0	4.0	26.0	30.0	18.0
		24	58	0	0	36.2	34.5	29.3
all-*trans*-RA	0.3	6	32	37.5	12.5	9.4	21.9	18.8
		24	34	0	5.9	17.6	47.1	29.4
	0.1	24	20	60.0	25.0	0	0	15.0
	0.6	4	36	22.2	77.8	0	0	0
		6	26	53.8	23.0	0	7.7	15.4
13-*cis*-RA	0.3	4	34	44.1	50.0	0	0	5.9
		6	41	43.9	7.3	26.8	2.4	19.5
	0.1	6	34	82.4	14.7	0	0	2.9
		24	31	48.4	51.6	0	0	0
13-*cis*-retinal	0.6	4	28	85.7	7.1	0	0	7.1
		6	32	87.5	12.5	0	0	0
all-*trans*-retinal	0.6	24	20	80.0	0	0	10.0	10.0
all-*trans*-retinol	0.6	24	20	100	0	0	0	0

the distal end. It was sensitive to actinomycin D and puromycin, although a trace of the activity was still observable (our unpublished data), suggesting the de novo synthesis of the enzyme. We do not know at present whether *Polyandrocarpa* aldehyde dehydrogenase gene is activated directly by retinoic acid. The gene of human alcohol dehydrogenase ADH3, which is involved in retinoic acid biosynthesis from retinol, has a retinoic acid-responsive element (Duester et al., 1991). This may explain why, in chick limb buds, the level of retinoic acid is induced 1000-fold above the normal endogenous level when analogs of retinoic acid are implanted into the anterior margin (for review, Tabin, 1991).

Polyandrocarpa aldehyde dehydrogenase purified partially has metabolized in vitro a large amount of retinal, as compared with the control lacking the enzyme. In contrast, the amount of retinoic acid as metabolite was very small. This discrepancy was improved to some extent by shortening the time length of reaction and reducing the concentration of the substrate. We assume that the inconsistency may have come partly from rapid oxidation of retinoic acid (Thaller and Eichele, 1988). Radiolabeled precursors of retinoic acid can be converted into retinoic acid in the chick limb bud tissue (Thaller and Eichele, 1988) and in the floor plate of the neural tube (Wagner et al., 1990), but the absolute amount of labeled retinoic acid is very low even in those in vivo experiments. Furthermore, our in vitro assay system was devoid of intrinsic factors, such as cellular retinoic acid-binding protein (CRABP; Takase et al., 1986; Maden et al., 1988, 1991), that may protect retinoic acid from oxidation. Nevertheless, all-*trans*-retinoic acid and 13-*cis*-retinoic acid were produced respectively from their precursors in the presence of the enzyme. Therefore, we conclude that *Polyandrocarpa* aldehyde dehydrogenase has the activity of retinoic acid synthase.

In multipotent epithelial cells of developing *Polyandrocarpa* buds, morphogenetic events proceed from day 1 as follows: change of cell shape from squamous to cuboidal form, swelling of the nucleus, increase in RNA contents (Kawamura and Nakauchi, 1984), onset of DNA synthesis (Kawamura et al., 1988), disappearance of cell surface antigen (Fujiwara and Kawamura, 1992), beginning of cell division and evagination of gut rudiment (Kawamura and Nakauchi, 1986; for review, Kawamura and Nakauchi, 1991). Expression of aldehyde dehydrogenase was much earlier than any of those morphogenetic events. The epidermis showed enzyme activity soon after bud extirpation, followed by mesenchymal cells. As mentioned above, they are candidates for retinoid-containing cells. Thus, there is a great possibility that, in developing buds, the metabolism of *cis*-retinal has begun immediately after the incision. Antagonists would be useful for further consideration of the biological function of the enzyme. Disulfiram is known to inhibit the aldehyde dehydrogenase activity (Sanny, 1987; Helander, 1989), but it turned out that the reagent is inappropriate because of low solubility in sea water. We have recently found that there is an endogenous aldehyde dehydrogenase inhibitor in *P. misakiensis* (our unpublished data). This inhibitor may afford direct evidence for the role of the enzyme in the initiation of bud development.

How do retinoic acid and its derivatives act on bud tissues?

The present work confirmed and extended our previous work which showed that retinoic acid is able to induce a new developmental field in the palleal bud of *P. misakiensis* (Hara et al., 1992). The minimum incubation time required for pattern duplication was one hour. Treatment for more than 3 hours gave desirable induction ratios, the value apparently higher than bead implantation. Exogenous 13-*cis*-retinoic acid at the concentration of 0.1 μg/ml caused pattern duplication in about 50% of the cases (Table 1). This concentration would be compatible with the endogenous level of retinoic acid, as mentioned above. As far as we examined, other retinoids had much weaker effect or no apparent effect on bud development, consistent with the

results of developing or regenerating limbs in vertebrates (Maden, 1982; Summerbell and Harvey, 1983).

In *P. misakiensis*, there are two kinds of multipotent cells, the atrial epithelium constituting the inner vesicle of the bud and the haemoblast in the mesenchymal space or haemocoel (for review, Kawamura and Nakauchi, 1991). As already discussed, the epithelial cells change their shape before the onset of organogenesis. This phenomenon took place ectopically when a bead soaked with retinoic acid was implanted into the mesenchymal space (Hara et al., 1992). The epithelium secretes lectin granules into the mesenchymal space to develop the extracellular matrix, which facilitates the migration and aggregation of haemoblasts toward the epithelium (Kawamura et al., 1991). The cell aggregation could also be induced by retinoic acid (Hara et al., 1992). These observations have suggested that retinoic acid can mimic major parts of the normal morphogenetic events restricted usually at the proximal end of bud.

Recently, we have found that *Polyandrocarpa* budding involves a transdifferentiation-like system (Fujiwara and Kawamura, 1992). The multipotent epithelium shared a cell surface antigen with a certain kind of apparently differentiated mesenchymal cell, suggesting that this antigen may be a differentiation marker. This antigen was lost when the epithelial cells were committed to organ rudiments. This was also the case when the secondary morphogenetic field was induced by retinoic acid (our unpublished data). It is uncertain at present whether or not in *P. misakiensis* retinoic acid acts as a transdifferentiation factor, as retinoic acid can act indirectly on the multipotent epithelium via mesenchymal cells (Hara et al., unpublished data).

In vertebrates, increasing evidence suggests that basic fibroblast growth factor (bFGF) is involved in the transdifferentiation of chick retinal pigment epithelium (Park and Hollenberg, 1989; Pittack et al., 1991). In axolotls, acidic FGF is present in regenerating limb blastemas and binds specifically to blastema tissues (Boilly et al., 1991). bFGF is stored within the basement membrane of bovine cornea (Folkman et al., 1988). In bovine capillary endothelial cells, it is released from the extracellular matrix through plasminogen activator-mediated proteolytic activity (Saksela and Rifkin, 1990). The plasminogen activator gene is one of the possible target genes of retinoic acid (Rickles et al., 1991). Thus, there is a possiblility that, in *Polyandrocarpa* buds, retinoic acid has influence on transdifferentiation via this line of developmental cascade, although there is no literature indicating that retinoic acid induces transdifferentiation in developing or regenerating vertebrate limbs (Tickle et al., 1982, 1985; Maden, 1983; Thoms and Stocum, 1984). We showed previously that bovine pancreas protease could induce the secondary bud axis in *P. misakiensis* (Kawamura and Watanabe, 1987).

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REFERENCES

Boilly, B., Cavanaugh, K. P., Thomas, D., Hondermarck, H., Bryant, S. and Brandshaw, R. A. (1991). Acidic fibroblast growth factor is present in regenerating limb blastemas of axolotls and binds specifically to blastema tissues. *Dev. Biol.* **145**, 302-310.

Colberg-Poley, A. M., Voss, S. D., Chowdhury, K. and Gruss, P. (1985). Structural analysis of murine genes containing homeo box sequences and their expression in embryonal carcinoma cells. *Nature* **314**, 713-718.

Deschamps, J., De Laaf, R., Joosen, L., Meijlink, F. and Destree, O. (1987). Abundant expression of homeobox genes in mouse embryonal carcinoma cells correlates with chemically induced differentiation. *Proc. Natl. Acad. Sci. USA* **84**, 1304-1308.

Duester, G., Shean, M. L., McBride, M. S. and Stewart, M. J. (1991). Retinoic acid response element in the human alcohol dehydrogenase gene ADH3: implications for regulation of retinoic acid synthesis. *Mol. Cell. Biol.* **11**, 1638-1646.

Durston, A. J., Timmermans, J. P. M., Hage, W. J., Hendriks, H. F. J., De Vries, N. J., Heideveld, M. and Nieuwkoop, P. (1989). Retinoic acid causes an anteroposterior transformation in the developing central nervous system. *Nature* **340**, 140-144.

Folkman, J., Klagsbrun, M., Sasse, J., Wadzinski, M., Ingber, D. and Vlodavsky, I. (1988). A heparin-binding angiogenic protein -basic fibroblast growth factor- is stored within basement membrane. *Am. J. Pathol.* **130**, 393-400.

Fujiwara, S. and Kawamura, K. (1992). Ascidian budding as a transdifferentiation-like system: multipotent epithelium is not undifferentiated. *Dev. Growth Differ.* **34**, 463-472.

Giguere, V., Ong, E. S., Segui, P. and Evans, R. M. (1987). Identification of a receptor for the morphogen retinoic acid. *Nature* **330**, 624-629.

Hara, K., Fujiwara, S. and Kawamura, K. (1992). Retinoic acid can induce a secondary axis in developing buds of a colonial asicidian, *Polyandrocarpa misakiensis. Dev. Growth Differ.* **34**, 437-445.

Helander, A. (1989). Inhibition of human erythrocyte and leukocyte aldehyde dehydrogenase activities by diethylthiocarbamic acid methyl ester. An *invivo* metabolite of disulfiram. *Biochem. Pharmacol.* **38,** 2195-2198.

Hunter, K., Maden, M., Summerbell, D., Eriksson, U. and Holder, N. (1991). Retinoic acid stimulates neurite outgrowth in the amphibian spinal cord. *Proc. Natl. Acad. Sci. USA* **88**, 3666-3670.

Kawamura, K., Fujiwara, S. and Sugino, Y. M. (1991). Budding-specific lectin induced in epithelial cells is an extracellular matrix component for stem cell aggregation in tunicates. *Development* **113**, 995-1005.

Kawamura, K. and Nakauchi, M. (1984). Changes of the atrial epithelium during the development of anteroposterior organization in palleal buds of the polystyelid ascidian, *Polyandrocarpa misakiensis*. *Mem. Fac. Sci. Kochi Univ.* **5** (Ser. D), 15-35.

Kawamura, K. and Nakauchi, M. (1986). Mitosis and body patterning during morphallactic development of palleal buds in ascidians. *Dev. Biol.* **116**, 39-50.

Kawamura, K. and Nakauchi, M. (1991). Homeostatic integration of stem cell dynamics during palleal budding of ascidians. *Zool. Sci.* **8**, 11-22.

Kawamura, K., Ohkawa, S.-I., Michibata, H. and Nakauchi, M. (1988). Autoradiographic studies on cell population kinetics in epithelial and haematopoietic stem cell lines in the process of palleal budding of ascidians. *Mem. Fac. Sci. Kochi Univ*. **9** (Ser. D), 1-12.

Kawamura, K. and Watanabe, H. (1983). The role of parental positional information in the determination of anteroposterior polarity during palleal budding in ascidians. *WilhelmRoux Arch. Dev. Biol.* **192**, 28-36.

Kawamura, K. and Watanabe, H. (1987). Possible role of the epidermis and mesenchyme in the determination of morphogenesis domain during palleal budding in ascidians. *Mem. Fac. Sci. Kochi Univ*. **8** (Ser. D), 15-27.

Lee, M. O., Dockham, P. A. and Slader, N. E. (1990). Identification of human liver aldehyde dehydrogenases that catalyze the oxydation of retinalaldehyde to retinoic acid. *Pharmacologist* **32**, 156.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the phenol reagent. *J. Biol. Chem*. **193**, 265-275.

Maden, M. (1982). Vitamin A and pattern formation in the regenerating limb. *Nature* **295**, 672-675.

Maden, M. (1983). The effect of vitamin A on limb regeneration in *Rana temporaria*. *Dev. Biol.* **98**, 409-416.

Maden, M., Hunt, P., Eriksson, U., Kuroiwa, A., Krumlauf, R. and Summerbell, D. (1991). Retinoic acid-binding protein, rhombomeres, and the neural crest. *Development* **111**, 35-44.

Maden, M., Ong, D. E., Summerbell, D. and Chytil, F. (1988). Spatial

distribution of cellular proteins binding to retinoic acid in the chick limb bud. *Nature* **335**, 733-735.

Manthey, C. L., Landkamer, G. J. and Slader, N. E. (1990). Identification of the mouse aldehyde dehydrogenases important in aldophosphamide detoxification. *Cancer Res.* **50**, 4991-5002.

McCaffery, P., Tempst, P., Lara, G. and Drager, U. C. (1991). Aldehyde dehydrogenase is a positional marker in the retina. *Development* **112**, 693-702.

Noji, S., Nohno, T., Koyama, E., Muto, K., Ohyama, K., Aoki, Y., Tamura, K., Ohsugi, K., Ide, H., Taniguchi, S. and Saito, T. (1991). Retinoic acid induces polarizing activity but is unlikely to be a morphogen in the chick limb bud. *Nature* **350**, 83-86.

Park, C. M. and Hollenberg, M. J. (1989). Basic fibroblast growth factor induces retinal regeneration *in vivo*. *Dev. Biol.* **134**, 201-205.

Pittack, C., Jones, M. and Reh, T. A. (1991). Basic fibroblast growth factor induces retinal pigment epithelium to generate neural retina *in vitro*. *Development* **113**, 577-588.

Rickles, R. J., Darrow, A. L. and Strickland, S. (1989). Differentiation-responsive elements in the 5′ region of the mouse tissue plasminogen activator gene confer two-stage regulation by retinoic acid and cyclic AMP in teratocarcinoma cells. *Mol. Cell. Biol.* **9**, 1691-1704.

Saksela, O. and Rifkin, D. B. (1990). Release of basic fibroblast growth factor-heparin surfate complexes from endothelial cells by plasminogen activator-mediated proteolytic activity. *J. Cell Biol.* **110**, 767-775.

Sanny, C. G. (1987). Inactivation of horse liver mitochondrial aldehyde dehydrogenase by disulfiram. Evidence that disulfiram is not an active-site-directed reagent. *Biochem. J.* **242**, 499-503.

Summerbell, D. and Harvey, F. (1983). Vitamin A and the control of pattern in developing limbs. *Prog. Clin. Biol. Res.* **110**, 109-118.

Tabin, C. J. (1991). Retinoids, homeoboxes, and growth factors: toward molecular models for limb development. *Cell* **66**, 199-217.

Takase, S., Ong, D. E. and Chytil, F. (1986). Transfer of retinoic acid from its complex with cellular retinoic acid-binding protein to the nucleus. *Arch. Biochem. Biophys.* **247**, 328-334.

Taylor, I. M., Wiley, M. J. and Agur, A. (1981). Retinoic acid-induced heart malformation in the hamster. *Teratology* **21**, 193-197.

Thaller, C. and Eichele, G. (1987). Identification and spatial distribution of retinoids in the developing chick limb bud. *Nature* **327**, 625-628.

Thaller, C. and Eichele, G. (1988). Characterization of retinoid metabolism in the developing chick limb bud. *Development* **103**, 473-483.

Thaller, C. and Eichele, G. (1990). Isolation of 3,4-didehydroretinoic acid, a novel morphogenetic signal in the chick wing bud. *Nature* **345**, 815-819.

Thoms, S. D. and Stocum, D. L. (1984). Retinoic acid-induced pattern duplication in regenerating urodele limbs. *Dev. Biol.* **103**, 319-328.

Tickle, C., Alberts, B., Wolpert, L. and Lee, J. (1982). Local application of retinoic acid to the limb bond mimics the action of the polarizing region. *Nature* **296**, 564-566.

Tickle, C., Lee, J. and Eichele, G. (1985). A quantitative analysis of the effect of all-*trans*-retinoic acid on the pattern of chick wing development. *Dev. Biol.* **109**, 82-95.

Wagner, M., Thaller, C., Jessell, T. and Eichele, G. (1990). Polarizing activity and retinoid synthesis in the floor plate of the neural tube. *Nature* **345**, 819-822.

Watanabe, H. and Tokioka, T. (1972). Two new species and one possibly new race of social styelids from Sagami Bay, with remarks on their life history, especially the mode of budding. *Publ. Seto Mar. Biol. Lab.* **14**, 327-345.

Wright, R. K. (1981). Urochordates. In *Invertebrate Blood Cells*, vol. 2 (eds. N. A. Ratcliffe and A. F. Rowley), pp. 563-626. London: Academic Press.

Zamboni, L. and DeMartino, C. (1967). Buffered picric acid-formaldehyde: A new, rapid fixative for electron microscopy. *J. Cell Biol.* **35**, 148A.

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