Activin-Treated Urodele Animal Caps: I. Mesoderm and Endoderm Differentiation of Salamander Animal Caps

Authors: Ariizumi, Takashi, Takano, Kazuhiro, Ninomiya, Hiromasa, and Asashima, Makoto

Source: Zoological Science, 15(6) : 887-892

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.15.887

ZOOLOGICAL SCIENCE **15**: 887–892 (1998)

Activin-Treated Urodele Animal Caps: I. Mesoderm and Endoderm Differentiation of Salamander Animal Caps

Takashi Ariizumi[1], Kazuhiro Takano[2], Hiromasa Ninomiya[3] and Makoto Asashima[1,2,3]*

[1]*CREST, Japan Science and Technology Corporation (JST), The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan*
[2]*Department of Life Sciences (Biology), Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan*
[3]*Department of Biological Sciences, Graduate School of Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8654, Japan*

ABSTRACT—The differentiation patterns of animal cap explants from the Japanese salamanders *Hynobius lichenatus* and *Hynobius nigrescens* were examined after exposure to various concentrations of activin A. A wide range of concentrations of activin A (0.5-100 ng/ml) induced various mesodermal tissues such as ventral mesoderm, somitic muscle, and notochord. At concentrations higher than 50 ng/ml, yolk-rich endodermal tissue was induced in many of the explants. Activin A is also known to have mesoderm- and/or endoderm-inducing activity on the animal caps of *Xenopus laevis* and *Cynops pyrrhogaster*, but their response patterns are slightly different. The mode of differentiation of activin-treated *Hynobius* animal caps was compared with that of *Xenopus* and *Cynops* in relation to the structure of the animal caps.

INTRODUCTION

The animal cap assay (Yamada and Takata, 1961), a simple system using isolated ectoderm (animal cap) as the responding tissue, has enabled remarkable advances in the identification of inductive factors in recent years (reviewed in Asashima, 1994). Peptide growth factors belonging to the transforming growth factor-β (TGF-β) family can induce animal caps to differentiate into mesodermal and/or endodermal tissues that normally arise in the vegetal half of the embryo (reviewed in Ariizumi and Asashima, 1995a). Most studies on such "vegetalization" of animal caps by growth factors have been carried out with *Xenopus laevis* (reviewed in Klein and Melton, 1994). For example, we previously reported that activin A, a member of the TGF-β family, is capable of inducing various mesodermal tissues in *Xenopus* blastula animal caps in a concentration-dependent manner (Ariizumi *et al*., 1991a, b). Low doses of activin A induced ventral mesoderm (mesenchyme and coelomic epithelium), while higher doses induced dorsal axial mesoderm (muscle and notochord). However, the response patterns of animal caps from different species to activin A may differ slightly. No clear concentration-dependent effect of activin A is observed in the Japanese newt, *Cynops pyrrhogaster*, and the frequency of mesoderm differentiation (e.g., muscle) is generally lower than in *Xenopus* animal caps (Moriya and Asashima, 1992). On the other hand, marked induction of yolk-rich endodermal tissues is observed in *Cynops* animal caps (Ariizumi and Asashima, 1995b; Ninomiya *et al*., 1998), especially at a high dose of activin A (100 ng/ml).

As shown in Fig. 1, the late blastula animal cap of *Xenopus* is composed of more than one layer (i.e., an outer epithelial cell layer and an inner blastocoelic layer of 2-3 cells thick) whereas that of *Cynops* consists of just one layer. These structural differences in the animal caps may be responsible for their different responses to activin A. Although the Japanese salamanders *Hynobius lichenatus* and *Hynobius nigrescens* are classified as urodeles, the same as *Cynops*, their late blastula or early gastrula animal caps consist of more than one layer, the same as the animal cap of *Xenopus*. In this study, we investigated the response patterns of *Hynobius* animal caps to exposure to activin A and compared them to those of *Xenopus* (Ariizumi *et al*., 1991a) and *Cynops* (Moriya and Asashima, 1992) in relation to the structure of the animal caps.

MATERIALS AND METHODS

Embryo and preoperative treatment

Egg capsules of *H. lichenatus* and *H. nigrescens* were collected in Yamagata Prefecture in the Tohoku district of Japan on April 26, 1998. They were stored at 4°C until the embryos reached the desired stage. *H. lichenatus* embryos were staged according to Sawano (1947), and *H. nigrescens* embryos according to Usui and Hamasaki

* Corresponding author: Tel. +81-3-5454-6632; FAX. +81-3-5454-4330.

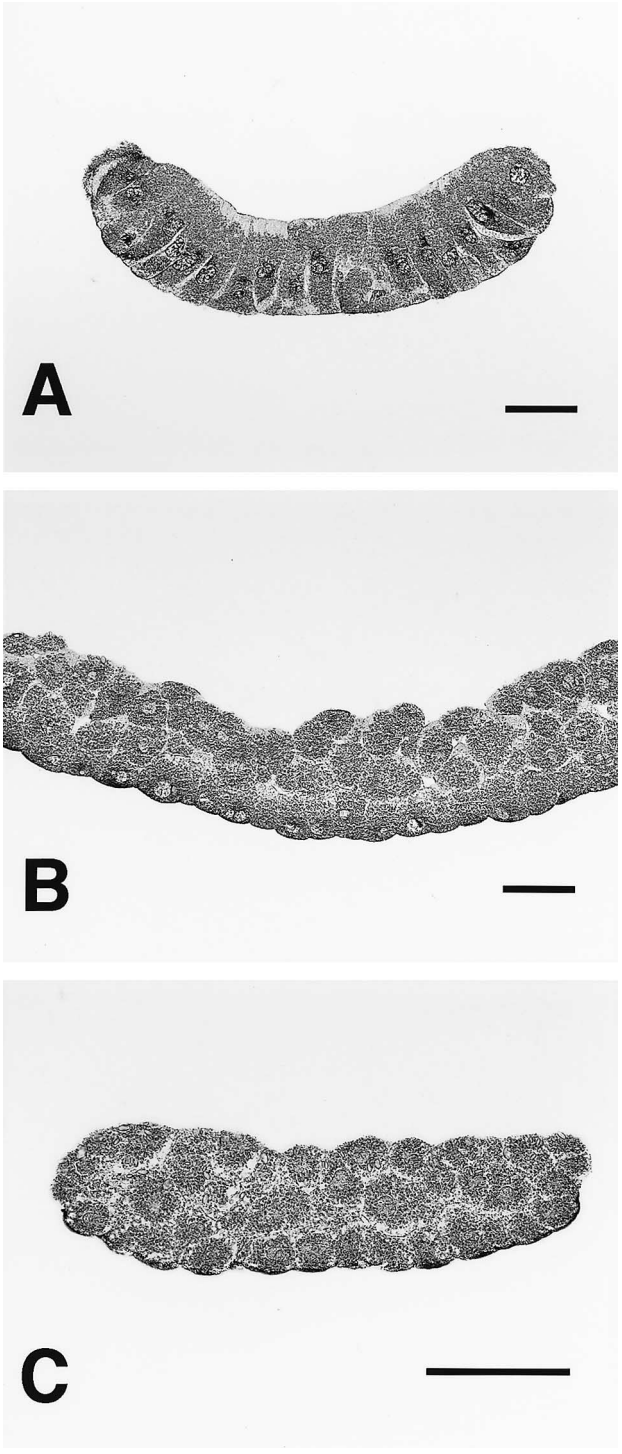

Fig. 1. Histological sections of late blastula animal caps. (**A**) *Cynops pyrrhogaster* (stage 10; Okada and Ichikawa, 1947). (**B**) *Hynobius nigrescens* (stage 10; Usui and Hamasaki, 1939). (**C**) *Xenopus laevis* (stage 9; Nieuwkoop and Faber, 1956). The *Cynops* animal cap is almost a single cell layer, while *Hynobius* and *Xenopus* animal caps consist of more than one layer. Transverse sections were cut 6 μm thick and stained with H/E. The surface cell side is at the bottom. Bar, 100 μm.

(1939). Egg capsules were opened with iridectomy scissors. Embryos were sterilized in 70% ethanol for 1 min and then washed with modified Holtfreter's solution (MHS; 60 mM NaCl, 0.7 mM KCl, 0.9 mM $CaCl_2$, 4.6 mM HEPES, 0.1 g/l kanamycin sulfate, pH 7.6). Jellycoats were chemically removed with MHS containing 1% sodium thioglycolate (pH 9.0).

Operation and culture

Operations were performed under sterile conditions at 10°C. After removing their jellycoats, the embryos were placed in 3% agar-coated Petri dishes filled with MHS. Vitelline membranes were peeled off the embryos with watchmaker's tweezers. The *H. lichenatus* animal cap was dissected from early gastrulae (stage 11; about 3.0 mm in diameter) with tungsten needles. It was 1.8 mm × 1.8 mm in size and contained 5046 ± 120 cells. The *H. nigrescens* animal cap was dissected from late blastulae (stage 10; about 2.6 mm in diameter). It was 1.4 mm × 1.4 mm in size and contained 1668 ± 65 cells. The animal caps were then transferred to the activin A solutions with their surface cell side face down.

Human recombinant activin A was kindly provided by Dr. Y. Eto (Central Research Laboratories, Ajinomoto Co. Inc., Japan) and dissolved in MHS containing 0.1% BSA (A-7888, Sigma, USA) at concentrations of 0, 0.1, 0.5, 1, 5, 10, 50, 100 and 500 ng/ml. The solutions were placed in 96-well plates (SUMILON®; MS-309UR, Sumitomo Bakelite, Japan). The animal cap explants were cultured in the activin A solutions at 10°C for 3 weeks, during which time both the *H. lichenatus* and *H. nigrescens* control embryos reached stage 41.

Histology

Explants were fixed in Bouin's fluid for 12 hr. They were then dehydrated through a graded series of ethanol, cleared in xylene, embedded in paraffin (Histprep 568; Wako, Japan), and cut into 6-μm thick sections. The sections were stained with Delafield's hematoxylin/eosin (H/E).

RESULTS

At the end of the culture period, when the control embryos had reached stage 41 (Fig. 2A), the animal cap explants of *H. lichenatus* exhibited four different patterns of morphological changes depending on the concentration of activin A (Fig. 2B-E). The *H. nigrescens* animal caps generally showed similar patterns. The results of the histological analysis of *H. lichenatus* and *H. nigrescens* explants are summarized in Table 1 and Table 2, respectively.

Control explants cultured without activin A became smaller and very wrinkled (Fig. 2B). They formed atypical epidermis without any other differentiation (Fig. 3A). Similar explants were obtained when treated with 0.1 ng/ml of activin A. At 0.5 ng/ml of activin A, part or all of the explant swelled as a result of absorbing fluid (Fig. 2C). Well-developed epidermis lined with ventral mesoderm (mesenchyme and coelomic epithelium) was found in addition to atypical epidermis (Fig. 3B). Dorsal mesoderm, i.e., notochord and muscle, was also seen in 44% and 38%, respectively, of the *H. lichenatus* explants and in 59% and 24% of the *H. nigrescens* explants. Explants often formed a trunk and tail with fins when cultured in 1 ng/ml of activin A (Fig. 2D). Axial organs such as the notochord, somitic muscle, and the neural tube were organized along the axis (Fig. 3C). Similar explants with axial structures were obtained by treatment with 5 ng/ml of activin A. At 10 ng/ml of activin A,

Table 1. Differentiation of *H. lichenatus* animal caps treated with activin A

Activin A (ng/ml)	0	0.1	0.5	1	5	10	50	100
Total no. of explants	16	16	16	16	16	16	18	17
Negative explants†	16	16	2	0	0	0	0	0
Positive explants‡	0	0	14	16	16	16	18	17
Epidermis	0*	0	63	100	38	13	6	0
Neural tissue	0	0	50	69	81	50	28	0
Ear vesicle	0	0	0	19	13	0	0	0
Notochord	0	0	44	94	100	100	100	35
Muscle	0	0	38	100	100	100	44	0
Mesenchyme	0	0	75	100	25	0	0	0
Coelomic epithelium	0	0	38	19	0	0	0	0
Endodermal tissue	0	0	6	6	13	0	56	100

†Explants with atypical epidermis alone.
‡Explants with any of the tissues listed below.
*Figures are percentages of the total number of explants.

Table 2. Differentiation of *H. nigrescens* animal caps treated with activin A

Activin A (ng/ml)	0	0.1	0.5	1	5	10	50	100	500
Total no. of explants	48	47	46	48	45	51	45	43	18
Negative explants†	48	47	4	1	3	2	0	0	0
Positive explants‡	0	0	42	47	42	49	45	43	18
Epidermis	0*	0	41	69	51	39	36	2	0
Neural tissue	0	0	74	58	82	80	82	51	0
Ear vesicle	0	0	11	15	20	18	9	0	0
Notochord	0	0	59	63	82	77	84	35	0
Muscle	0	0	24	44	76	49	44	21	0
Mesenchyme	0	0	41	65	36	29	31	0	0
Coelomic epithelium	0	0	9	15	9	4	11	0	0
Endodermal tissue	0	0	0	0	29	24	80	100	100

†Explants with atypical epidermis alone.
‡Explants with any of the tissues listed below.
*Figures are percentages of the total number of explants.

notochord and muscle were still induced at high frequencies (100% and 100% in *H. lichenatus* explants; 77% and 49% in *H. nigrescens* explants), however, most of them were not surrounded by epidermis (Fig. 2E, Fig. 3D). At concentrations of activin A higher than 50 ng/ml, yolk-rich endodermal tissue was frequently induced in addition to dorsal mesoderm (Fig. 3E). It occurred in most of the explants treated with 100 ng/ml of activin A (Fig. 3F). No other differentiation besides endodermal tissue could be seen in the *H. nigrescens* explants when incubated in 500 ng/ml of activin A (Table 2). The differentiation pattern of *H. nigrescens* explants was basically the same as that of *H. lichenatus* explants. However, the frequency of neural differentiation tended to be higher, and that of dorsal mesoderm (notochord and muscle) to be slightly lower, than in the *H. lichenatus* explants (Table 1). Although the frequency was relatively low, ventral mesoderm (mesenchyme and coelomic epithelium) was induced by a wider range of concentrations of activin A (0.5-50 ng/ml) in the *H. nigrescens* explants.

DISCUSSION

The array of tissues induced in *Hynobius* animal caps depended largely on the concentration of activin A added to the culture medium. As the concentrations increased, activin A induced ventral mesoderm, then dorsal mesoderm accompanied by neural tissue, and finally yolk-rich endoderm (Table 1 and Table 2). Activin A thus appears to have dose-dependent mesoderm- and endoderm-inducing activity on *Hynobius* animal caps, the same as in other species previously examined (Ariizumi *et al*., 1991a, b; Moriya and Asashima, 1992). Activin A had an effect on *Hynobius* animal caps even at 0.5 ng/ml, a minimum dose similar to that required to induce *Xenopus* and *Cynops* animal caps.

The differentiation pattern of activin-treated *Hynobius* explants, however is slightly different from that of *Xenopus*. In *Xenopus*, various mesodermal tissues, from ventral to dorsal, are induced at high frequencies at clear dose thresholds (Ariizumi *et al*., 1991a). In *Hynobius*, dorsal mesoderm was consistently induced at high frequencies by activin A at a broader range of concentrations (0.5-50 ng/ml). On the other hand, the frequency of ventral mesoderm differentiation was

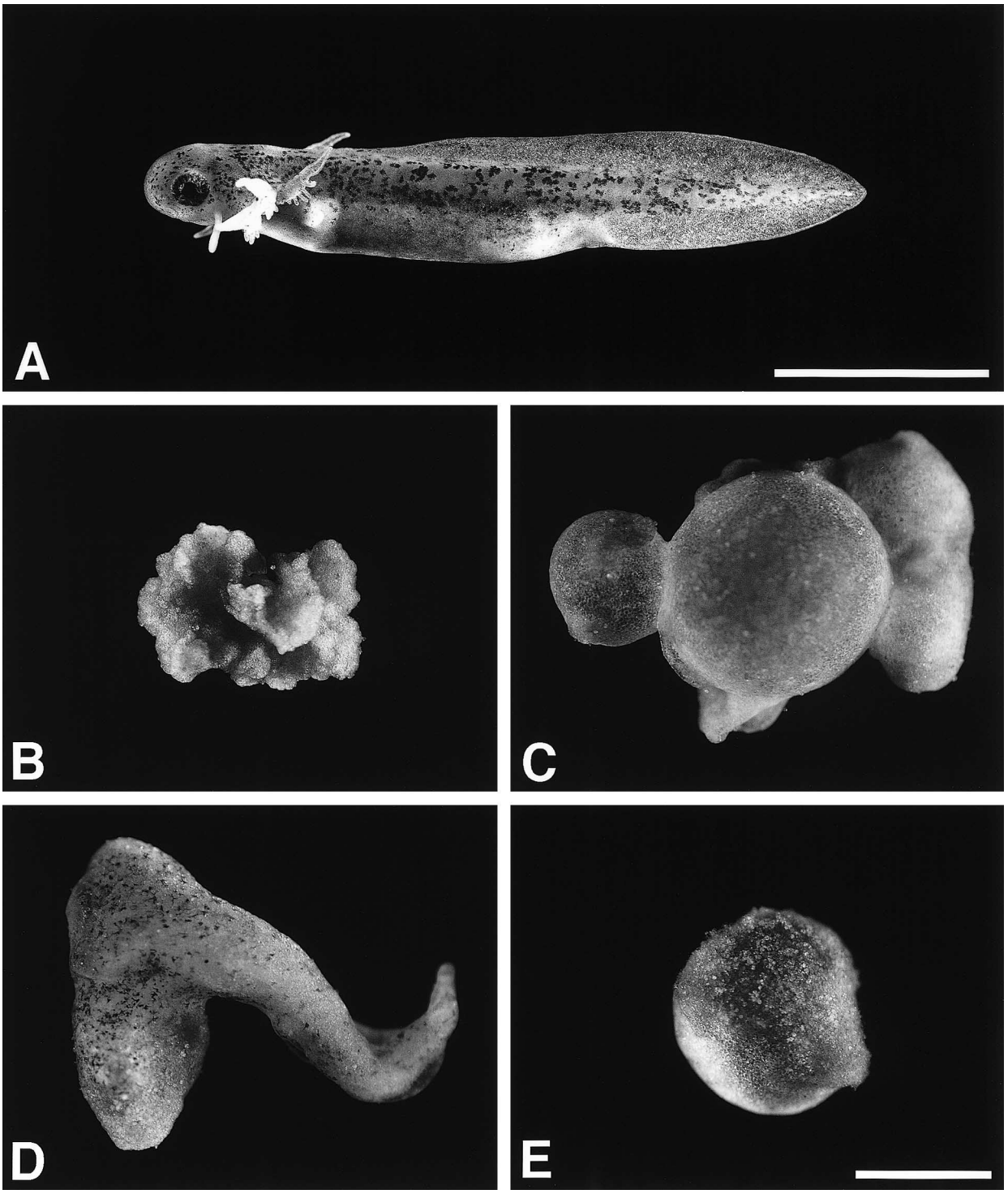

Fig. 2. External views of *H. lichenatus* animal caps treated with various concentrations of activin A. (**A**) Control embryos reached stage 41 after 3 weeks of culture at 10°C. (**B**) Animal caps cultured without activin A became very wrinkled. (**C**) Animal caps cultured in 0.5 ng/ml of activin A solution had smooth surfaces. (**D**) At 1 ng/ml of activin A, explants often formed a trunk and tail. (**E**) At activin A concentrations of 10 ng/ml and above, explants became fragile with a ragged outline. Bar, 5 mm in (**A**) and 1 mm in (**B**)-(**E**).

relatively low (e.g., coelomic epithelium was induced in less than 38% of *H. lichenatus* explants and less than 15% of *H. nigrescens* explants). Furthermore, the concentration range required for ventral mesoderm differentiation largely overlapped the range for dorsal mesoderm differentiation. These results imply that the *Hynobius* animal caps are in a more "dorsalized" state than *Xenopus* animal caps. In *Xenopus*, it is known that animal cap cells on the prospective dorsal side

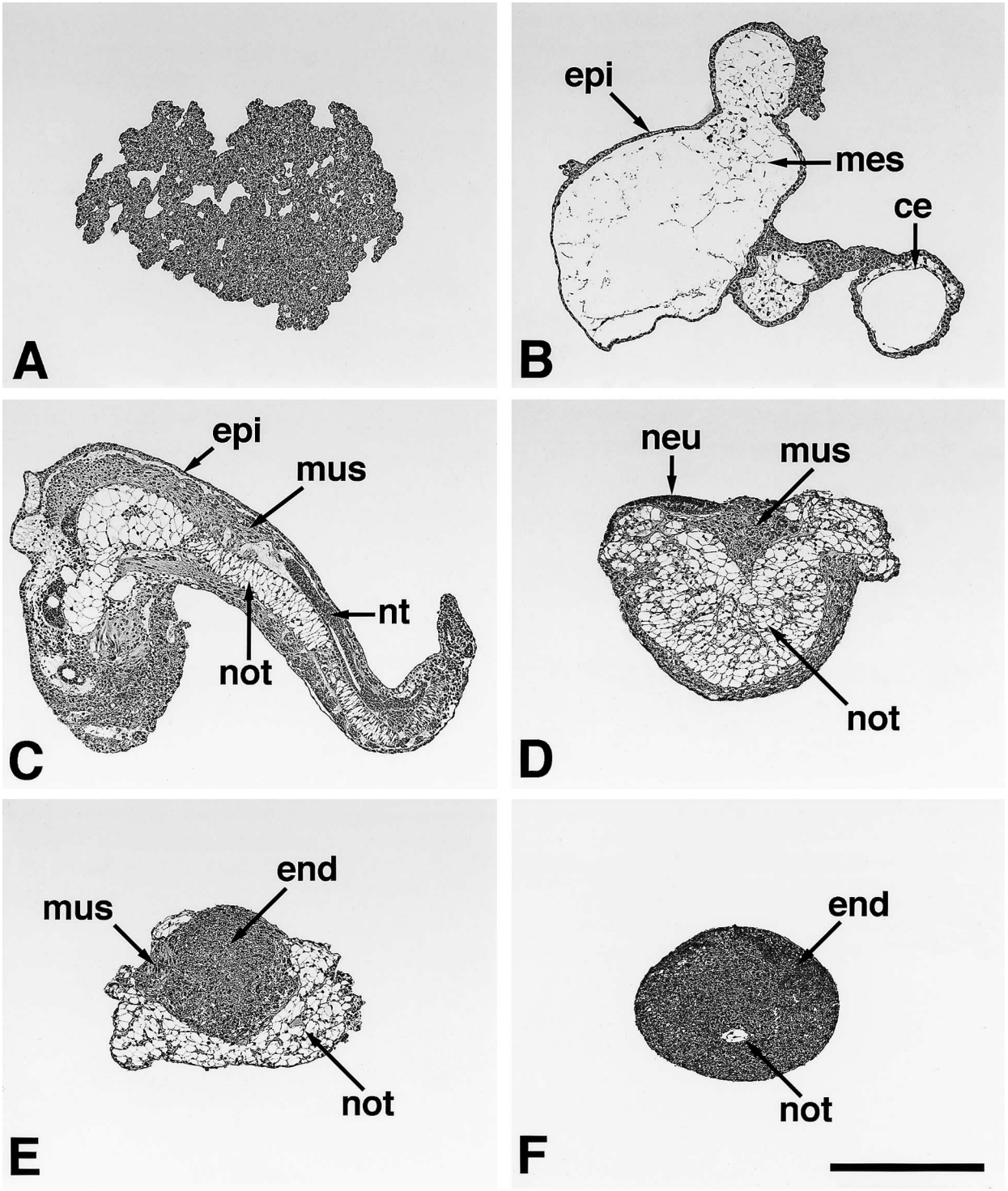

Fig. 3. Histological sections of *H. lichenatus* animal caps treated with various concentrations of activin A. (**A**) Control explants cultured without activin A formed irregular-shaped atypical epidermis. (**B**) Ventral mesoderm (mesenchyme and coelomic epithelium) was found in explants cultured in 0.5 ng/ml of activin A. (**C**) The same explant with trunk and tail structures as shown in Fig. 2D. Axial organs such as notochord, somitic muscle, and neural tube were formed. (**D**) At 10 ng/ml of activin A, a large amount of notochord accompanied by nonspecific neural tissue and muscle was induced in the animal caps. (**E**) At 50 ng/ml of activin A, nonspecific endodermal tissue was induced in addition to notochord and muscle. (**F**) Endodermal tissue was predominant in the 100 ng/ml activin A-treated animal caps. ce, coelomic epithelium; end, nonspecific endodermal tissue; epi, epidermis; mes, mesenchyme; mus, muscle; neu, nonspecific neural tissue; not, notochord; nt, neural tube. Bar, 1 mm.

tend to form dorsal mesoderm when exposed to activin A, whereas cells on the ventral side form ventral mesoderm when they are exposed to the same concentration of activin A (Sokol and Melton, 1991, 1992; Ariizumi and Asashima, unpublished). The high sensitivity of *Hynobius* animal caps to induction of dorsal mesoderm differentiation can be ascribed to their dorsalized state, the same as the prospective dorsal region of *Xenopus* animal caps. This tendency was clear in the *H. lichenatus* animal caps, in which dorsal mesoderm was always induced at high frequencies (Table 1).

The differentiation pattern of *Hynobius* explants is very different from that of *Cynops* explants, in which the frequency of mesoderm differentiation is very low (Moriya and Asashima, 1992). The structural differences in animal caps seem to be related to the mode of their response to activin A. Although the *Cynops* animal cap consists of a single layer of homogeneous cells, *Hynobius* and *Xenopus* animal caps consist of more than one layer containing different types of cells (Fig. 1). Activin A cannot act on the deeper or superficial cells, only on the blastocoelic cells lining the animal cap. The high frequencies of mesoderm differentiation, especially of dorsal mesoderm, in *Hynobius* animal caps thus appear to be due to their heterogeneous structure. We recently confirmed that mesodermal tissues are frequently induced even in the activin-treated *Cynops* animal caps (Ariizumi *et al*., 1999). The degree of mesoderm differentiation depended on the size of the animal caps. As the size of animal caps increased, more types of mesodermal tissue were induced in addition to endodermal tissue. Furthermore, a well-organized axial structure composed of dorsal mesoderm and a central nervous system was frequently induced in the "heterogeneous" explants in which activin-treated animal caps (fated to form endoderm) were combined with untreated animal caps. These findings suggest that further inductive interactions between induced and non-induced cells should be considered when the animal cap assay is performed with heterogeneous animal caps such as those of *Xenopus*, *Ambystoma*, and *Hynobius*.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, and by CREST (Core Research for Evolutional Science and Technology) of the Japan Science and Technology Corporation.

REFERENCES

Ariizumi T, Moriya N, Uchiyama H, Asashima M (1991a) Concentration-dependent inducing activity of activin A. Roux's Arch Dev Biol 200: 230–233

Ariizumi T, Sawamura K, Uchiyama H, Asashima M (1991b) Dose and time-dependent mesoderm induction and outgrowth formation by activin A in *Xenopus laevis*. Int J Dev Biol 35: 407–414

Ariizumi T, Asashima M (1995a) Control of the embryonic body plan by activin during amphibian development. Zool Sci 12: 509–521

Ariizumi T, Asashima M (1995b) Head and trunk-tail organizing effects of the gastrula ectoderm of *Cynops pyrrhogaster* after treatment with activin A. Roux's Arch Dev Biol 204: 427–435

Ariizumi T, Komazaki S, Asashima M (1999) Activin-treated urodele animal caps: II. Inductive interactions in newt animal caps after treatment with activin A. Zool Sci 16, in press

Asashima M (1994) Mesoderm induction during early amphibian development. Dev Growth Differ 36: 343–355

Klein PS, Melton DA (1994) Hormonal regulation of embryogenesis: The formation of mesoderm in *Xenopus laevis*. Endocrine Rev 15: 326–341

Moriya N, Asashima M (1992) Mesoderm and neural inductions on newt ectoderm by activin A. Dev Growth Differ 34: 589–594

Nieuwkoop PD, Faber J (1956) Normal table of *Xenopus laevis* (Daudin). North-Holland Publishing Company, Amsterdam

Ninomiya H, Ariizumi T, Asashima M (1998) Activin-treated ectoderm has complete organizing center activity in *Cynops* embryos. Dev Growth Differ 40: 199–208

Okada YK, Ichikawa M (1947) Normal stages of development of the Japanese newt, *Triturus pyrrhogaster* (Boie). Jpn J Exp Morphol 3: 1–6 (in Japanese)

Sawano J (1947) Normal stages of development of the Japanese salamander, *Hynobius lichenatus* (Boulenger). Gurusu-bunko, Sapporo (in Japanese)

Sokol S, Melton DA (1991) Pre-existent pattern in *Xenopus* animal pole cells revealed by induction with activin. Nature 351: 409–411

Sokol S, Melton DA (1992) Interaction of Wnt and activin in dorsal mesoderm induction in *Xenopus*. Dev Biol 154: 348–355

Usui M, Hamasaki M (1939) Tafeln zur Entwicklungsgeschichte von *Hynobius nigrescens* (Stejneger). Zool Mag 51: 195–206 (in Japanese)

Yamada T, Takata K (1961) A technique for testing macromolecular samples in solution for morphogenetic effects on the isolated ectoderm of the amphibian gastrula. Dev Biol 3: 411–423

(Received July 13, 1998 / Accepted September 1, 1998)