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In vitro Control of Embryonic Axis Formation by Activin A, Concanavalin A, and Retinoic Acid in *Xenopus laevis*

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ABSTRACT—We have demonstrated that the tissue differentiation patterns along the dorsoventral and anteroposterior axes can be controlled by a combination of activin A, concanavalin A (Con A), and retinoic acid. *Xenopus* blastula animal caps, normally fated to form epidermal tissues, differentiated into ventral mesoderm tissues such as coelomic epithelium and blood-like cells following treatment with activin A (0.5 ng/ml). Dorsal mesoderm tissues like muscle and notochord, were induced by graded addition of Con A. Conversely, Con A (1 mg/ml) induced anterior neural tissues, forebrain accompanied by eyes and cement glands, in the animal caps. Posterior neural tissues, hindbrain with ear vesicles and spinal cord, were induced by graded addition of activin A. Retinoic acid was also capable of shifting the Con A-induced anterior neural tissues to more posterior tissue phenotypes, however, its caudalizing activity was slightly different from that of activin A. These results suggest that the concentration gradients of these three factors can regulate the differentiation patterns along the embryonic axes. The present study provides a suitable test system for analyzing the establishment of the fundamental body plan in early vertebrate development.

INTRODUCTION

The establishment of the embryonic axes during vertebrate development has been widely studied in amphibians. After the discovery of Spemann's organizer (Spemann and Mangold, 1924), many investigators grappled with analysis of axis formation (reviewed by Sasai and De Robertis, 1997). As a result of these attempts, several models based on concentration gradients of inducer molecules were proposed. In the "two-gradient hypothesis" proposed by Toivonen and Saxén (1955), concentration gradients of two distinct agents, a neuralizing agent (N) and a mesodermalizing agent (M), were postulated to occur in early amphibian embryos. According to this hypothesis, each region of the embryo has a different set of concentrations of the two agents, which leads to the establishment of anteroposterior (A-P) and dorsoventral (D-V) axes. In A-P patterning, anterior neural tissues such as the forebrain, the eye, and the nose are induced by the N agent alone, while varying N/M ratios determine the fate of posterior neural tissues (hindbrain and spinal cord). Another appealing model for A-P specification of the central nervous system (CNS) was proposed by Nieuwkoop and his colleague (1952). In the "two-signal model", the anterior CNS is induced by the first signal (activator) emanating from the dorsal mesoderm. It may be shifted towards more posterior CNS under the influence of a second signal (transformer) released by the axial mesoderm.

Recently, several factors have been identified as candidates for these agents or signals. Noggin, chordin, and follistatin are the most likely candidates for the N agent or the activator. They are secreted by the dorsal mesoderm before and during gastrulation, and can regulate neural induction and dorsalization of ventral mesoderm explants like Spemann's organizer (Lamb *et al*., 1993; Hemmati-Brivanlou *et al*., 1994; Sasai *et al*., 1995, 1996; Piccolo *et al*., 1996; Zimmerman *et al*., 1996). Although concanavalin A (Con A) is not an endogenous factor, it also induces anterior neural tissues in animal cap explants (Takata *et al*., 1981, 1984; Grunz, 1985) and dorsalizes ventral mesoderm explants in *Cynops* embryos (Diaz *et al*., 1990). One of the candidates for the M agent, activin, is known to induce mesodermal tissues along the D-V axis in a dose-dependent manner (Asashima *et al*., 1990; Green and Smith, 1990; Ariizumi *et al*., 1991), however, its role in A-P patterning remains unclear. On the other hand, basic fibroblast growth factor (bFGF; Kimelman and Kirschner, 1987; Slack *et al*., 1987; Cox and Hemmati-Brivanlou, 1995; Kengaku and Okamoto, 1995; Lamb and Harland, 1995), Wnt3A (McGrew *et al*., 1995) and retinoic acid (RA; Durston *et al*., 1989; Ruiz i Altaba and Jessell, 1991; Lopez and Carrasco, 1992) are considered candidates for the "transformer''. Recent molecular studies have revealed that these molecules can enhance the expression of posterior markers

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in anterior tissues. These findings suggest that certain factors regulate the establishment of embryonic axes. However, most of the experiments were carried out using whole embryos or regions that can differentiate by themselves. Further analysis of the patterning of tissue differentiation along the body axes will require a simpler experiment system.

In this study, we employed *Xenopus* animal caps, which are fated to become atypical epidermis unless influenced by inducing factors. They were treated with various ratios of concentrations of activin A, Con A, and RA to examine whether cell and tissue differentiation along the D-V and A-P axes could be controlled by concentration gradients of these factors *in vitro*. First, the dorsalizing activity of Con A on the activin-induced ventral mesoderm was examined (series I), and second, the caudalizing activities of activin A and RA on the Con A-induced anterior neural tissues were compared (series II and III). The results provide a simple system suitable for analysis of pattern formation along the embryonic axes *in vitro*.

MATERIALS AND METHODS

Eggs and embryos

Xenopus laevis eggs were obtained by injecting the dorsal lymph sacs of males and females with 600 IU of hCG (GESTRON; Denka Seiyaku, Japan). Late blastulae (stage 9; Nieuwkoop and Faber, 1956) were dejellied with 4.5% cysteine hydrochloride (pH 7.8) dissolved in Steinberg's solution (SS; 58.00 mM NaCl, 0.67 mM KCl, 0.34 mM $Ca(NO_3)_2$, 0.83 mM $MgSO_4$, 3.00 mM HEPES and 100 mg/l kanamycin sulfate, pH 7.4). The vitelline membranes were manually removed with a pair of watchmaker's tweezers.

Test solutions

Human recombinant activin A (kindly provided by Dr. Y. Eto, Central Research Laboratories of Ajinomoto Co. Inc., Kawasaki, Japan) and concanavalin A (Con A; L-1104, E-Y LABS, USA) were dissolved in SS. Bovine serum albumin (BSA; A-7888, Sigma, USA) was added to SS to a final concentration of 1 mg/ml to prevent adsorption of activin A and Con A onto the plastic surfaces. Retinoic acid (RA; R2625, Sigma, USA) was dissolved in absolute ethanol at a concentration of 10^{-2} M. These three solutions were mixed as follows (final concentrations):

Series I	activin A:	0.1, 0.5 ng/ml
	Con A:	0, 50, 100 and 300 μg/ml
Series II	Con A:	0, 500 μg/ml
	activin A:	0, 0.5 and 1 ng/ml
Series III	Con A:	1 mg/ml
	RA:	0, 10^{-8}, 5×10^{-8}, 10^{-7}, 5×10^{-7}, 10^{-6}, 5×10^{-6}, 10^{-5} and 10^{-4} M

Surgical operation and histology

Animal caps dissected from late blastulae (stage 9) to a size of 0.5 mm × 0.5 mm were treated with the test solutions for 3 hr at 20°C. After two washes with SS, they were cultured for 4 days at 20°C in

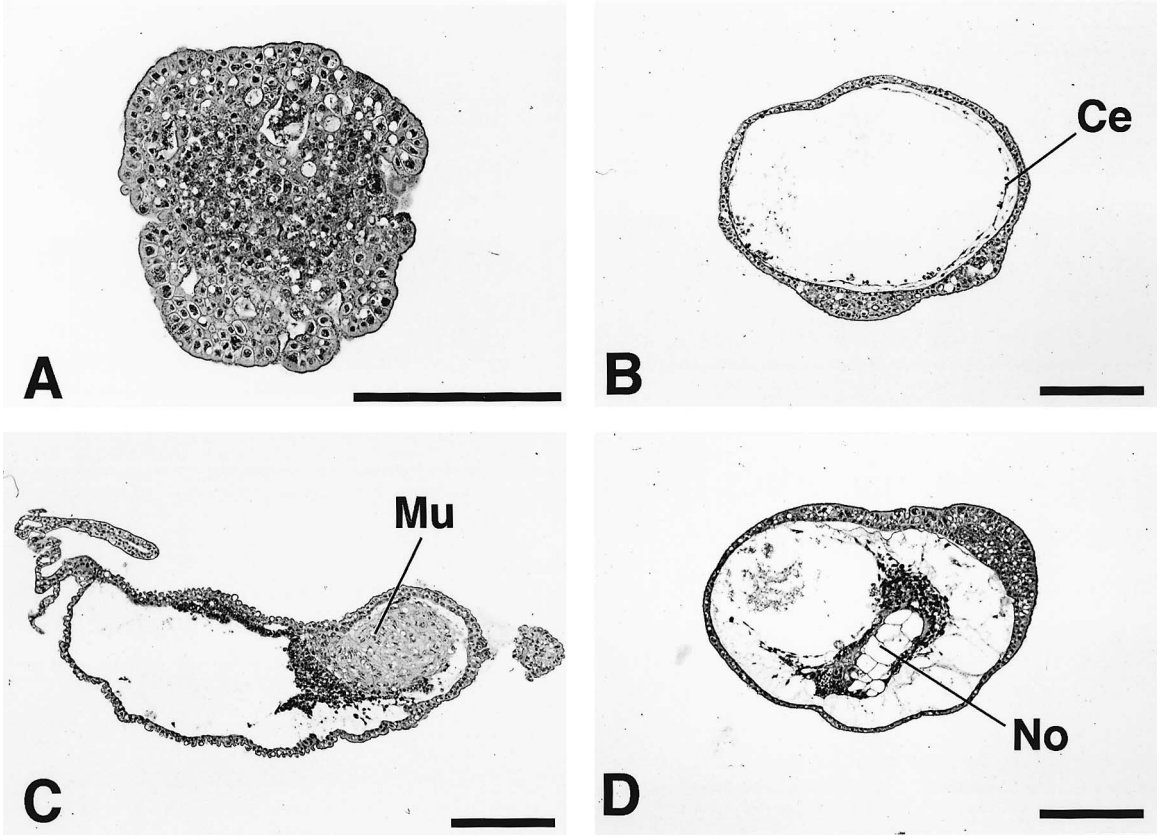

Fig. 1. Con A dorsalized activin A-induced ventral mesoderm. (**A**) Control explant cultured without activin A and Con A. (**B**) Activin A at 0.5 ng/ml induced ventral mesoderm such as coelomic epithelium (Ce). (**C**) Activin A (0.5 ng/ml) with Con A (50 μg/ml) induced a mass of muscle cells (Mu). (**D**) Activin A (0.5 ng/ml) with Con A (100 μg/ml) induced notochord (No). Bars: 200 μm.

SS. The animal cap explants were fixed with Bouin's fluid for 3 hr. After dehydration, they were embedded in paraffin and cut into 6 μm sections. The sections were then stained with hematoxylin and eosin.

RT-PCR analysis

Extraction of total RNA, RT-PCR reactions, and subcloning and sequencing of RT-PCR fragments were performed as previously described, with one modification, the RT-PCR reaction was carried out in a 50 μl volume (Yokota *et al*., 1998). N-CAM (Kintner and Melton, 1987), XAG1 (Sive *et al*., 1989), Xotx2 (Pannese *et al*., 1995), En2 (Hemmati-Brivanlou *et al*., 1991), Krox20 (Bradley *et al*., 1993), XlHbox 6 (Wright *et al*., 1990), alpha skeletal muscle actin (muscle-specific actin; ms-actin; Stutz and Spohr, 1986) and EF1-α (Kreig *et al*., 1989) were analyzed as previously described (Hemmati-Brivanlou and Melton, 1994; Hatada *et al*., 1995; Lai *et al*., 1995; Sasai *et al*., 1995).

RESULTS

Series I: Con A dorsalizes activin A-induced ventral mesoderm

Control animal caps, incubated in SS without activin A and Con A, formed atypical epidermis (Fig. 1A). Ventral mesoderm tissues such as the coelomic epithelium and blood-like cells (77%) were induced by 0.5 ng/ml of activin A (Fig. 1B and Table 1). These tissues were often accompanied by small blocks of muscle cells (80%), and all explants were surrounded by the epidermis (100%). As the concentrations of Con A added to SS plus 0.5 ng/ml activin A increased (50-300 μg/ml), increasingly dorsal types of mesoderm were induced in a concentration-dependent manner. Addition of 50 μg/ml of Con A markedly reduced the frequency of ventral mesoderm (24%). The frequency of muscle remained high (90%), and well-developed muscle cells were seen in the explants (Fig. 1C). The most dorsal mesoderm, the notochord, was also induced at a high frequency (83%). At 100 μg/ml of Con A, the frequency of muscle formation was decreased (60%), while that of notochord formation remained high (87%) (Fig. 1D). At 300 μg/ml of Con A, no muscle was induced, and the frequency of notochord formation was reduced (50%). The frequencies of neural tissue formation were concentration-dependently increased by Con A and reached a plateau (97%) at 100 μg/ml. No endodermal tissues were induced in any specimens.

Activin A at 0.1 ng/ml also induced ventral mesodermal tissues in the animal caps. However, the frequencies were relatively low (30%), and most explants remained undifferentiated (atypical epidermis, 70%). The dorsalizing effects of Con A on the mesoderm could not be seen in these explants, except for a reduction in the frequency of ventral mesoderm. Neural tissues and a cement gland were induced by the addi-

Table 1. Differentiation of animal caps treated with activin A plus Con A (series I)

activin A [ng/ml]	0.1				0.5			
Con A [μg/ml]	0	50	100	300	0	50	100	300
Number of specimens	10	9	10	10	30	29	30	30
Atypical epidermis	7 (70)	6 (67)	9 (90)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Epidermis	3 (30)	1 (11)	0 (0)	4 (40)	30 (100)	27 (93)	29 (97)	24 (80)
Cement gland	0 (0)	0 (0)	5 (50)	4 (40)	0 (0)	0 (0)	0 (0)	0 (0)
Eye or eye fragments	0 (0)	0 (0)	0 (0)	2 (20)	0 (0)	0 (0)	0 (0)	0 (0)
Ear vesicle	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	4 (14)	6 (20)	7 (23)
Neural tissues	0 (0)	2 (22)	1 (10)	10 (100)	6 (20)	26 (90)	29 (97)	29 (97)
Notochord	0 (0)	0 (0)	0 (0)	0 (0)	2 (7)	24 (83)	26 (87)	15 (50)
Muscle	0 (0)	0 (0)	0 (0)	0 (0)	24 (80)	26 (90)	18 (60)	0 (0)
Pronephric tubules	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Ventral mesoderm	3 (30)	0 (0)	0 (0)	0 (0)	23 (77)	7 (24)	3 (10)	5 (17)

Figures in parentheses indicate the percentage of the number of specimens.

Table 2. Differentiation of animal caps treated with Con A plus activin A (series II)

Con A [μg/ml]	0			500		
activin A [ng/ml]	0	0.5	1	0	0.5	1
Number of specimens	19	19	19	40	19	19
Atypical epidermis	19 (100)	13 (68)	1 (5)	2 (5)	1 (5)	1 (5)
Epidermis	0 (0)	6 (32)	17 (89)	34 (85)	18 (95)	18 (95)
Cement gland	2 (11)	0 (0)	1 (5)	37 (93)	11 (58)	1 (5)
Eye or eye fragments	0 (0)	0 (0)	0 (0)	7 (18)	4 (21)	0 (0)
Ear vesicle	0 (0)	0 (0)	1 (5)	0 (0)	2 (11)	1 (5)
Neural tissues	0 (0)	0 (0)	12 (63)	38 (95)	18 (95)	18 (95)
Notochord	0 (0)	0 (0)	3 (16)	0 (0)	6 (32)	17 (89)
Muscle	0 (0)	2 (11)	18 (95)	0 (0)	0 (0)	0 (0)
Pronephric tubules	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Ventral mesoderm	0 (0)	6 (32)	4 (21)	0 (0)	0 (0)	1 (5)

Figures in parentheses indicate the percentage of the number of specimens.

tion of Con A (Table 1).

Series II: Activin A caudalizes Con A-induced anterior neural tissues

Neural tissues and a cement gland were induced by 500 μg/ml of Con A at high frequencies (95% and 93%, respectively, Table 2). The neural tissue exhibited a forebrain-like appearance in histological sections (Fig. 2A) and was accompanied by eyes or eye fragments in some specimens (18%). As the concentrations of activin A added to Con A (500 μg/ml) increased (0.5-1 ng/ml), the frequency of cement gland formation decreased, while that of notochord formation increased in a concentration-dependent manner. When activin A (0.5 ng/ml) was added, ear vesicles were induced (11%), but they were not induced by Con A alone. At a high concentration of activin A (1 ng/ml), the eye and eye fragments failed to develop. Although neural tissues were always induced at high frequencies (95% in each combination), the types of neural tissues changed from forebrain-type to spinal cord-type depending on the concentration of activin A (Fig. 2E).

Series III: RA also caudalizes Con A-induced anterior neural tissues

Neural tissues were induced at every combination of concentrations of Con A and RA (Table 3). Animal caps treated with 1 mg/ml of Con A solely differentiated into the forebrain accompanied by eyes (tapetum with or without lens, 29%) and a cement gland (95%). The addition of a low concentration of RA (10^{-8} M) to the Con A solution increased the frequencies of formation of eyes or eye fragments (45%) (Fig. 2B). At a higher concentration of RA (10^{-7} M), differentiation of both the cement gland and the eye were decreased (45% and 0%, respectively). A hindbrain with ear vesicles was induced (38%) when 10^{-6} M of RA was added to the solution (Fig. 2C). As the RA concentrations increased further (10^{-5} and 10^{-4} M), the frequencies of ear vesicle formation gradually diminished. Although neural tissues were frequently induced at higher concentrations of RA, they did not exhibit any of the characteristics found in the spinal cord of normal embryos (Fig. 2D).

RT-PCR analysis was performed to confirm that RA treatment affected patterns of gene expression in the explants.

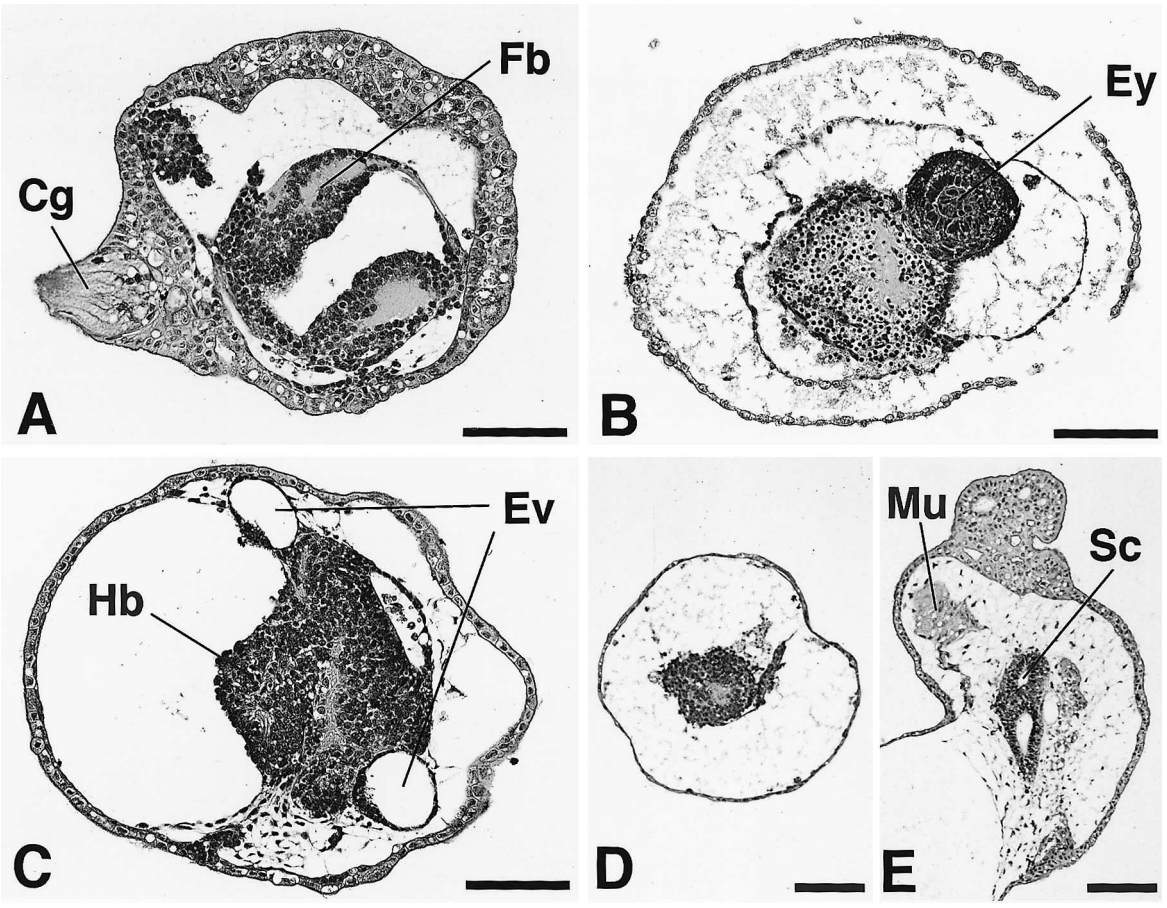

Fig. 2. Activin A and RA caudalized Con A-induced anterior neural tissue. (**A**) Con A (0.5-1 mg/ml) alone induced forebrain (Fb) accompanied by cement gland (Cg). (**B**) Con A (1 mg/ml) with a low concentration of RA (10^{-8} M) induced forebrain with an eye (Ey). The explants treated with Con A and a low concentration of activin A (0.5 ng/ml) also differentiated into these structures. (**C**) Con A (1 mg/ml) with a middle concentration of RA (10^{-6} M) induced hindbrain (Hb) with ear vesicles (Ev). The explants treated with Con A and higher concentrations of activin A (0.5-1 ng/ml) also differentiated into these structures. (**D**) A high concentration of RA (10^{-4} M) with Con A induced nonspecific neural tissue. (**E**) A high concentration of activin A (1 ng/ml) with Con A induced spinal cord (Sc). Mu: muscle. Bars: 100 μm.

Table 3. Differentiation of animal caps treated with Con A plus RA (series III)

Con A [mg/ml]	1								
RA [M]	0	10^{-8}	5×10^{-8}	10^{-7}	5×10^{-7}	10^{-6}	5×10^{-6}	10^{-5}	10^{-4}
Number of specimens	21	11	11	11	11	21	12	20	11
Atypical epidermis	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Epidermis	21 (100)	10 (91)	10 (91)	10 (91)	10 (91)	19 (90)	12 (100)	17 (85)	3 (27)
Cement gland	20 (95)	10 (91)	8 (73)	5 (45)	3 (27)	0 (0)	0 (0)	0 (0)	0 (0)
Eye or eye fragments	6 (29)	5 (45)	1 (9)	0 (0)	0 (0)	1 (5)	0 (0)	0 (0)	0 (0)
Ear vesicle	0 (0)	0 (0)	0 (0)	0 (0)	1 (9)	8 (38)	4 (33)	4 (20)	0 (0)
Neural tissues	21 (100)	11 (100)	11 (100)	11 (100)	11 (100)	21 (100)	12 (100)	20 (100)	11 (100)
Notochord	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Muscle	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Pronephric tubules	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Ventral mesoderm	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Figures in parentheses indicate the percentage of the number of specimens.

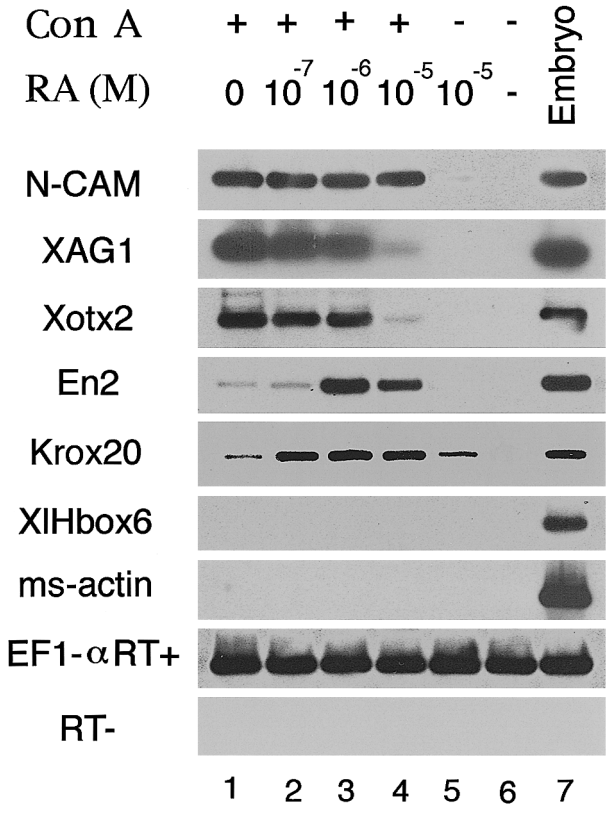

Fig. 3. RA caudalized gene expression patterns induced by Con A. Ectoderm at stage 9 was treated with Con A and RA for 3 hr, cultured for 4 days, and subjected to the RT-PCR analysis. Con A alone or with RA at low concentrations induced the anterior markers, XAG1 (cement gland) and Xotx2 (forebrain) (lanes 1-3). The addition of RA reduced the expression of these anterior markers and enhanced posterior markers, En2 (midbrain-hindbrain boundary) and Krox20 (hindbrain rhombomeres 3 and 5) (lanes 1-4). The most posterior marker, XlHbox 6 (spinal cord), and ms-actin (muscle) were not induced by Con A and RA (lanes 1-5). EF1-α RT+ is a loading control and RT- is a negative control. Lanes 1-4: Con A (1 mg/ml) plus RA (0, 10^{-7}, 10^{-6}, 10^{-5} M). Lane 5: RA (10^{-5} M) alone. Lane 6: control. Lane 7: whole embryo.

Con A (1 mg/ml) induced expression of the panneural marker, N-CAM, regardless of the dose of RA added (Fig. 3, lanes 1-4). The anterior markers, XAG1 and Xotx2, were strongly induced by treatment with Con A alone (Fig. 3, lane 1). Expression of these markers was reduced by graded addition of RA, while expression of the posterior neural markers, En2 and Krox20, was enhanced by high concentrations of RA (Fig. 3, lanes 1-4). En2 expression was most strongly induced by 10^{-6} M of RA (Fig. 3, lane 3). XlHbox 6 and ms-actin, the most posterior neural and mesodermal markers, respectively, were not induced by treatment with Con A and RA (Fig. 3, lanes 1-5). Control explants did not express any markers tested (Fig. 3, lane 6). Although explants treated with RA alone expressed Krox20, the expression was very weak as compared with the explants treated with both Con A and RA (Fig. 3, lanes 4, 5).

DISCUSSION

Dorsalizing activity of anterior neural inducers

Con A was originally identified as a neural inducer in isolated *Cynops* (Takata *et al*., 1981, 1984) and *Xenopus* (Grunz, 1985) animal caps. Neural tissues induced in animal caps have characteristics of the forebrain of normal embryos. The same molecule was later found to dorsalize *Cynops* ventral marginal zone (VMZ) explants. The VMZ explants differentiated into blood cells, pronephros, muscle, and notochord depending on the concentrations of Con A added (Diaz *et al*., 1990). In the present study, the activin-treated *Xenopus* animal caps fated to differentiate into ventral mesoderm were also dorsalized by the addition of Con A (series I). Other potent neural inducers, noggin, chordin, and follistatin, induce the expression of panneural marker and anterior neural marker genes in the animal caps (Lamb *et al*., 1993; Hemmati-Brivanlou *et al*., 1994; Sasai *et al*., 1995). Noggin and chordin enhance the expression of muscle actin in *Xenopus* VMZ explants (Lamb *et al*., 1993; Piccolo *et al*., 1996). The VMZ explants isolated from follistatin-mRNA-injected embryos exhibit an elongation movement that mimics the convergent extension of the dorsal mesoderm during gastrulation (Sasai *et al*.,

1995). These findings suggest that the anterior neural inducers may also act as dorsalizing factors on the ventral mesoderm.

The bone morphogenetic protein (BMP) signal ventralizes the mesoderm and inhibits the formation of dorsal mesoderm, Spemann's organizer, in early *Xenopus* embryos (Dale *et al*., 1992; Jones *et al*., 1992; Re'em-Kalma *et al*., 1995; Schmidt *et al*., 1995). This signal also acts as an anti-neuralizing agent on the ectoderm (Sasai *et al*., 1995). The neuralizing/dorsalizing activities of noggin, chordin, and follistatin may result from inhibition of the BMP signal on the ectoderm/mesoderm. Chordin and noggin are known to bind to BMP-4 directly and inhibit the anti-neuralization and ventralization mediated by the BMP signal (Sasai *et al.,* 1995; Piccolo *et al*., 1996; Zimmerman *et al*., 1996). Although it is unknown whether Con A can bind to BMP, Con A has been reported to bind to the subunit of the Ca^{2+} channel and increase intracellular Ca^{2+} concentration in ectoderm explants. When the increase in Ca^{2+} was inhibited, neural induction did not occur (Moreau *et al*., 1994). Thus, there may be a close connection between the concentration of Ca^{2+} and neural differentiation. Although Con A is not a natural neural inducer, it is a useful reagent for investigating the mechanisms of neuralization and dorsalization. It can induce well-differentiated neural tissue similar to that of the normal embryo, and it possesses neuralizing and dorsalizing activity similar to the natural neural inducers.

Caudalizing activity of activin A and RA

As described in previous reports (Takata *et al*., 1981, 1984; Grunz, 1985), Con A-treated animal caps differentiated into anterior neural tissues such as the forebrain and the eye. The regional specification of these neural tissues was shifted posteriorly by the addition of activin A (series II). Activin A itself induces a variety of mesodermal tissues along the D-V axis in a concentration-dependent manner (Green and Smith, 1990; Ariizumi *et al*., 1991). In this study, a low concentration of activin A, insufficient to induce dorsal mesoderm (muscle and notochord) alone, induced these tissues when Con A was added (series I). Takaya (1977) reported that the A-P patterning of the CNS in *Cynops* embryos is affected by the quality and quantity of surrounding mesodermal tissues. Formation of the forebrain is always accompanied by a small amount of mesenchyme, while the hindbrain and the spinal cord are formed in the presence of the notochord and somitic muscle. In the present study, differentiation of the ear vesicle and the spinal cord was always accompanied by notochord formation. It has been reported that mesodermalized ectoderm induces neural tissues as a secondary induction (Suzuki *et al*., 1986). It is likely that the caudalization of Con A-induced anterior CNS is caused by the dorsal mesoderm initially induced by activin A and Con A. The signal released by activin A / Con A-induced dorsal mesoderm is unknown at present. However, several candidate caudalizing agents have been reported. bFGF can modify the patterns of gene expression from the anterior to posterior type in animal caps fated to form anterior neural tissues by treatment with noggin or cell-dissociation (Cox and Hemmati-Brivanlou, 1995; Kengaku and Okamoto, 1995; Lamb and Harland, 1995). Wnt3A and β-catenin also enhance the expression of posterior marker genes in anterior neural tissue induced in the animal cap explants (McGrew *et al*., 1995).

Another candidate caudalizing signal, RA, affects the A-P patterning of the CNS. RA and its receptor are localized in the posterior region of *Xenopus* embryos (Ellinger-Ziegelbauer and Dreyer, 1991; Lopez and Carrasco, 1992; Chen *et al*., 1994). RA-treated *Xenopus* embryos have reduced anterior neural structures, as RA transforms the CNS from anterior to the posterior type (Ruiz i Altaba and Jessel, 1991; Lopez and Carrasco, 1992). However, the degree of caudalizing effect of RA is slightly different from that of the mesoderm inducers, as shown above. In the present study, RA led the Con A-induced anterior neural tissue to form hindbrain with ear vesicles. Recently, it was reported that hindbrain is the most sensitive region of the CNS to RA (Kolm *et al*., 1997; van der Wees *et al*., 1998). The most posterior CNS region, the spinal cord, was not induced even when the RA concentration was increased up to 10^{-4} M (series III). Spinal cord formation may require additional factor(s) such as bFGF and Wnt3A. In addition to the caudalizing activity, we have reported a lateralizing activity of RA on activin-induced dorsal mesoderm (Moriya *et al*., 1993). Therefore, RA may be capable of modifying the differentiation of neural and mesodermal tissues toward the posterior and lateral sides *in vitro*.

We have demonstrated that three factors, activin A, Con A, and RA, can modify inductive activity of each other on the animal cap cells and that these modifications are concentration-dependent. These results provide a simple and suitable system for analysis of the establishment of the fundamental body plan *in vitro*.

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