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Original Article

Dose and time-dependent mesoderm induction and outgrowth formation by activin A in *Xenopus laevis*

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ABSTRACT We examined the quality of mesoderm induced by the action of activin A on the *Xenopus* presumptive ectoderm when various concentrations and treatment times were employed. The minimum concentration of activin A to induce mesodermal tissues was inversely proportional to its treatment time. The explants differentiated into different types of mesodermal tissues, from ventral-type to dorsal-type depending on the concentration of activin A and its treatment time. To confirm whether activin A has a role in establishing axial organization, activin A was injected into the blastocoel of late blastulae. About 70% of the injected embryos formed secondary tail-shaped outgrowths in which muscle and neural tube differentiated. The amount of activin A to form secondary outgrowths was 0.5-2.5 pg, roughly consistent with the amount estimated from *in vitro* experiments. As we have detected almost the same amount of activin homologue in the early embryos (Asashima *et al.*, 1991a), we speculate that activin A may be the natural mesodermal inducer, and that it is responsible for establishing axial organization in the *Xenopus* embryo.

KEY WORDS: *activin A, mesoderm induction, dorso-ventral axis, microinjection, Xenopus laevis*

Introduction

In amphibian development various tissues and organs differentiate from the fertilized egg. One of the most important research projects in contemporary developmental biology is the elucidation of the mechanisms of morphogenesis and cell differentiation comprising this process. Following the discovery of the organizer by Spemann and Mangold (1924), many investigators grappled with this problem. It has, however, been difficult to approach and elucidate the phenomenon at the molecular level, because of the complexity of the process and insufficiency of the methods for analysis. In contrast, substantial information on morphological changes has accumulated.

Early amphibian development was at first considered as a chain of inductions originating from the organizer. However, the presumptive endoderm at very early stages was discovered to precede organizer action and induce mesoderm when combined with the presumptive ectoderm (Nieuwkoop, 1969; Nakamura *et al.*, 1971; Asashima, 1975). Further, Nieuwkoop and Ubbels (1972) found that the dorsal endoderm of *Xenopus* blastula induced notochord and muscle, whereas the ventral and lateral endoderm preferentially induced blood cells and pronephric tubules. There also exists a regional difference within the presumptive endoderm at earlier stages; *i.e.*, at the 64-cell stage (Gimlich and Gerhart, 1984), the 32-cell stage (Dale and Slack, 1987), and even at the 8-cell stage (Kageura and Yamana, 1984, 1986). Therefore, the first induction in the amphibian embryo is considered to be mesoderm induction mediated by some substance(s) released from the vegetal endodermal cells. To help explain the mechanism of this phenomenon several models have been proposed, such as the «three-signal model» (Smith *et al.*, 1985).

Some peptide growth factors belonging to the FGF and TGF-ß families have been found to possess mesoderm-inducing activity on the *Xenopus* presumptive ectoderm (Knöchel *et al.*, 1987, 1989; Slack *et al.*, 1987; Rosa *et al.*, 1988; Green *et al.*, 1990a). We previously reported that activin A, a member of the TGF-ß superfamily, also has a potent mesoderm-inducing activity (Asashima *et al.*, 1989, 1990a). Following our findings, many different mesoderm-inducing factors turned out to be identical with or closely related to activin A: XTC-MIF from conditioned medium of XTC cells (Smith *et al.*, 1990; van den Eijnden-Van Raaij *et al.*, 1990); WEHI-MIF from murine leukemia cells (Albano *et al.*, 1990); vegetalizing factor from calf kidney and chicken embryo (Asashima *et al.*, 1990b, 1991b); and PIF from mouse macrophage cells (Thomsen *et al.*, 1990). Activin A induces almost all mesodermal tissues in a concentration-dependent manner (Ariizumi *et al.*, 1991; Green *et al.*, 1990a,b), Low dose-induced ventral-type mesoderm such as blood-like cells, mesenchyme, and high dose-induced dorsal-type such as muscle and notochord.

Abbreviations used in this paper: FGF, fibroblast growth factor; TGF-ß, transforming growth factor ß; MIF, mesoderm-inducing factor.

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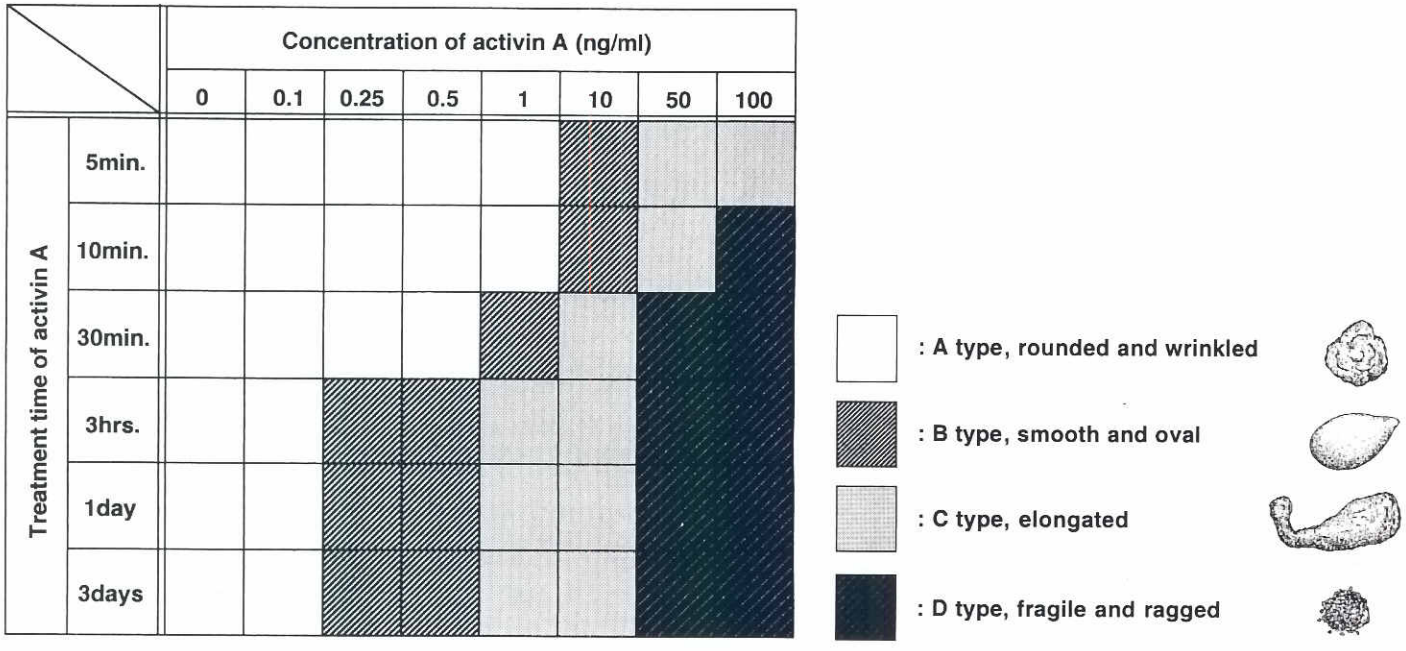

Fig. 1. Correlation between the concentration and the treatment time of activin A on the presumptive ectoderm. *About 25 pieces of presumptive ectodermal region were tested in each combination, and the type of explant (A-D type) usually obtained in each case is shown.*

In the implantation experiment, animal cap cells treated with conditioned medium of XTC cells (XTC-CM) can induce a secondary axis. The secondary anterodorsal structures were induced when the presumptive ectoderm treated with XTC-CM was implanted into the blastocoel of host embryos, whereas posteroventral structure was induced by the implantation of basic FGF-treated ectoderm (Cooke, 1989; Ruiz i Altaba and Melton, 1989). On the other hand, Cooke and Smith (1989) observed the abnormalities of the larval body after the injection of XTC-CM into the blastocoel before gastrulation. However, in addition to XTC-MIF (= activin A), the conditioned medium of XTC cells contains some other substances such as TGF-ß2 and -ß5 (Roberts *et al.*, 1990). It is still a matter for discussion whether all these phenomena result solely from the action of activin A.

To examine the role of activin A for the establishment and organization of embryonic axis in early *Xenopus* development, we extended the *in vitro* study on the mesoderm-inducing activity of activin A by changing the concentration and the time of activin A treatment. We also injected pure activin A into the blastocoel of *Xenopus* late blastula and compared this result with the previous work on the injection of XTC-CM into the blastocoel by Cooke and Smith (1989).

Results

Correlation between the concentration and the treatment time of activin A on the presumptive ectoderm

The animal cap explants treated with activin A underwent four types of morphological change depending on the concentration of activin A. The control explants cultured without activin A rounded up and became wrinkled (A type in Fig. 1). At low concentrations of activin A, the explants swelled to become smooth and oval (B type). At middle concentrations, the explants became elongated (C type). At high concentrations, the explants became fragile giving a ragged outline (D type).

In this experiment, the explants were treated with various concentrations of activin A (0-100 ng/ml) for various times (5 minutes-3 days). In each combination, more than 80% of the explants showed a similar morphological change, which could be classified into the above four types (Fig. 1). The concentration of activin A that can induce morphological change of the explants was inversely proportional to the time of activin A treatment. If the explants were treated with activin A briefly (*e.g.*, 5 minutes), a high concentration (10 ng/ml) was needed to cause morphological changes. Conversely, if the treatment time was longer (*e.g.*, 3 hours) the required concentration was lower (0.25 ng/ml). Morphological changes did not occur under 0.25 ng/ml, even if the treatment time was longer than 3 hours.

Activin A can induce mesodermal tissues in a concentration-dependent manner on the presumptive ectoderm

Fig. 1 indicates that the type of explants changed in a dose-dependent manner in each treatment time. We examined this change of the explants histologically. Sections of the four typical explants mentioned above are shown in Fig. 2. The control explant differentiated solely into atypical epidermis, without forming any mesodermal derivatives. Activin A-treated explants showing morphological changes contained mesodermal derivatives. At low concentrations of activin A, the explants differentiated into ventral mesoderm, such as blood-like cells, coelomic epithelium, and mesenchyme. At middle concentrations, muscle and neural tissue were induced as well. And at high concentrations, notochord, the most dorsal mesoderm was induced.

The results of the histological examination of the activin A-

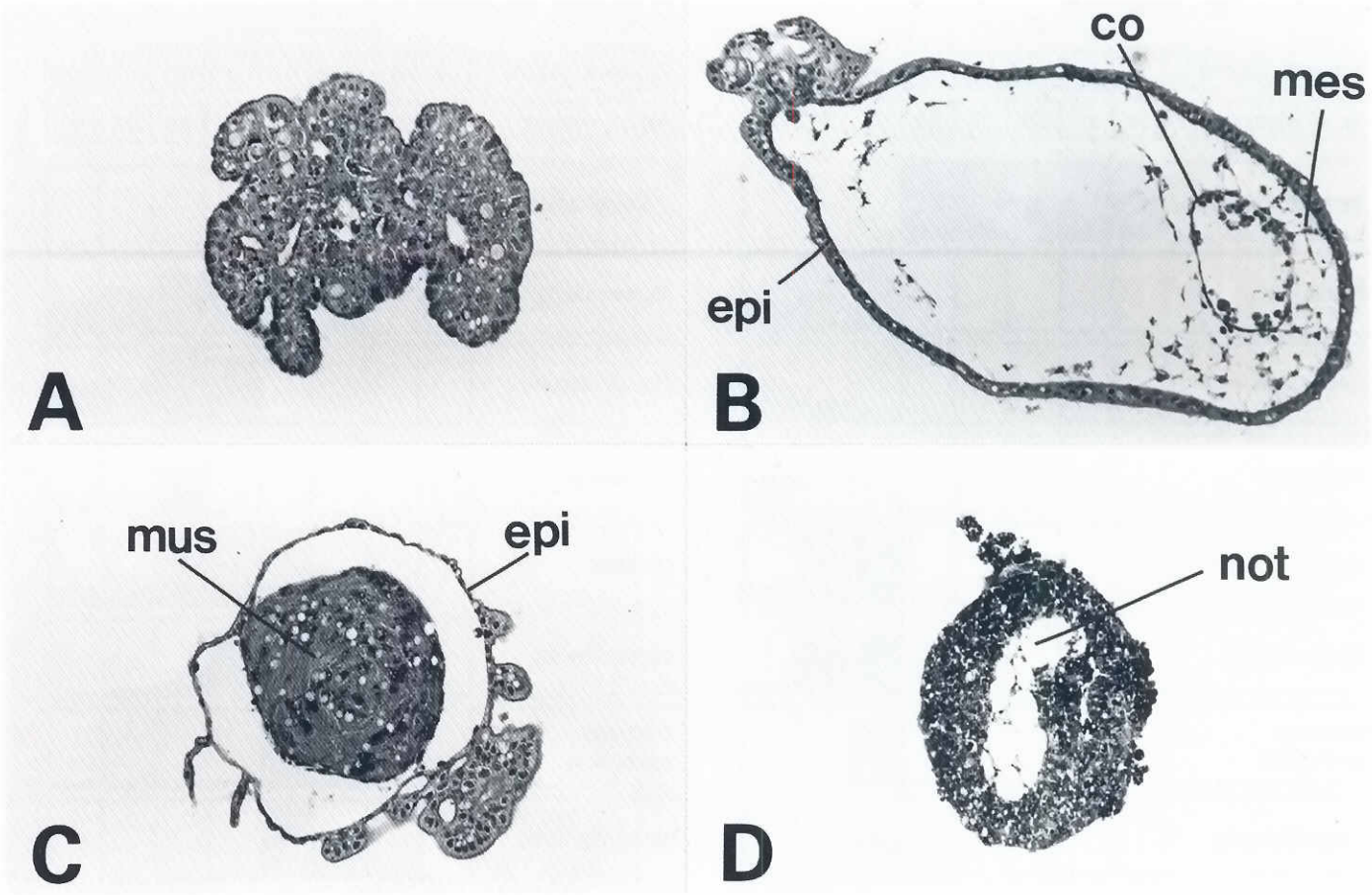

Fig. 2. Histological sections of the explants treated with various concentrations of activin A for 3 days. **(A)** *The control explant cultured without activin A differentiated into atypical epidermis.* **(B)** *The explant treated with a low concentration of activin A (0.5 ng/ml) differentiated ventral-type mesodermal tissues such as mesenchyme and coelomic epithelium (50% and 93% of the total explants, respectively).* **(C)** *At a middle concentration of activin A (10 ng/ml), a large block of muscle was differentiated (100%).* **(D)** *The most dorsal-type mesoderm, notochord, was differentiated (62%) at a high concentration of activin A (50 ng/ml). epi, epidermis; not, notochord; mus, muscle; mes, mesenchyme; co, coelomic epithelium.*

treated explants are expressed diagrammatically in Fig. 3. Fig. 3A is the result of the examination of the explants which were treated for 5 minutes, representing a brief treatment of activin A. Fig. 3B is that of 1-day treatment, representing a long-term treatment. At 1-day treatment, the minimum concentration of activin A needed for mesoderm induction is much lower than that of 5-minute treatment (0.25 ng/ml and 10 ng/ml, respectively). But in both cases, the explants differentiated into ventral to dorsal mesodermal tissues, depending on the concentration of activin A.

Outgrowth formation by the injection of activin A into the blastocoel

In order to confirm whether activin A has a role in establishing the axial organization of the *Xenopus* embryo, we injected about 10nl of activin A – in Steinberg's solution (0-250 ng/ml) – into the blastocoel of late blastula stage embryos (Stage 9). The relation between the amount of injected activin A and the frequency of the outgrowth formation is shown in Fig. 6.

All of the control embryos injected with 10 nl of 0.1% BSA-containing Steinberg's solution underwent normal development. The embryos injected with 0.1 pg of activin A also developed normally. 0.5-1 pg of activin A caused outgrowth formation in the injected embryos. Most of the outgrowths had a tail-like shape, and fused with the primary axis in its trunk region (Fig. 4A). At a dose of 2.5 pg of activin A, the injected embryos survived until the early tailbud stage (stage 22-23) and showed secondary axis-like formations in some cases, but most of them died before stage 42. At doses of more than 2.5 pg, all injected embryos died before neurulation. As the amount of injected activin A was increased, headless embryos with a tail-like outgrowth appeared (Fig. 4B). These embryos lacked eyes and cement glands in their head region.

Histological examination of the activin A-injected embryos

Most of the injected embryos that formed outgrowths contained two sets of muscle and neural tube. However, notochord was not formed in the outgrowth (Fig. 5B). The outgrowth does not, therefore, represent a true secondary axis. The secondary neural tube (tissue) was separated from the primary neural tube at the hindbrain region and extended to the distal end of the outgrowth. The secondary muscle also extended to the distal end of the outgrowth along with neural tube.

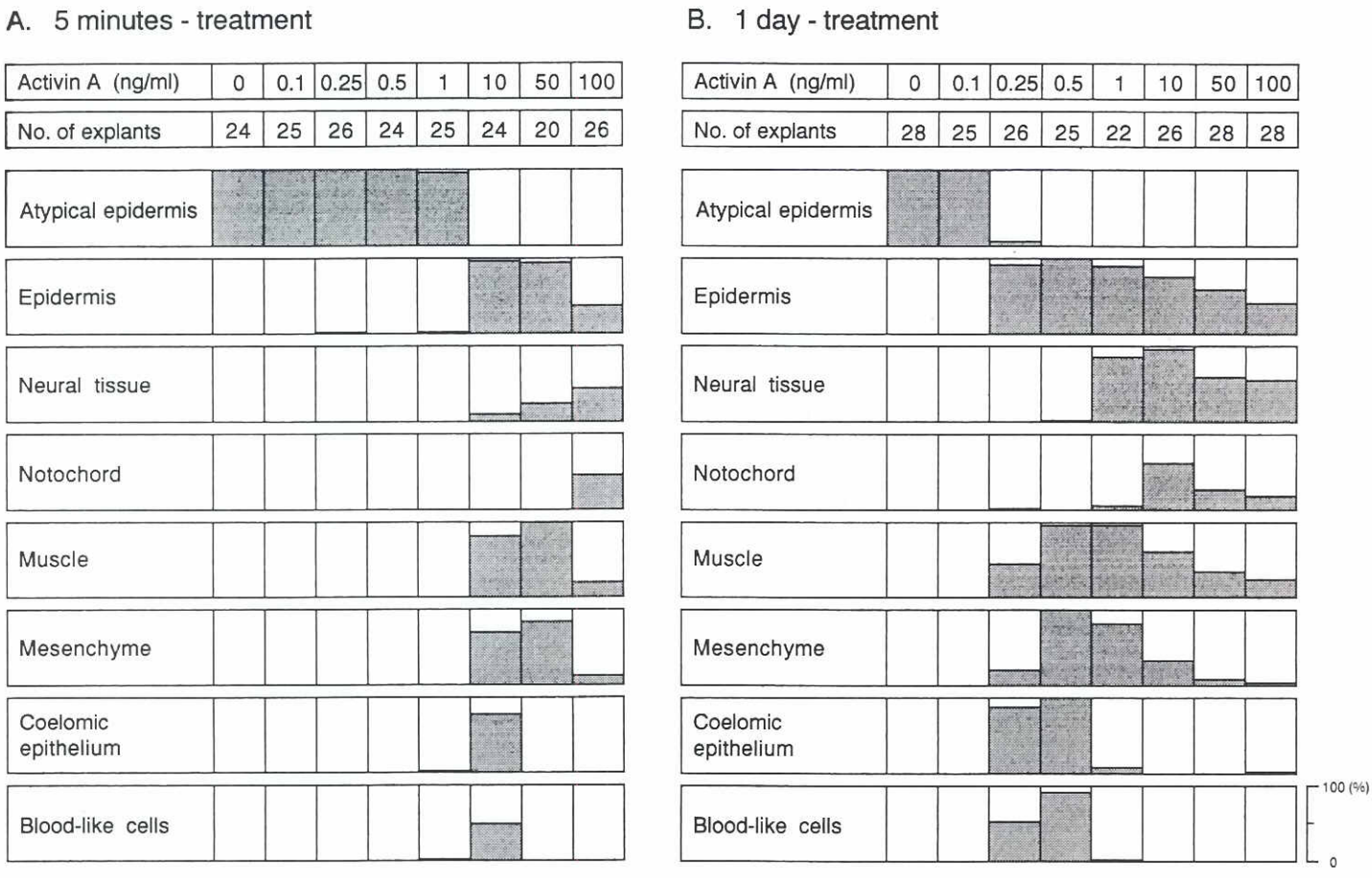

Fig. 3. Schematic representation of the differentiation of explants. *Bar charts indicate the percentage of differentiated explants in the total explants treated with each concentration of activin A. One column length represents 100% of induction. The minimum concentration of activin A to cause mesoderm induction is higher in a short time treatment* (**A:** *5 minutes) than that in a long time treatment* (**B:** *1 day). As the concentration of activin A increased, the differentiation of the explant changed from ventral-type (situated on the bottom side) to dorsal-type (situated on the top side) in each case.*

The headless embryo did not have head structures such as brain, eye, ear vesicle, or cement gland. Fragments of neural tissues were scattered in the head region of this embryo. Instead, a large block of muscle was formed in this region (not shown). The outgrowth consisted of neural tube and muscle. A notochord was not observed.

Discussion

In the present study, it was demonstrated that the minimum concentration of activin A required to cause mesoderm induction was inversely proportional to the time of activin A treatment. If we assume that the occurrence or non-occurrence of mesoderm induction is determined by the number of activin molecules binding to its predicted receptor-site, the above result seems very plausible.

We also demonstrated that activin A has a mesoderm-inducing activity at a concentration as low as 0.25 ng/ml when the explant was treated for a sufficient time. Almost all mesodermal tissues from ventral-type to dorsal-type could be induced by changing its concentration. A potent mesoderm-inducing factor, *Xenopus* basic FGF, can induce ventral mesoderm at low concentration but notochord is never induced even if its concentration is increased (Green *et al.*, 1990a). Other molecules that have been reported to have mesoderm-inducing activity include TGF-ß1 and TGF-ß2 (Knöchel *et al.*, 1987, 1989; Rosa *et al.*, 1988). These molecules have less potent mesoderm-inducing activity and cannot induce as many mesodermal tissues as activin A does. Therefore, activin A could conceivably function in the embryo in a concentration-dependent manner, with the highest concentration on the dorsal side of the *Xenopus* embryo. Recently, Sokol and Melton (1991) reported that prospective dorsal and ventral regions of the ectoderm respond differently to the same concentration of PIF, which is an activin A homologue derived from the P388DI cell line. They demonstrated that the prospective dorsal region of the ectoderm differentiated into dorsoanterior structures such as eye, neural tissue, and notochord when treated with PIF, whereas the ventral region preferentially differentiated into ventrolateral structures such as muscle and mesenchyme. Therefore, the patterning of the mesoderm should be discussed from dual aspects: the localization of the inducer and the differential competence of the responding tissues.

Cooke and Smith (1989) examined the abnormalities of the larval body caused by the injection of XTC-conditioned medium (XTC-

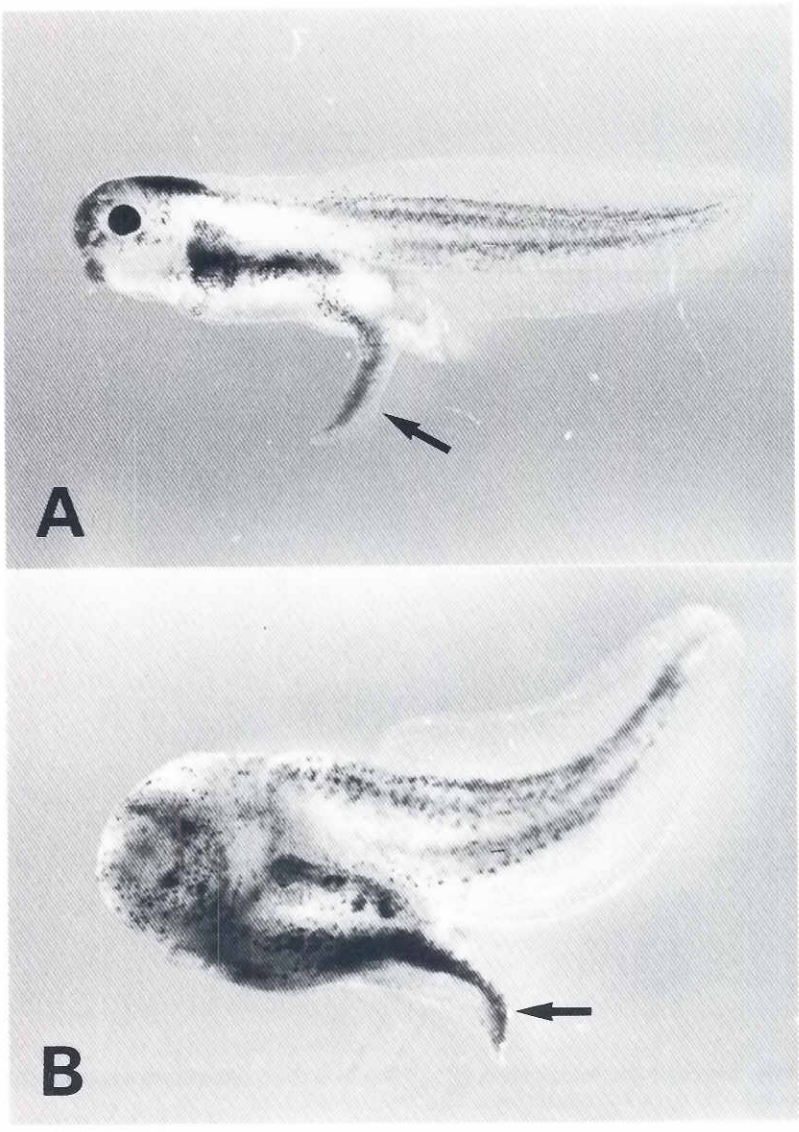

Fig. 4. Embryos with an outgrowth obtained by the injection of activin A into the blastocoel. (A) *At a dose of 1 pg of activin A, embryo formed a tail-shaped outgrowth.* **(B)** *At a dose of 2.5 pg, headless embryo with an outgrowth was obtained.*

CM) into the blastocoel before gastrulation. They reported that the anterior mesodermal and endodermal pattern were lost progressively as the dose of XTC-CM was increased. At a dose of 0.005-0.006 units, which corresponds to less than 2.5-3 pg of pure protein (Smith *et al.*, 1988), the headless embryos with an accessory tail were obtained. These embryos had much the same characters as the headless embryos we obtained by the injection of a high dose of activin A (*e.g.*, 2.5 pg) . They explain that the loss of anterior structures is because of the lack of a substrate for the spread of prechordal mesoderm, rather than because of a direct effect of XTC-MIF in the blastocoel. They observed, however, that the presence of the accessory tail was highly dependent upon individual egg batch.

In our microinjection results, about 70% of the injected embryos formed tail-shaped outgrowths after the injection of 1 pg of activin A. Further, embryos lacking head structures appeared at a high frequency as the amount of activin A was increased. Our speculations concerning the mechanisms of outgrowth formation and loss of head structures are as follows: if the gastrulation movement is completed before the injected activin A sufficiently mesodermalizes the blastocoel roof, the head structures will develop normally as a result of the neural induction by the prechordal mesoderm. At a high concentration of activin A, the blastocoel roof would be mesodermalized and lose competence for neural induction. As a result, muscle would develop in the head region instead of brain and eye. On the other hand, the future ventral epidermal region still has competence for activin A even if the gastrulation movement has progressed and will be differentiated into muscle and some neural tissues by the action of residual activin A trapped at the ventral-most region. Many headless embryos were obtained when 2.5 pg of activin A was injected into the blastocoel. Assuming the volume of the blastocoel to be 250 nl, the concentration of injected activin A in the blastocoel will be 10 ng/ml. Consistently, in the *in vitro* experiment, activin A induces muscle at a high frequency rate (67% of the total explants) around this concentration if the presumptive ectoderm is treated with activin A for 3 hours.

In contrast to these microinjection results, it is reported that a secondary axis with anterodorsal (or head) structures was induced

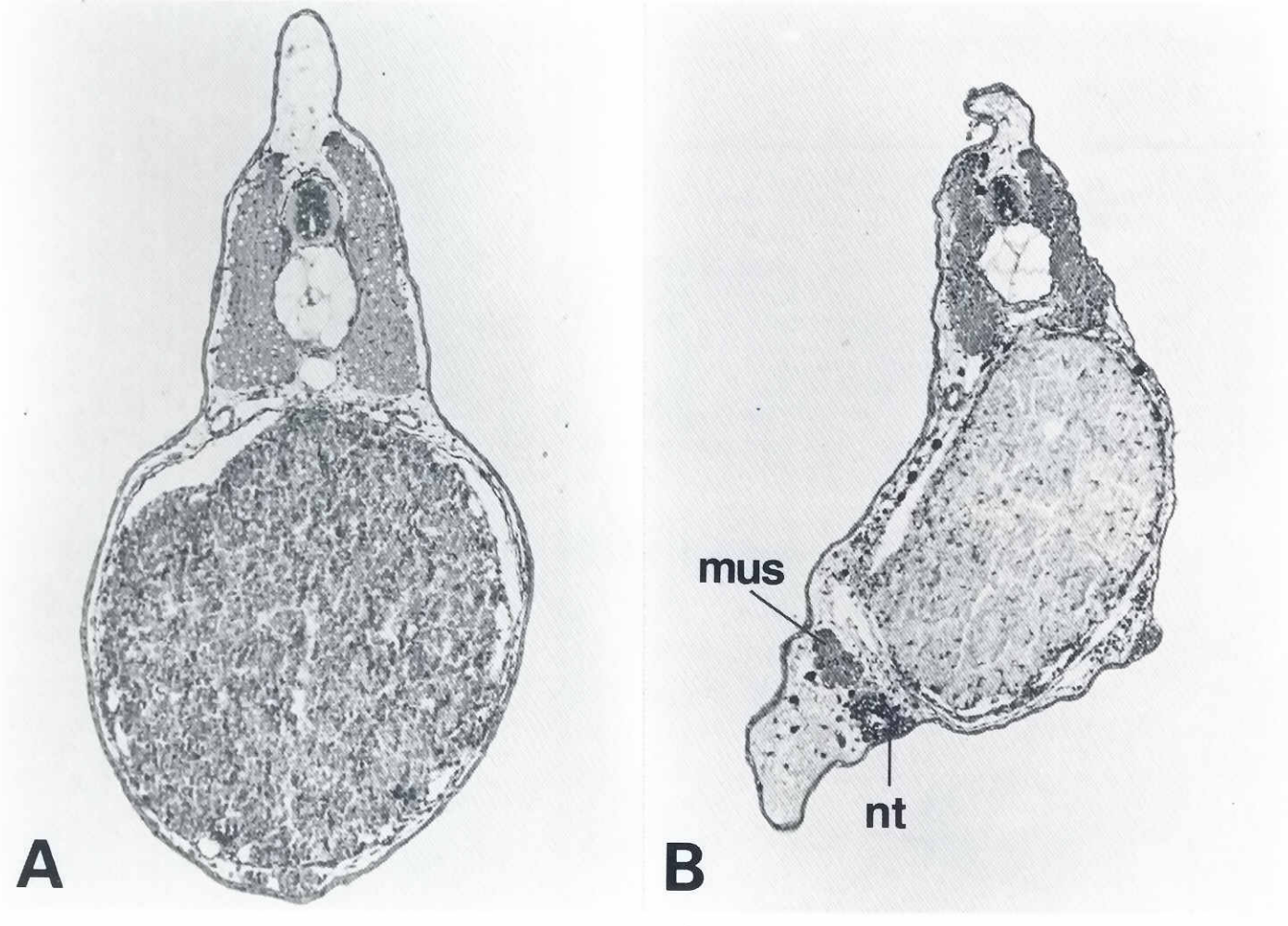

Fig. 5. Transverse sections of the activin-injected embryos. (A) *The control embryo injected with 0.1% BSA-containing Steinberg's solution.* **(B)** *The embryo injected with 1 pg of activin A. Muscle and neural tube are induced in the outgrowth. mus, muscle; nt, neural tube.*

when the XTC-CM-treated ectodermal explant was implanted into the blastocoel of the host blastula (Ruiz i Altaba and Melton, 1989). This discrepancy may be due to the difference of the experimental design. In the case of the implantation of the explant, activin A could mesodermalize the dissected explant at first, then the mesodermalized explant would act as Spemann's organizer in the blastocoel and induce secondary head structures as the organizer does. On the other hand, in the case of the injection of activin A into the blastocoel, injected activin A would directly mesodermalize the ectoderm of blastocoel roof as mentioned above.

Recently, we confirmed that activin homologue is already present in unfertilized eggs and blastulae of *Xenopus laevis* and estimated that there is at least 1 pg/egg or 500 pg/ml of activin homologue (Asashima *et al.*, 1991a). In the present study, about the same amount or concentration of activin A induced mesodermal tissues in the animal cap assay and formed the outgrowths when injected into the blastocoel. Although it is not certain which kind of activin (A, B, AB) this homologue belongs to, we speculate that activin(s) may be the natural mesodermal inducer and responsible for establishing axial organization in the *Xenopus* embryo. One appealing model would employ a gradient of activin(s) along the dorsoventral axis with high dose in the dorsal-most region.

Materials and Methods

Eggs and embryos

Eggs of *Xenopus laevis* were obtained by injecting males and females with human chorion gonadotropin (Gestron; Denka Seiyaku Co., Kawasaki, Japan). Males were injected with two doses of 400 IU each and females with one dose of 800 IU. After the jelly coat of the fertilized eggs was removed in 4.5% cystein hydrochloride- Steinberg's solution (pH 7.4) the jelly-free eggs were transferred to culture dishes containing Steinberg's solution (pH 7.4). The embryos were allowed to develop to stage 9 (Nieuwkoop and Faber,1956).

Treatment of presumptive ectoderm with activin A

At stage 9, the vitelline membrane was removed with fine forceps under a binocular microscope. The presumptive ectoderm region was then isolated at an angle of about 10 degrees around the animal pole using fine tungsten needles. Human recombinant activin A (= EDF, Erythroid Differentiation Factor; Murata *et al.*, 1988) was kindly provided by Dr. Eto from the Central Research Laboratories of Ajinomoto Co. Inc. (Kawasaki 210, Japan). Activin

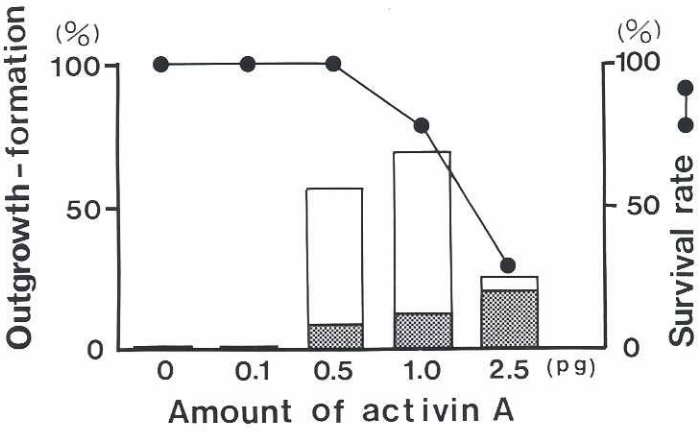

Fig. 6 Percentage of outgrowth formation by the injection of activin A. *Number of cases are 51, 10, 50, 40, 40 for 0, 0.1, 0.5, 1, 2.5 pg of activin A, respectively. Bar charts indicate the percentage of the embryos with an outgrowth out of the total embryos injected with each amount of activin A, and stippled region in each bar represents that of the headless embryos with an outgrowth.*

A was dissolved in 0.1% BSA-containing Steinberg's solution (pH 7.3) at concentrations of 0, 0.1, 0.25, 0.5, 1, 10, 50, 100 ng/ml and put in non-surface-treated polystyrene wells (SUMILON 24-well plate MS-8024, Sumitomo Bakelite Co., Japan). Presumptive ectodermal sheets were transferred to these test solutions with the inner surface facing up, and were treated for various times (5, 10, 30, 180 minutes, 1 day, 3 days). About 25 explants were treated in each treatment condition. After the treatment with activin A, explants were cultured in 0.1% BSA-containing Steinberg's solution (pH 7.3) at 20°C until control embryos reached stage 40.

Microinjection of activin A into the blastocoel

Jelly-free eggs (Stage 9, with vitelline membrane) were lined up along the edge of a microscope slide. About 10 nl of human recombinant activin A, which was dissolved in 0.1% BSA-containing Steinberg's solution at concentrations of 0, 10, 50, 100, 250 ng/ml, was injected into the blastocoel. Likewise, alkaline phosphatase-conjugated anti-mouse immunoglobulins (Tago, Burlingame, CA, USA) were microinjected into the blastocoel. Immediately, these embryos were homogenized in PBS and centrifuged at 10,000 x g for 5 minutes. The volume of the injected solution was estimated from the alkaline phosphatase activity of the supernatant by using paranitrophenylphosphate as a substrate. Injected embryos were incubated in 5% Ficoll-Steinberg's solution. After several hours the medium was changed to 20% Steinberg's solution. Embryos were cultured in this medium at 20°C until controls reached stage 42.

Histological examination

Explants and injected embryos were fixed in Bouin's fluid for 3 hours. They were dehydrated through ethanol series, embedded in paraffin and sectioned at 5 μm. The sections were stained with hematoxylin-eosin.

Acknowledgments

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