Original Article

The location of the third cleavage plane of *Xenopus* embryos partitions morphogenetic information in animal quartets

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ABSTRACT Analysis of the developmental potential of animal quartets (the set of four animal blastomeres isolated from the 8-cell stage *Xenopus* embryo) provided insight into the manner in which morphogenetic information is distributed along the animal-vegetal axis. Gravity treatments were employed to alter the partitioning plane. Animal quartets isolated from embryos exposed to simulated weightlessness had larger animal blastomeres, and they formed structures such as a groove and a protrusion more often than 1g-control animal quartets. Animal quartets with an unusual non-horizontal third cleavage plane were also found to have a higher frequency of protrusion formation than animal quartets with a typical horizontal cleavage plane. The increase in the frequency seen in simulated weightlessness animal quartets was not due to their increased size. Fusing two animal quartets isolated from hypergravity (3g) exposed embryos (small blastomeres and low incidence of protrusions) did not affect the frequency of protrusion formation. Molecular analyses revealed that a partial induction was associated with the protrusion formation. Transcripts of the dorsal lip specific homeobox gene, *goosecoid*, and α-*cardiac actin* were detectable by PCR amplification in the animal quartet with a protrusion, and α-*cardiac actin* mRNA was found by whole-mount *in situ* hybridization to be localized in the protrusion. Taken together, all these results are consistent with the notion that both animal and vegetal information is necessary for normal development and the partitioning of morphogenetic information into animal quartets results in gravity-dependent differential morphogenesis and gene regulation.

KEY WORDS: *animal quartet, cleavage plane, gravity treatment, morphogenetic information, Xenopus embryo*

Introduction

The amphibian embryo provides a favorable model system for analyzing the effects of external forces such as gravity on early development (reviewed in Malacinski and Neff, 1984). The location of the third cleavage furrow of *Xenopus* embryos along the animal-vegetal axis can be shifted by clinostat-simulated weightlessness (μg) or centrifuge-simulated hypergravity (Neff *et al.*,1992 and 1993b). Compared to ground controls under Earth's gravitational field (1g), μg-treatment shifts the third cleavage plane towards the vegetal pole, and three times Earth's gravity (3g) relocates the cleavage plane towards the animal pole. As a consequence, μg-treated embryos produce larger animal blastomeres and 3g-treated embryos generate smaller animal blastomeres at the 8-cell stage than 1g-controls (see Fig. 1A).

In order to quantitate the amount of alteration in the location (along the animal/vegetal axis) of the third cleavage plane, an animal-vegetal cleavage ratio (AVCR) has been defined previously as the ratio of the height of the animal blastomere to the height of the embryo at the 8-cell stage (Fig. 1A). Although there was a substantial variation in AVCR values from spawning to spawning and from embryo to embryo (Yokota *et al.*, 1992), the mean AVCR was 0.45 for μg-treated embryos, 0.38 for 1g-controls and 0.29 for 3g-treated embryos (Neff *et al.*, 1992 and 1993b). Since embryo inversion and D_2O immersion increased AVCR (like μg-treatment) and cold shock reduced AVCR (like hypergravity treatment), the primary cause of AVCR changes was postulated to be an alteration in the distribution of dense cytoplasmic components such as yolk platelets and the rearrangement of microtubule arrays.

AVCR alterations were exploited in this report for analyzing the developmental potential of animal and vegetal blastomeres at the early embryonic stages. The specific question posed in this report is: does the location of the third cleavage plane affect the segregation of morphogenetic information along the animal-vegetal axis? Background information (reviewed in Kimelman *et al.*, 1992; Slack

Abbreviations used in this paper: AVCR, animal-vegetal cleavage ratio; PCR, polymerase chain reaction; μg, clinostat-simulated weightlessness; 1g, Earth's gravitational acceleration (9.8 m/sec^2); 3g, centrifuge-simulated three times Earth's gravitational acceleration (29.4 m/sec^2).

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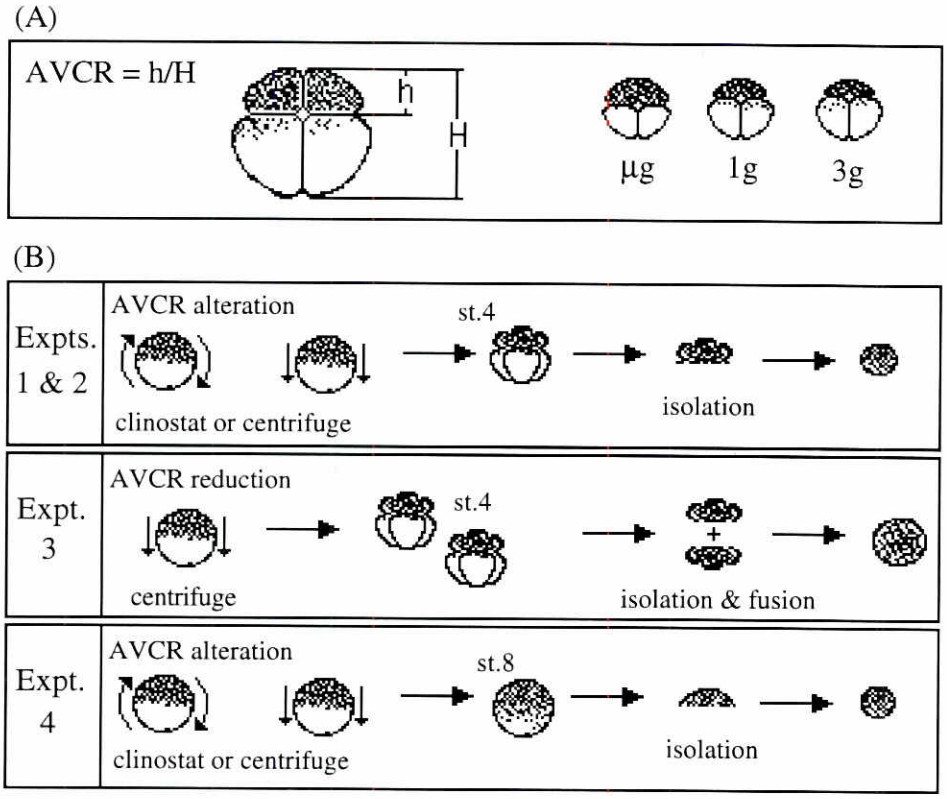

Fig. 1. Definition of AVCR and experimental protocols for animal quartet assay. (A) *AVCR (animal-vegetal cleavage ratio) is defined as the ratio of the height of the animal blastomere to the height of the embryo at the 8-cell stage. Please refer to earlier publication (Neff et al., 1992 and 1993b).* **(B)** *In Expts. 1 and 2, embryos were either clinostated to increase AVCR or centrifuged to decrease AVCR. At the 8-cell stage, animal quartets (four animal blastomeres) were isolated and isolated animal quartets were cultured for two days. In Expt. 3, two animal quartets isolated from centrifuged embryos were fused together by placing one upon the other. In Expt. 4, embryos were clinostated or centrifuged until they reached stage 8. At the blastula stage, the animal cap was isolated and cultured for three days.*

and Tannahill, 1992) would predict that a positive answer would be obtained. To answer the question, an assay for developmental potential of the animal quartet was developed. In this assay, embryos are first either clinostated to simulate weightlessness (μg) or centrifuged to simulate hypergravity (3g). An animal quartet (the set of four animal blastomeres) was then isolated at the 8-cell stage from a group of embryos which displayed an enormous variation in the location of the third cleavage plane. It is reasoned that if the type of morphogenetic information included in an animal quartet is related to the size and shape of the blastomeres, by relocating the third cleavage plane morphogenesis of isolated animal quartets would be altered.

A similar isolation experiment was conducted by Henry *et al.* (1989) using sea urchin embryos. A significant number of isolated blastomeres (9-14%) were reported to differentiate endoderm and mesenchymal cells even when the isolated mesomeres were formed within the animal hemisphere. However, when the third cleavage plane was shifted toward the vegetal pole and the resulting animal blastomeres inherited a fraction of the vegetal hemisphere, more isolated blastomeres (39%) differentiated endodermal and mesenchymal cell types.

By removing various sets of blastomeres, Kageura and Yamana (1983 and 1984) demonstrated that for complete *Xenopus* embryogenesis, a combination of two animal blastomeres together with one dorsal and one ventral vegetal blastomeres is minimally essential at the 8-cell stage. When either an animal quartet or a vegetal quartet was cultured separately, normal embryogenesis failed to occur. Their experimental analysis, however, concentrated on the morphogenesis of a reduced set of blastomeres derived only from 1g-control embryos.

Experimental protocols for our animal quartet assay are illustrated in Fig. 1B. In experiments (Expts.) 1 and 2, embryos were either clinostated to increase AVCR or centrifuged to decrease AVCR. At the 8-cell stage, an animal quartet (set of intact four animal blastomeres) was isolated and cultured to the gastrula stage for morphological analysis. In Expt. 3, two animal quartets isolated from centrifuged embryos (3g hypergravity) were fused together by placing one animal quartet upon the other. It will be demonstrated that relocating a third cleavage plane by gravitational treatment does not provide normal embryogenesis from isolated animal quartets, but their morphogenesis is affected in terms of a frequency of forming a groove and a protrusion. Molecular analyses revealed that a muscle specific gene (*α-cardiac actin*) is expressed in the protrusion, indicating that muscle developmental potential of isolated animal quartets is altered by the position of the third cleavage plane.

Results and Discussion

Animal quartets from embryos exposed to weightlessness simulation (μg) showed altered morphogenesis

In Expt. 1, morphogenesis of animal quartets isolated from gravity-treated embryos was examined. Fertilized eggs were either clinostated to simulate weightlessness (μg) or centrifuged to simulate hypergravity (3g), in order to relocate the third cleavage plane. It has been demonstrated (Neff *et al.*, 1992, 1993b) that μg-treatment shifts the third cleavage plane closer to the vegetal pole and 3g-treatment relocates it towards the animal pole (see Fig. 1A). At the 8-cell stage, embryos were removed from the clinostat or the centrifuge, and intact animal quartets consisting of four animal

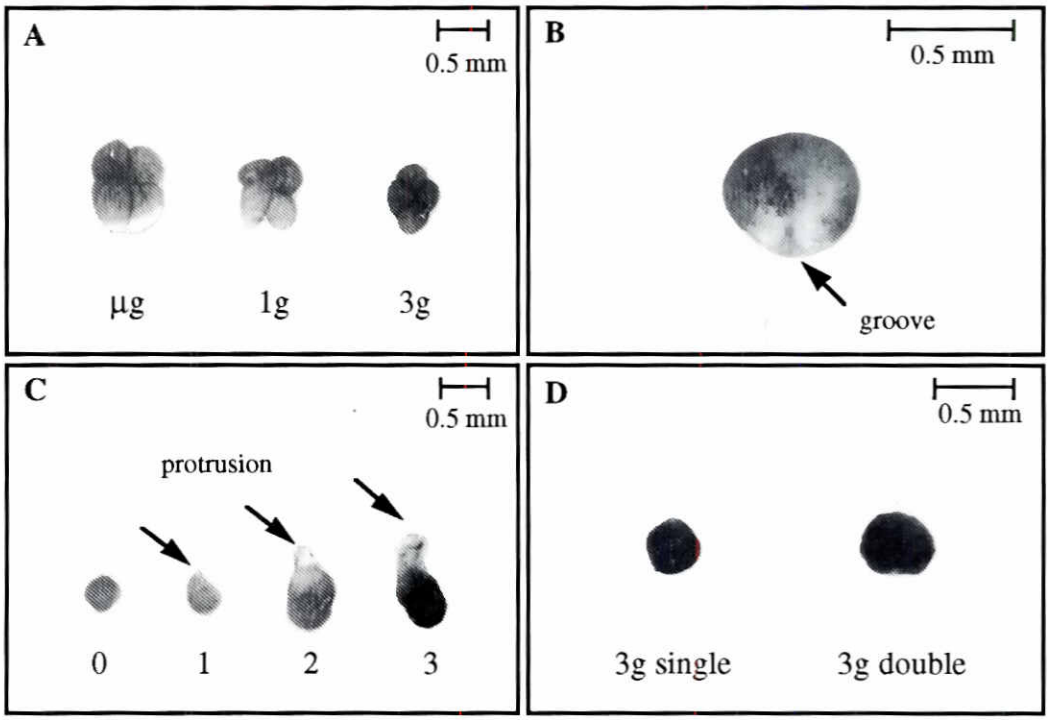

Fig. 2. Isolated animal quartets and their morphogenesis. **(A)** *Animal quartets isolated from μg-treated embryo, 1g-control embryo and 3g-treated embryo;* **(B)** *isolated animal quartet with a groove (darkly pigmented) one day after isolation (at 13°C);* **(C)** *a sample of isolated animal quartets two days after isolation with protrusions of varying prominence ("0" - no protrusion, "1" - minimum detectable protrusion, "2" - intermediate protrusion and "3" - large protrusion); and* **(D)** *single animal quartet from 3g-exposed embryo (left) and a double set of animal quartets from 3g-exposed embryos (right).*

blastomeres were isolated. Embryos utilized for isolation had a nearly horizontal (third) cleavage plane. Figure 2A illustrates how isolated animal quartets from an embryo exposed to μg-simulation are larger than the animal quartet from the embryo exposed to 3g-simulation as well as the control (1g) animal quartet.

One day after isolation when intact control embryos were gastrulae (stage 11), a darkly pigmented groove shown in Fig. 2B was observed in 1/3 of animal quartets isolated from μg-exposed embryos. This groove always appeared in the unpigmented area. The pigmentation pattern was related to the groove suggesting that neighboring cells around the groove were involuting into it. Although the external morphology of the groove resembles that of the dorsal lip of the blastopore, a genuine dorsal lip is rounder than these grooves. In an intact control embryo, a close relationship between the lower fifth (horizontal) cleavage plane in the vegetal hemisphere and the location of the dorsal lip is reported (Gimlich and Gerhart, 1984; Dale and Slack, 1987). If the groove represents an abnormal dorsal lip (involution site), then the morphogenetic information for dorsal lip formation must be more broadly distributed along the animal-vegetal axis than reported by Gimlich and Gerhart (1984) and Dale and Slack (1987).

The frequency of groove formation was clearly affected by gravitational treatments. In Fig. 3, 39% of animal quartets isolated from μg-exposed embryos formed a darkly pigmented groove, while only 5% of 1g-control animal quartets and no animal quartets isolated from 3g-treated embryos formed a similar groove (data based on 6 spawnings, 108 animal quartets).

Two days after isolation, when intact control embryos were at stage 13, 2/3 animal quartets isolated from μg-treated embryos formed a protrusion (Fig. 2C). The length of the protrusions varied, and some protrusions had a groove at their tip. A size scoring system was developed classifying protrusions from "0" (no protrusion), "1" (minimum detectable protrusion), "2" (intermediate protrusion) to "3" (large protrusion). Animal quartets isolated from μg-treated embryos formed a protrusion more often than control animal quartets and animal quartets isolated from 3g-exposed embryos. Figure 4 displays a frequency of various sized protrusions with an average size score of 1.18 for μg animal quartets, 0.05 for 1g control animal quartets and 0.04 for 3g animal quartets (data based on 10 spawnings, 165 animal quartets). Regardless of groove or protrusion formation, no animal quartets developed grossly observable axial structures.

The third cleavage plane (first horizontal) normally divides an embryo into four animal blastomeres and four vegetal blastomeres.

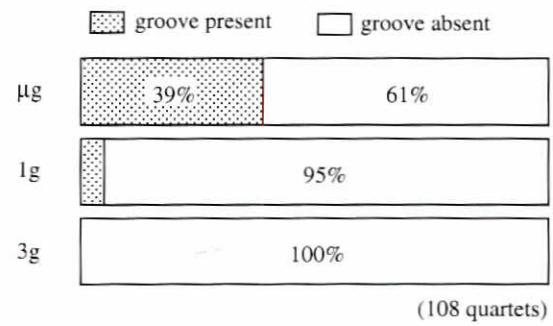

Fig. 3. Effects of gravity treatment on the frequency of groove formation. *Animal quartets were isolated from gravity-treated embryos at the 8-cell stage, and the presence of a groove was scored one day after isolation. μg-treatment clearly increased the frequency of groove formation (data based on 6 spawnings).*

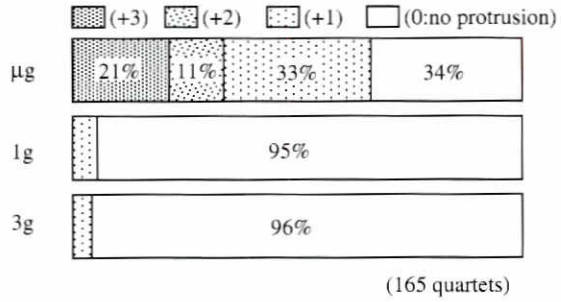

Fig. 4. Effects of gravity treatment on the frequency and the size of protrusions. *Based on the protrusion size, isolated animal quartets were scored from "0" to "3" two days after isolation. Out of 70 quartets, 66% of animal quartets isolated from μg-treated embryos formed various sized protrusions, and 21% of them were a large protrusion. Conversely, a majority of 1g-controls (95%) and animal quartets isolated from 3g-treated embryos (96%) did not form any protrusion (data based on 10 spawnings).*

A cleavage plane shifted toward the vegetal pole by μg-treatment produces larger animal blastomeres, while a cleavage plane shifted toward the animal pole by 3g-treatment produces smaller animal blastomeres than 1g-controls. Two hypotheses which could explain an increased frequency of groove and protrusion formation in animal quartets isolated from μg-treated embryos are: (i) the "vegetal information hypothesis" — a larger animal quartet generated by μg-treatment contains a greater amount of vegetal materials than 1g-control and animal quartets generated by 3g-treatment. The vegetal morphogenetic information enhances the developmental potential of isolated animal quartets, thus forming a groove and a protrusion (Dale and Slack, 1987); and (ii) the "community effect hypothesis" — Green and Smith (1990) proposed a positive autocatalytic or community effect to explain the embryonic cell fate of animal pole blastomeres treated with XTC-MIF. Likewise, the size of isolated animal quartets could explain the difference in groove and protrusion frequency by a community effect. A larger size may increase the chance of inductive events, and as a result it elevates the frequency of forming grooves and protrusions. In order to test the "vegetal information hypothesis" and the "community effect hypothesis", Expts. 2 and 3 were designed.

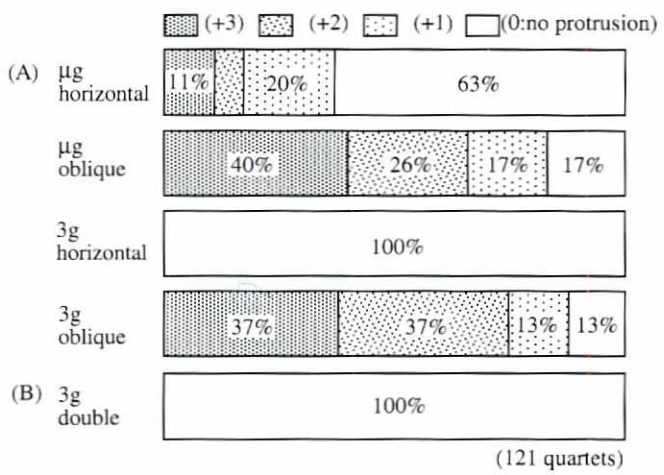

Fig. 5. Effects of an orientation of the third cleavage plane and the size of animal quartet on the frequency of protrusion. (A) *Frequency of protrusion scored two days after isolation for a horizontal cleavage plane and an oblique cleavage plane: dramatic increase in protrusion formation was observed for animal quartets isolated from embryos with an oblique cleavage plane (data based on 6 spawnings).* **(B)** *Frequency of protrusion scored for a double animal quartet from 3g-treated embryos: fusing two animal quartets did not change the frequency of protrusion formation (data based on 3 spawnings).*

Tilting the third cleavage plane to an animal-vegetal axis affected morphogenesis of isolated animal quartets

In *Xenopus* embryos, the third cleavage plane is typically horizontal. However, there exist a number of cases in which the third cleavage plane is tilted (oblique) towards the animal-vegetal axis. Yet those embryos develop normally. In Expt. 2, embryos were either clinostated or centrifuged to relocate the third cleavage plane, and then embryos with a non-horizontal third cleavage plane were selected. Animal quartets were isolated from each group of embryos, and the frequency of protrusion formation was compared in two groups. The results in Fig. 5A clearly demonstrate that a non-horizontal cleavage plane increased the frequency of protrusion formation for animal quartets isolated from both μg- and 3g-treated embryos (μg: from 37% to 83%, and 3g: 0% to 87%; data based on 6 spawnings, 106 animal quartets).

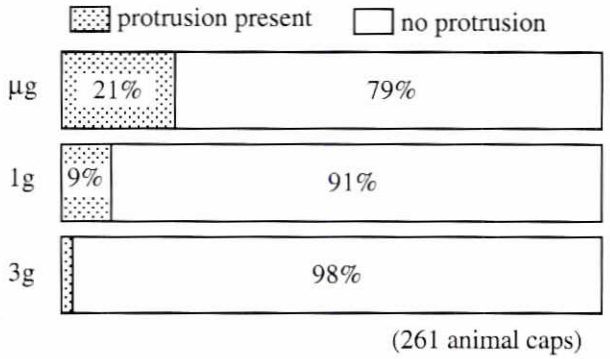

Fig. 6. Effects of gravity treatment on the frequency of protrusions. *Animal caps were isolated from gravity-treated embryos at the blastula stage and scored for the presence of protrusions after three days of culture. μg-treatment increased the frequency of protrusions (data based on 8 spawnings).*

The results in Expt. 2 are consistent with the "vegetal information hypothesis" rather than with a "community effect hypothesis". The orientation of the third cleavage plane alone does not affect the size of animal quartets, but it affects the partitioning of vegetal materials into four animal blastomeres. An animal quartet with a non-horizontal third cleavage plane therefore inherits a larger amount of vegetal morphogenetic information than an animal quartet with a horizontal third cleavage plane. The oblique third cleavage plane also affects the partitioning of dorsalizing materials. Results from egg inversion (Neff *et al.*, 1984) suggest that the asymmetric distribution of vegetal materials was requisite for inverted embryos to establish the dorso-ventral polarity and to succeed in normal development. Since the orientation of the tilted

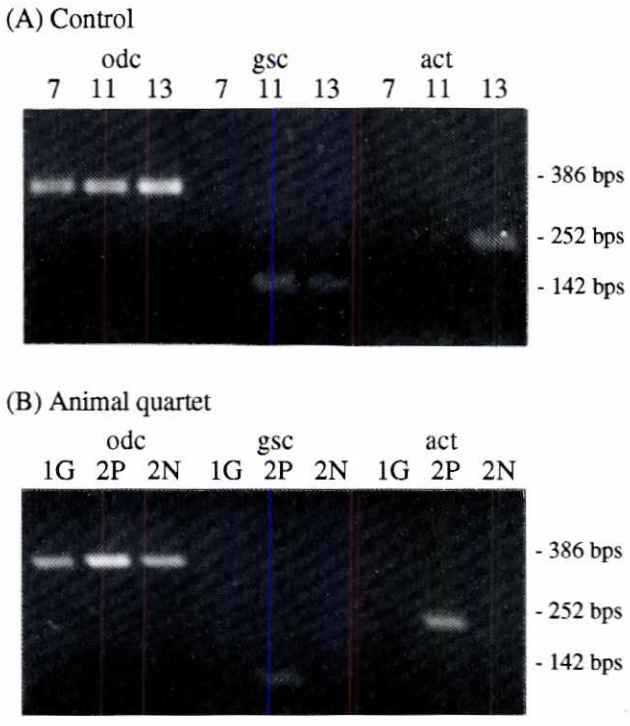

Fig. 7. Ethidium bromide-stained agarose gel containing PCR amplification products (30 cycles) primed by *ornithine decarboxylase, goosecoid* and *α-cardiac actin*. (A) *Control panel: PCR amplified cDNAs from intact control embryos at stages 7, 11 and 13 primed by ornithine decarboxylase (odc), goosecoid (gsc) and α-cardiac actin (act) primers. The expected size of PCR products is 386 bps for odc, 252 bps for gsc and 142 bps for act.* **(B)** *Experimental (animal quartet) panel: PCR amplified cDNAs from animal quartets (1G: one day after isolation with a groove, 2P: two days after isolation with a protrusion, and 2N: two days after isolation without a protrusion).*

plane was random, however, Expt. 2 did not provide information on the dorsoventral polarity of the morphogenetic components.

Masho and Kubota (1986) and Masho (1988) conducted a lineage analysis of 8-cell stage *Xenopus* embryos. They compared the fates of animal blastomeres with a horizontal third cleavage plane to those with a non-horizontal third cleavage plane and demonstrated that the fates of animal-dorsal blastomeres varied according to the orientation of the third cleavage plane. Their analysis is consistent with the results obtained in Expt. 2 where differential third cleavage planes gave rise to variations in morphogenesis of isolated animal quartets.

The position of the third cleavage plane normally shows some variation from spawning to spawning and from embryo to embryo within a single spawning (Yokota *et al.*, 1994). Gravitational treatments apparently can exaggerate this natural variation. In similar analyses carried out by Kageura and Yamana (1983) on 1g-embryos, they obtained 47% as the frequency of protrusion formation as opposed to 5% in this report (Fig. 4). Conceivable reasons for explaining a difference in frequencies in those independent studies are: (i) the culture media were different (supplemented by 0.1% bovine serum albumin in our report and by 10% fetal calf serum in Kageura and Yamana, 1983): preliminary studies support the idea that an animal quartet cultured in the presence of activin A increases the frequency of protrusion formation (Yokota and Chung, unpublished results). Fetal calf serum may contain trace amounts of growth factors which may have a positive effect on protrusion formation; (ii) culturing time: longer culturing time may enhance protrusion formation; (iii) natural variation from spawning to spawning: in some spawnings isolated animal quartets did not form any protrusion, whereas others gave rise to a higher frequency of protrusion formation (under μg-treatment, percent frequency of protrusion formation ranged from 0% to 100%). The variation in frequency may result from the differential distribution of morphogenetic information and/or the natural shift in AVCR values; and (iv) orientation (horizontal/non-horizontal) of the third cleavage plane: the inclusion of embryos with a non-horizontal cleavage plane would increase protrusion formation, as shown in Expt. 2.

Combining two 3g-treated animal quartets did not affect their morphogenesis

In order to provide a direct test of the "community effect hypothesis", two animal quartets isolated from 3g-treated embryos were fused together (Expt. 3) and the frequency of protrusion formation was examined. An animal quartet from a 3g-exposed embryo was approximately half the volume of an animal quartet isolated from a μg-treated embryo, so that fusing two animal quartets isolated from 3g-treated embryos made the size nearly equivalent to a single animal quartet isolated from a μg-exposed embryo (Fig. 2F). Figure 5B clearly shows that fusing two animal quartets from 3g-exposed embryos did not increase the frequency of protrusion formation. Thus, the "community effect hypothesis", based on data from 3 spawnings (43 animal quartets), is disfavored.

Animal caps from embryos exposed to weightless simulation (μg) also showed altered morphogenesis

The natural derivative of the animal quartet is the animal cap of the blastula stage embryo. Lineage label studies utilizing colloidal gold injected into blastomeres of gravity-treated 8-cell embryos indicate that little particular movement occurs between the blastomeres; label injected into animal blastomeres is subse-

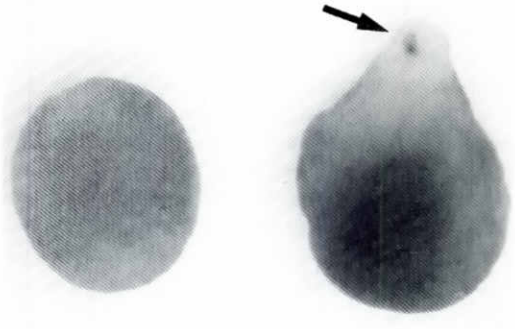

Fig. 8. Whole mount *in situ* hybridization of isolated animal quartets using digoxigenin-labeled *α-cardiac actin* antisense cDNA probe: *left, representative animal quartet explant from a 1g (control) embryo. Right, representative cultured animal quartet from a μg-treated embryo showing a protrusion and localized expression of α-cardiac actin message (arrow). Animal quartets were isolated at the 8-cell stage and cultured for two days at 13°C. Explants were depigmented with H_2O_2.*

quently found only in the animal cap (unpublished data). As the size of the blastomeres in the animal quartets of gravity-treated embryos differs, so do the size and number of cells in the animal caps of gravity-treated embryos. Cells of μg-treated animal caps are usually larger that normal and form an average of four cell layers instead of the normal two layers. Cells of 3g-treated animal caps are usually smaller than normal and form only a single layer (Neff *et al.*, 1993b). It was thought that animal caps isolated from embryos subjected to simulated weightlessness or hypergravity conditions would display similar behavior to animal quartets isolated from gravity-treated embryos. In Expt. 4, the morphogenesis of animal caps isolated from gravity treated embryos was examined (Fig. 1B). As in Expt. 1, fertilized eggs were either clinostated to simulate weightlessness (μg) or centrifuged to simulate hypergravity (3g) in order to relocate the third cleavage plane. The embryos were subjected to the various gravity treatments for 12 h until they reached the midblastula stage (stage 8). At this time, the animal caps were isolated and cultured. Within 2 h after the animal caps were isolated and placed in culture, they began to round up to form a sphere. The explants began to exhibit signs of morphologic changes within 48 h. These changes included elongation of the explant and the formation of protrusions from the wound site. Although the size scoring system was not utilized in analysis of these explants, typically the protrusions rated as "2" (intermediate protrusion). It was found that explants isolated for μg-exposed embryos had a 2.3-times higher incidence of mesoderm autoinduction compared to 1g controls. The percent autoinduction observed was 21% for μg-explants, 9% for 1g-control explants, and 2% for 3g-explants (see Fig. 6).

Preliminary studies involving the combination of two 1g-treated animal caps also failed to support the "community effect hypothesis". Fusing two 1g-treated caps formed explants at least equivalent to the size of a μg-treated explants. No (0/6) 1g double explants formed protrusions, further disfavoring the "community effect hypothesis".

The dorsal lip-specific homeobox gene, goosecoid, was detected in animal quartets which displayed a protrusion

The morphology of the groove formed in some animal quartets resembled that of the dorsal lip of the blastopore. In order to characterize the groove and compare it to the dorsal lip of the blastopore at a molecular level, expression of the dorsal lip-specific homeobox gene *goosecoid* was tested. Blumberg *et al.* (1991) have demonstrated that *goosecoid* expression is first detectable at stage 8.5, peaks at stage 10.5 and is down-regulated at stage 13. It was reported by those workers that the dorsal blastopore lip of the early *Xenopus* gastrula can organize a complete secondary body axis when transplanted to another embryo, and the microinjection of goosecoid mRNA into the ventral side of *Xenopus* embryos, where *goosecoid* is normally absent, leads to cell movement and the formation of an additional, complete body axis (Cho *et al.*, 1991; Niehrs *et al.*, 1993).

Poly(A)$^+$RNA was isolated from three cultured animal quartets (lane 1G: 1 day after isolation with a groove, lane 2P: 2 days after isolation with a protrusion, and lane 2N: 2 days after isolation without a protrusion). The isolated RNAs were reverse transcribed using random hexamers. The reverse-transcribed cDNAs were then amplified by a polymerase chain reaction (PCR-30 cycles) using a *goosecoid* primer together with an *ornithine decarboxylase* primer. *Ornithine decarboxylase* cDNA served as a standard marker (Osborne *et al.*, 1991). As controls, poly(A)$^+$RNA was also isolated from normal intact embryos at stages 7, 11 (corresponding to the animal quartet one day after isolation) and 13 (corresponding to the animal quartets two days after isolation). The same reverse transcriptase reaction and PCR procedures were applied.

The results in Fig. 7A confirmed that *goosecoid* was undetectable at stage 7 and detectable at stage-11 and -13 intact control embryos. In cultured animal quartets (Fig. 7B), *goosecoid* was not detectable in the animal quartet with a groove (one day after isolation) but it was detectable in the animal quartet with a protrusion (2 days after isolation). The fact that an animal quartet with a groove did not express a detectable level of *goosecoid* indicates that it is unlikely that the groove is a functional dorsal lip. However, since goosecoid was detectable in the animal quartet one day later, *goosecoid* expression may be delayed in cultured animal quartets or down-regulated one day after isolation.

α-cardiac actin mRNA was present in animal quartet explants with projections

Ariizumi *et al.* (1991) demonstrated that when animal cap explants were cultured in the presence of activin A, explants underwent dose-dependent morphological changes. At a low concentration the explants swell to become smooth and oval, and at higher concentrations the explants became elongated. The morphology of elongated animal cap explants resembles that of cultured animal quartets with a protrusion. Since activin A can induce mesoderm and form muscle (Asashima *et al.*, 1990; Thomsen *et al.*, 1990), the protrusions in the animal quartets observed in those studies were hypothesized to contain muscle.

The following specific question was posed: Can *α-cardiac actin* mRNA, a marker for mesoderm induction and subsequent muscle differentiation (Mohun *et al.*, 1986), be detected by PCR in isolated animal quartets containing a protrusion? The PCR conditions were identical to the procedure for *goosecoid* detection, and the reverse-transcribed cDNAs from the same reactions were utilized for amplifying *ornithine decarboxylase* cDNA, goosecoid cDNA and *α-cardiac actin* cDNA. Figure 7A and 7B shows that *α-cardiac actin*, nominally detectable in stage-13 control embryo, was detected in an animal quartet with a protrusion (lane 2P) but undetectable in two animal quartets without protrusions (lanes 1G and 2N). This observation supports the idea that animal quartets containing a protrusion differentiate, at least partly, into muscle.

In order to detect the spatial pattern of α-cardiac actin expression, whole-mount *in situ* hybridization was performed using a digoxigenin-labeled antisense *α-cardiac actin* cDNA probe. 56% (n=9) of the μg-treated animal quartets cultured for two days exhibited clear positive labeling. Figure 8 shows a representative animal quartet exhibiting a protrusion clearly displaying *α-cardiac actin* transcripts within its protrusion. This provides direct evidence that muscle is formed in a protrusion. 1g (control) animal quartets displayed no protrusions and no labeling (n=9). Internal-control (whole stage-18 and -22 embryos included in the hybridization) showed labeling restricted to the myotomes (data not shown). This *in situ* result is consistent with a previous histological analysis by Kageura and Yamana (1983) where a protrusion in a cultured animal quartet consisted mainly of muscle, melanophores and a cement gland as well as epidermis.

Taken together, all the results presented in this report support the notion that the altered partitioning of vegetal morphogenetic information in animal quartets is a primary cause of differential morphogenesis and gene regulation. Since embryos treated with various gravitational forces apparently eventually develop nor-

mally (Neff *et al.*, 1993b) despite significant AVCR alteration, regulation must play a pivotal role in organizing the differential partitioning of morphogenetic information.

Materials and Methods

Embryo preparation

Xenopus laevis embryos were obtained by artificially fertilizing eggs from chorionic-gonadotropin-injected females by standard methods. Fertilized eggs were placed in Cultusak™ 6"X9" 5-chamber units (Falcon). Microgravity (μg) was simulated by placing chambers on the horizontal clinostat with a 0.5 cm radius at 6 rpm (Neff *et al.*, 1985, 1989; Yokota *et al.*, 1992). Hypergravity (3g: three times Earth's gravity) was simulated by placing chambers on a centrifuge with a 17.6 cm radius at 120 rpm. Ground controls (1g) were incubated on the desktop adjacent to the clinostats and centrifuges. Embryos were maintained in 20% Steinberg's solution and were staged according to Nieukwoop and Faber (1956).

Animal quartet culture

G-treated embryos were dejellied by 3.5% cysteine (Sigma C-7880) at the early 8-cell stage. Dejellied embryos were then immersed in Ca^{++} and Mg^{++}-free,100% Steinberg's solution for 20 min, and transferred to 67% Leibovitz solution (L-15) with a supplement of 0.1% bovine serum albumin. Using a pair of watchmaker's forceps, the vitelline membrane was removed and an animal quartet consisting of four animal blastomeres was isolated at the late 8-cell stage or the beginning of the 16-cell stage in agarose-coated dishes in L-15. Five hours after isolation, animal quartets were transferred to 10% Steinberg's solution and were incubated at 13°C. The morphogenesis of cultured animal quartets was examined one day and two days after isolation.

Animal cap culture

The animal caps from G-treated blastula embryos were cultured in MMR medium [MMR contains: 0.1 M-NaCl, 2 mM-KCl, 1mM-$MgSO_4$, 2 mM-$CaCl_2$, 5 mM-HEPES, 0.1 mM-EDTA, pH 7.8, and 1 ml/100 ml of Sigma A-7292 antibiotic/antimycotic (Kimelman and Kirschner, 1987)]. After manual removal of the jelly coats and vitelline membranes, the animal caps were dissected from the embryos using sharpened tungsten needles. To prevent natural mesoderm induction, care was taken to remove all adherent vegetal hemisphere cells from the explants. The explants were then transferred to 96-well plastic culture plates containing MMR medium and cultured at 15°C for three days.

PCR

Poly(A)$^+$ RNA was isolated from intact control embryos or cultured animal quartets using oligo(dU)-messenger affinity paper (Amersham) by a standard procedure. Isolated RNA was then reverse-transcribed by Moloney murine leukemia virus reverse transcriptase (BRL) using random hexamers. Reverse-transcribed cDNA was amplified by a routine PCR procedure for 30 cycles using a programmable thermal controller (MJ Research Inc.). The PCR temperature profile was: denaturation at 94°C for 30 seconds, primer annealing at 60°C for 30 seconds, and primer extension at 72°C for 30 seconds with 1.5 mM $MgCl_2$. An *ornithine decarboxylase* cDNA fragment (386 bps) was primed by 5'-gtcaatgatggagtgtatggatc-3' (upstream) and 5'-tccattccgctctcctgagcac-3' (downstream) (Bassez *et al.*, 1990), an *α-cardiac actin* cDNA fragment (252 bps) was primed by 5'-tccctgtacgcttctggtcgta-3' (upstream) and 5'-tctcaaagtccaaagccacata-3' (downstream) (Rupp and Weintraub, 1991), and a *goosecoid* cDNA fragment (142 bps) was primed by 5'-gcagaaaaagcggacgaacag-3' (upstream) and 5'-acactctatgtacagatcccac-3' (downstream) (Blumberg *et al.*, 1991). PCR products were electrophoresed on 2% agarose gels, and stained by ethidium bromide.

In situ hybridization

In situ hybridization with whole animal quartets was carried out by a modified procedure (Neff *et al.*, 1993a) of Hemmati-Brivanlou *et al.* (1990). Animal quartets were fixed in 3.7% formaldehyde. A digoxigenin-labeled antisense *α-cardiac actin* cDNA probe (252 bps) was synthesized by asymmetric PCR amplification with a primer concentration ratio of (50 downstream: 1 upstream).

Acknowledgments

We would like to thank K-J. Lee and C-Y. Ko for embryo preparations and in situ analysis; Susan Duhon for helpful discussion; Betsy Osborne for in situ analysis and photography; and Lawrence Washington for oligonucleotide synthesis.

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Accepted for publication: April 1994