Experimentally Provoked Double Axes Formation in
Xenopus laevis Embryos

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Xenopus laevis Embryo에서 실험적으로 유도한 2중체축의 형성

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요   약

*Xenopus laevis*의 수정란의 한천층을 cysteine용액으로 용해시킨후 다시 예리
한 forceps으로 vitelline membrane을 제거하였다. 막을 제거한 수정란을 제1
분열이 일어나기 직전 미래의 복측 (vental side)부분을 중력에 대하여 반대방
향인 위쪽으로 향하게 하거나 또는 중력에 마주 대하도록 아랫쪽으로 배열하
였다. 그후 tailbud 시기까지 발생시켜 2중체축 (double axis) 형성률을 조사
하였다. 그 결과 수정란을 동물 반구가 위로 향하는 자연상태와 다르도록 배
열하는 것은 2중체축 형성에 큰 요인으로 작용하지 못하였고 오히려 수정란의
외형을 유지시키는 물리적 구조(편평형 또는 구형)가 더 큰 요인임이 밝혀
졌다.
이와같은 관찰은 무미양서류 수정란의 극성 결정 메카니즘과 배의 제1차
유도작용을 연구하는데 좋은 자료를 제공하여 준다.

INTRODUCTION

The determination of the polarity of the amphibian egg has historically been considered one of the most important research problems in experimental emryology. Since the turn of the century, beginning with Born's classical studies on crescent formation (Born, 1885), attention has focused on the mechanism employed by the egg to regulate the pattern of early embryogenesis. From the earlier observations (e.g., Schultze, 1894; Penners and Schleip, 1928 a, b; Motomura, 1935; Dalcq and Pasteels, 1937) a model emerged to account for the behavior of eggs held in novel orientations re. gravity. That

model has been thoroughly reviewed by Pasteels (1964). Its main feature is the proposal that following sperm penetration a rearrangement of the egg cytoplasm occurs to form a so called "vitelline wall" of vegetal yolk in the subcortical region of the dorsal marginal zone. That cytoplasmic localization is considered to have a causative effect on the subsequent induction of the dorsal mesoderm.

Support for that proposal has recently been offered by Gerhart *et al.* (1981). Those workers rolled de-membranated *Xenopus* eggs onto their sides so that the sperm entrance site (i.e., ventral side) faced upwards. Substantial proportions (20% in one experiment, 35% in a second experiment) of those eggs subsequently developed double axes. When 2 sets of eggs were similarly treated, but rolled onto their sides so that the ventral (sperm entrance site) side faced downwards, no twins developed. Out of a total of 34 eggs, from two different female frogs none were observed to develop double axes. Those observations were reinforced by a set of centrifugal force manipulations on eggs in various orientations.

As a distinct alternative to the above vitelline wall model, originally developed by Dalq and Pasteels (1937), is a recent proposal (Weyer *et al.*, 1980) that a diffusion mechanism accounts for mesoderm induction in amphibian embryos. The diffusion of a single morphogen, accompanied by a continual decay process has been mathematically simulated by those authors.

Needless to say, those two types of models (distinct cytoplasmic localization vs. diffusion gradient) need not be considered mutually exclusive. First, the gradient may be produced by elements contained in the "vitelline wall" localization. Second, the diffusion model has been proposed for the urodele egg, and the cytoplasmic localization model for the anuran egg. Perhaps those two orders of amphibia have indeed evolved separate mechanisms for the polarity determination which leads to polarization of the mesoderm.

In order to further investigate the phenomenon of twinning in amphibian embryos the present studies were carried out. Several goals were established at the outset, including the following: (1) to collect data which would contribute to an understanding of the extent to which either of the above types of models for polarity determination might be appropriate, and (2) to develop non-surgical methods which might be routinely employed for inducing double axes in amphibian embryos. Such methods could be useful in studying cytoplasmic distribution in early embryogenesis (e.g., Dreyer *et al.*, Malacinski *et al.*, 1980).

METHODS AND MATERIALS

Xenopus laevis eggs were obtained using standard procedures – injection of human chorionic gonadotropic hormone (500~1,000 IU) into gravid females – for artificial insemination. About 20 minutes after fertilization when the eggs finished rotation i.e., anmial hemisphere facing opposing gravity and vegetal hemisphere gravity side, they were de-
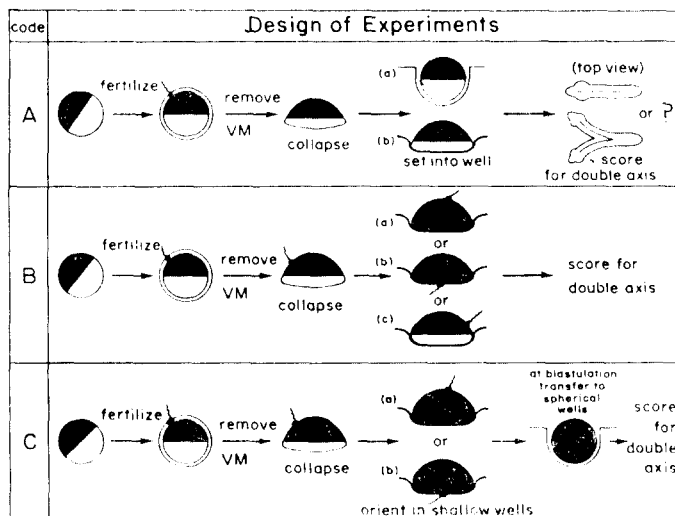
Fig. 1. Design of the experimental manipulations which provoked double axes formation.

jellied in a 1.75% cysteine solution (pH 7.6) and immersed in 10% Steinberg's solution. Using watchmaker's forceps the vilelline membrane was removed shortly before the first cleavage furrow was formed, or just after it formed. Eggs were oriented in either round shaped or flat wells formed in a plasticine (modeling clay) platform in 100% Steinberg's solution containing 400 mg of penicillin and 400 mg of streptomycin per liter. Unless stated otherwise, the data from eggs obtained from different female frogs was not pooled.

Double axes were easily recognized and were virtually always of "substantial" type (see Malacinski *et al.*, 1980, for a description of *Xenopus* secondary axis).

RESULTS

The experimental manipulations were designed to answer a series of questions, each of which pertains to one or another aspect of the conditions which provoke twinning in *Xenopus* embryos:

Does removal of the vitelline membrane (VM) and the subsequent collapse of the egg suprastructure lead to twinning of axial structures (Expt. A)?

The VM was removed at about the first cleavage and the eggs were transferred to either of two types of wells (Fig. 1, A). The deep well (a) cosntricted the egg slightly, so that its natural spherical shape was retained throughout development from the early cleavage stages to early neurulation. The shallow flat well (b) permitted the developing embryo to proceed through gastrulation in a somewhat collapsed condition. At the tailbud stage embryos were scored for the presence of double axes. As the data in Fig. 2 reveal, double axes frequently developed from embryos which were maintained in a collapsed state,

Expt. A	axis development	shape of developing embryo	
		spherical (a)	collapsed (b)
	single	121	43
	double	7	19
	excessive leakage (discarded)	19	37

Expt. B	axis development	orientation		
		ventral side up (a)	dorsal side up (b)	animal (c) hemisphere up
	single	2 (22)	3 (13)	19 (52)
	double	11 (8)	10 (4)	10 (5)
	excessive leakage (discarded)	11 (38)	12 (41)	2 (18)

Expt. C	axis development	orientation	
		ventral side up (a)	dorsal side up (b)
	single	20 (5)	20 (4)
	double	5 (6)	7 (3)
	excessive leakage (discarded)	17 (17)	13 (20)

Fig. 2. Data for experiements A-C. Numbers in parenthesis refer to results of duplicate experiments with eggs from different female frogs.

but seldom developed in embryos which were maintained in their natural spherical condition. The absence of the physical constraint normally provided by the vitelline membrane appears, therefore, to be responsible for the development of double axes.

Figure 3 contains photographs of embryos which manipulated according to the protocols for experiments A, B, and C. The embryos which developed in deep wells cleaved normally, (Fig. 3 a-e) while those in shallow wells usually bifurcated at first cleavage (Fig. 3 f-j). The data for experiment A (Fig. 2) represent the results of manipulations on eggs from three different female frogs. Excessive leakage was occasionally observed to follow the removal of the VM, and was probably due to damage inflicted to the egg cortex by the experimenter's forceps. Such damaged eggs appeared more frequently among the eggs which were maintained in shallow wells. They were routinely discarded.

Is the dorsal/ventral orientations of the embryo important for generating double axes in collapsed eggs (Expt. B)?

Collapsed eggs were set into shallow wells in either of 3 different orientations (Fig. 1, B): on their side with the sperm entrance site (ventral side) up (a); on their side
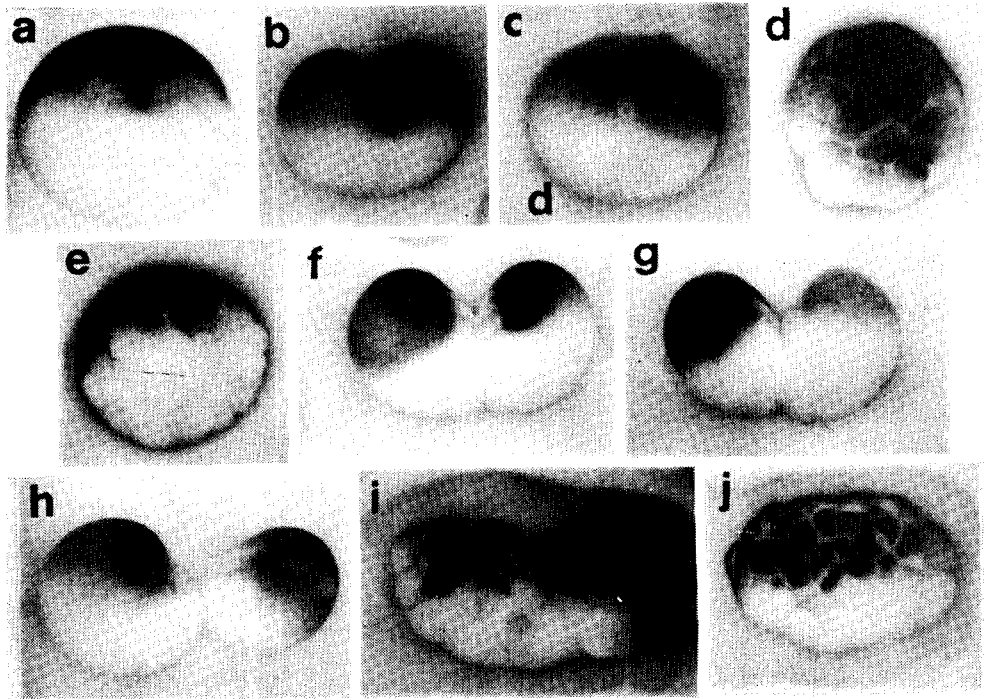
Fig. 3. Typical development of eggs in deep (a-e) and shallow (f-j) wells: (a) eggs oriented with the sperm entrance site "up" (opposing gravity); (b) first cleavage in egg with the sperm entrance site "up"; (c) first cleavage in egg with the sperm entrance site "down" (facing gravity); (d) early cleavage; (e) later cleavage. At the early gastrulation stage a few embryos displayed 2 dorsal lips. They developed into tail bud stage embryos which exhibited twinned axial structures (Fig. 4). Eggs which developed in shallow wells typically displayed a bifurcation at the first cleavage stage; (f) first cleavage in an egg oriented with the sperm entrance site "up"; (g) sperm entrance site "down"; (h) completion of first cleavage; (i) early cleavage; (j) later cleavage. Typically, at the blastula stage the extent of the initial bifurcation seen in (f)-(h) had diminished substantially. Eggs which developed in shallow wells (f-j) more frequently developed double axes than eggs which developed in deep wells (a-e) (Fig. 5).
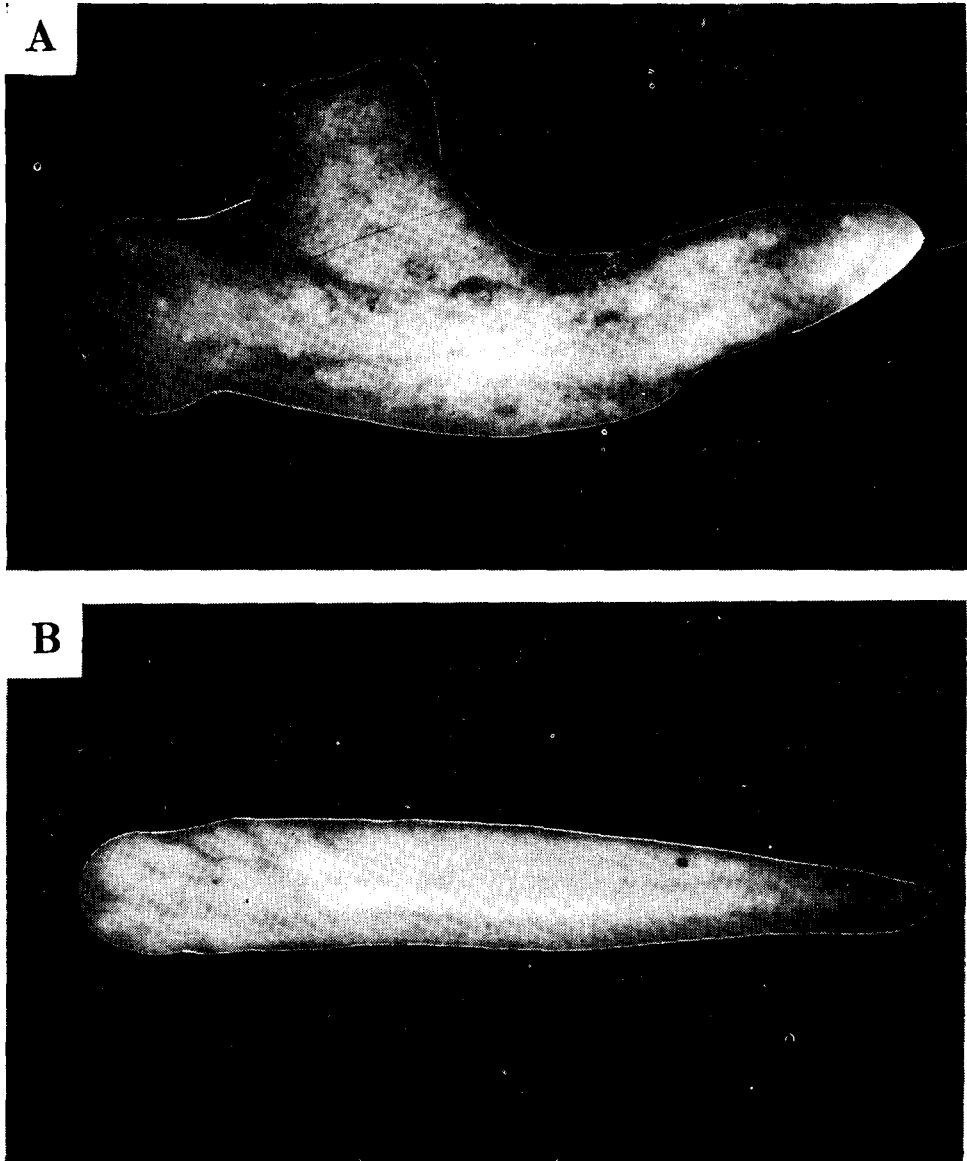
Fig. 4. Examples of embryos which displayed double axes (A) and normal control (B) at the tailbud stage ×20.

with the dorsal side up (sperm entrance site down) (b); or with the entire animal hemisphere facing upwards (natural orientation) (c). Embryos which developed on their side (a) and (b), *regardless of the orientation* of the dorsal/ventral axis, displayed a greater tendency to develop double axes than did embryos which were oriented in their natural (albeit collapsed) animal/vegetal hemisphere configuration(Fig. 2, B). The significant observation was made that there was no difference between the frequency of twinning in ventral vs. dorsal side up eggs.

Is there substantial variability in the extent of leakage and the frequency of double axes formation among different clutches of eggs?

The variability in the 2 sets of data obtained for Expt. B was substantial. Although the data for each of the 2 sets was clearly internally consistent, the variability was further examined by repeating Expt. B with the eggs of 4 other females (Fig. 5). The frequency of double axes formation varied from 5% to 24%. Likewise, the tendency of uncleaved, demembranated eggs to leak cytoplasm varied widely (15% to 73%) among the 4 clutches of eggs. Those variabilities do not weaken the conclusions that are drawn from data which is internally consistent (e.g., Fig. 2, Expt. B). Those observations do, however, underscore the need for evaluating the data from eggs of different female frogs separately.

Is the frequency of double axes for eggs in either dorsal or ventral orientation altered if they are permitted to gastrulate in a natural (spherical) state (Expt. C)?

The frequency of double axes formation in embryos from which the VM was removed could be substantially diminished if the embryos were inserted into spherical wells at the late blastula stage (data not shown). A determination of the influence of dorsal/vantral orientation on twinning frequencies was carried out. Collapsed eggs were placed in shallow wells and rolled onto their sides (Fig. 1, C) so that the sperm entrance site (future ventral side) faced either up (a), or down (b). At the early blastula stage they were

female ##	single axis (## embryos)	double axis (## embryos)	excessive leakage (discarded)	double axes / single + double axes
1	13	4	47	.24
2	56	6	11	.10
3	52	3	18	.05
4	21	6	40	.22

Fig. 5. Comparison of the frequency of double axes formation among eggs from different female frogs. Since the dorsal/ventral orientation of collapsed eggs (shallow wells) was not an important factor in determining the extent of twinning (Fig. 2, B), the eggs were oriented on their sides in a random fashion.

transferred to deep (spherical) wells so that they would maintain a natural spherical shape during gastrulation and subsequent organogenesis. The data in Fig. 2 further reveal that the dorsal/ventral orientation of the egg is *not* an important factor in generating double axes, as was first demonstrated in experiment B. The data in Fig. 2, C represent 2 extreme cases of variability: one set of data displayed a low frequency of double axes, while the other set displayed a very high frequency. In both cases, however, no significant difference between the dorsal/ventral orientations was observed.

DISCUSSION

From these studies it is clear that the frequency of twinning varies substantially among different batches of eggs. Likewise, the ability of different batches to withstand the manual removal of the vitelline membrane varied considerably among batches of eggs. Nevertheless, the frequency of twinning did not depend upon whether the ventral side faced up or down. A remarkably similar proportion of eggs oriented either ventral side up or dorsal side up developed double primary embryonic axes. It is difficult to reconcile those observations with models developed by Pasteels (reviewed in Pasteels, 1964) which involve a distinct cytoplasmic localization of vegetal yolk on the dorsal side of the egg. That localization would necessarily have to be divided into two segments in eggs which were rolled onto their sides with the sperm entrance site (ventral side) facing up (opposing gravity). Some support for that prediction was reported by Gerhart *et al.* (1981). In the present studies, however, no such distinction between the results of ventral vs. dorsal side up orientations was observed. The present observations do not, therefore, support the cytoplasmic locali-zation (vitelline wall) concept.

By examining the eggs which developed after manual removal of the vitelline membrane (Fig. 3) it is clear that "shallow well" embryos displayed a mechanical bifurcation. Although they remained connected by a cytoplasmic bridge, "shallow well" eggs resembled the bifurcated eggs originally described by Schultze (1894). Inversion experiments were carried out by Schustze which led to the interpretation (by Schultze) that twins were the product of the separation of the 2 blastomeres at first cleavage—each blastomere eventually developing a separate set of axial structures. That is, the mechanical separation of the blastomeres was the causative event on the eventual development of double axes. Penners and Schleip (1982 a, b), however, offered the idea that twinning in inverted embryos was the product of yolk movements. Pasteels (1983) later also reinter-preted the Schultze experiments and developed the concept that a gradient of yolk inter-acted with the cortical cytoplasm.

Clearly, the results of the present investigation favor the interpretation originally proposed by Schultze (1894)—that simple mechanical constraints act to generate twins in eggs exposed to novel gravity orientations.

Furthermore, recent investigation clearly revealed that Xenopus eggs inverted before fertiliation (PreFo inverted eggs) have capacity to develop normally without formation of a distinct vitelline wall (Neff *et al.*, 1983). In those eggs drastic yolk (or cytoplasm) shift occurred before first cleavage. However, cytoplasmic mobilty is somewhat different from batch to batch. Some eggs have fast cytoplasmic mobility (high mobility of cytoplasm) and others have low or intermediate mobility (Neff *et al.*, 1984) Therefore, it might be guite interesting to examine the possibility that a correlation exists between the cytoplasmic mobility and the freguency of twin formation.

The resolution of the distinction between the segregation of discrete cytoplasmic localizations (e.g·, vitelline wall) and more subtle morphogen diffusion mechanisms must await further experimentation. Perhaps a precise cytological identification and characterization of the vitelline wall and its localization in eggs exposed to novel gravity orientations would be useful.

ABSTRACT

Xenopus laevis eggs were de-jellied and manually manipulated to remove their vitelline membranes. They were then positioned in a variety of orientations re. gravity. That is the future ventral side was located upwards (opposed gravity) or downwards (faced gravity). Development through the tailbud stage was observed and the frequency of double axes formation recorded. Orientation of the egg re. its natural polarity was not an important factor in generating double axes. Its physical structure (flattened re. spherical), however, appeared to be important in determining the frequency of twinning.

These observations give insights into the mechanism of polarity establishment in anuran eggs, and provide methods which should be useful for studies on primary embryonic induction.

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