ZOOLOGICAL SCIENCE 4: 489–496 (1987)


# Twin Formation in *Xenopus laevis* Eggs Centrifuged before First Cleavage

MUTSUKI KUNIEDA and MASAMI WAKAHARA[1]

*Zoological Institute, Faculty of Science, Hokkaido University, Sapporo 060, Japan*

**ABSTRACT**—Twin formation was studied in the fertilized *Xenopus* eggs by means of low speed centrifugation (15–30×*g*) for a short period (20 sec to 5 min) before first cleavage. Dejellied eggs were embedded in gelatin with a vertical orientation (animal pole up), but randomly oriented with reference to the sperm entrance site. They were then spun with the centrifugal force vector at right angles to the animal-vegetal axis. When the eggs were centrifuged at T=0.3–0.6 (30–60% of the time interval to first cleavage), a large number of twin embryos developed, whereas when centrifuged after T=0.7 twin embryos were almost never obtained. Morphological features of the twin embryos were briefly described. They showed a rather continuous spectrum from almost normal to completely double-axes embryos. The twin embryos were tentatively grouped into 6 categories based on the degree of twinning. Twin embryos with clear double-axes were divided into 3 groups in regard to the spatial relationships between the two axes. All double-axes embryos developed from the gastrulae which had shown two blastoporal lips. Possible mechanisms of twinning are discussed in terms of a postcentrifugal modification of the localization of the "dorsal determinants" which specify the future dorsal side of the embryos.

## INTRODUCTION

It is well known that an amphibian egg can produce two complete embryos after separation of the blastomeres at the 2-cell stage [1]. This leads us to conclude that an amphibian egg has an ability to produce two complete sets of axial structures, including such dorsal axial structures as notochord, neural tube and somites. In fact, several experimental treatments of amphibian eggs are known to cause twin formation: Delayed fertilization (overripeness of eggs) in *Rana pipiens* [2]; rotation of eggs in *R. nigromaculata* [3] and *Xenopus laevis* [4–6]; and centrifugation of eggs in *Bufo vulgaris* [7] and *X. laevis* [5, 8, 9]. If an amphibian egg has the ability to produce two complete sets of axial strucutres, a regulation must naturally occur to insure that a single axial structure rather than two axes normally develops. Thus, analysing the twin by means of centrifugation or egg rotation will aid in understanding the development of dorsal-ventral polarity and also will provide information concerning the regulation of pattern formation in normal development.

We have recently succeeded to demonstrate possible existence of the "dorsal determinants", which specify the future dorsal side of the embryos, in fertilized *Xenopus* eggs by means of cytoplasmic withdrawal experiments [10]. At present, it seems much important to know the behavior of the "determinants" during the first cell cycle to elucidate the mechanisms of the establishment of the dorsal-ventral polarity in amphibian eggs. Twin formation by centrifugation is expected to be a biological tool for analysing the localization of the "dorsal determinants".

In this paper, we first describe the morphological features of twin embryos developed from the centrifuged *Xenopus* eggs, and then describe the conditions of centrifugation for obtaining twin embryos. The results obtained support the view that the postcentrifugal modification of the localization of the "dorsal determinants" which specify the future dorsal side of the embryos [10] causes

Accepted January 21, 1987
Received November 8, 1986
[1] To whom reprints should be requested.

the twin formation.

## MATERIALS AND METHODS

*Source of embryos*

The two colonies of *Xenopus laevis* which are maintained in our laboratory, J and HD groups [11] were used. Eggs from hormone-stimulated female were rinsed briefly with Steinberg's solution and then inseminated with a testis homogenate. Eggs and embryos were usually maintained at 15°C unless mentioned otherwise [12]. Eggs before first cleavage were staged according to the fraction of the interval between fertilization (T=0) and first cleavage (T=1.0) at the time of the relevant manipulation. Embryos were staged according to Nieuwkoop and Faber [13].

*Embedment of eggs in gelatin*

Twenty minutes after fertilization eggs were dejellied in 2.5% thioglycolic acid (pH 8.2) and washed four times in 10% Steinberg's solution. Nine percent gelatin was prepared according to Black and Gerhart [14], with some modifications: penicillin G potassium (100 IU/ml) and streptomycin sulfate (0.1 mg/ml) were added instead of gentamycin. Approximately 40 eggs were pipetted at once into molten 9% gelatin (25°C) and then tranferred to a plastic dish (Falcon 1008) containing 2.5 ml molten gelatin. Almost all eggs maintained their normal, vertical orientation (animal pole up) in the molten gelatin. The fewer eggs which had rotated or inverted when tranferred were quickly oriented with forceps to orient the animal pole up. Eggs were aligned approximately along the center line of the dish so that all the eggs could be exposed to the similar centrifugal forces. After the eggs were arranged the dish was placed on an ice-chilled copper plate for 2 min and then placed in a 15°C water bath for 10 min to complete the solidification of the gelatin.

*Centrifugation of embedded eggs*

Dishes with eggs were mounted on a holder made from a plastic centrifuge tube. The holders were placed on the swing rotor of a clinical centrifuge and spun at 15°C. The duration of centrifugation indicated below included only the time while the given centrifugal forces were operating (i.e. acceleration and deceleration periods of approximately 1 min were not included in the "duration"). The centrifugal force vector was at right angles to the animal-vegetal axis of the eggs. Since the eggs were not oriented to bring the sperm entrance site (SES) to the same direction when embedded in the gelatin, the centrifugal force vector varied from egg to egg with respect to the SES.

*Observation of embryos*

After centrifugation eggs were incubated at 15°C in the normal, vertical orientation (animal pole up) in gelatin. When the embryos developed to stage 4 (8-cell stage) they were freed from the gelatin by incubating the dish in 35°C water bath for a short period (1–2 min). Embryos freed from gelatin were washed twice with 10% Steinberg's solution. Developing embryos were periodically observed until they developed to stage 29. Dead and abnormal (except for twinning) embryos were discarded. Eggs and embryos were fixed with a modified Bouin-Holland or Smith's fixative. Serial sections of 8 $\mu$m thick were stained with Delafield's hematoxylin and eosin.

## RESULTS

*Brief description of morphological features of twin embryos which developed from centrifuged eggs*

As a starting point for experimental analysis, a general description of the morphological features of twin embryos which developed from centrifuged *Xenopus* eggs was given. Although Black and Gerhart [9] described in detail the conditions of centrifugation for obtaining twin embryos, there has been no systematic description on twin embryos developed from the centrifuged eggs.

Approximately 400 "twin embryos" (which included complete and incomplete double-axes embryos and single-axis embryos with a "wide" sucker; cf. "imbalanced embryos" of Cooke [6]) were obtained. They showed a continuous spectrum in external and internal morphology from

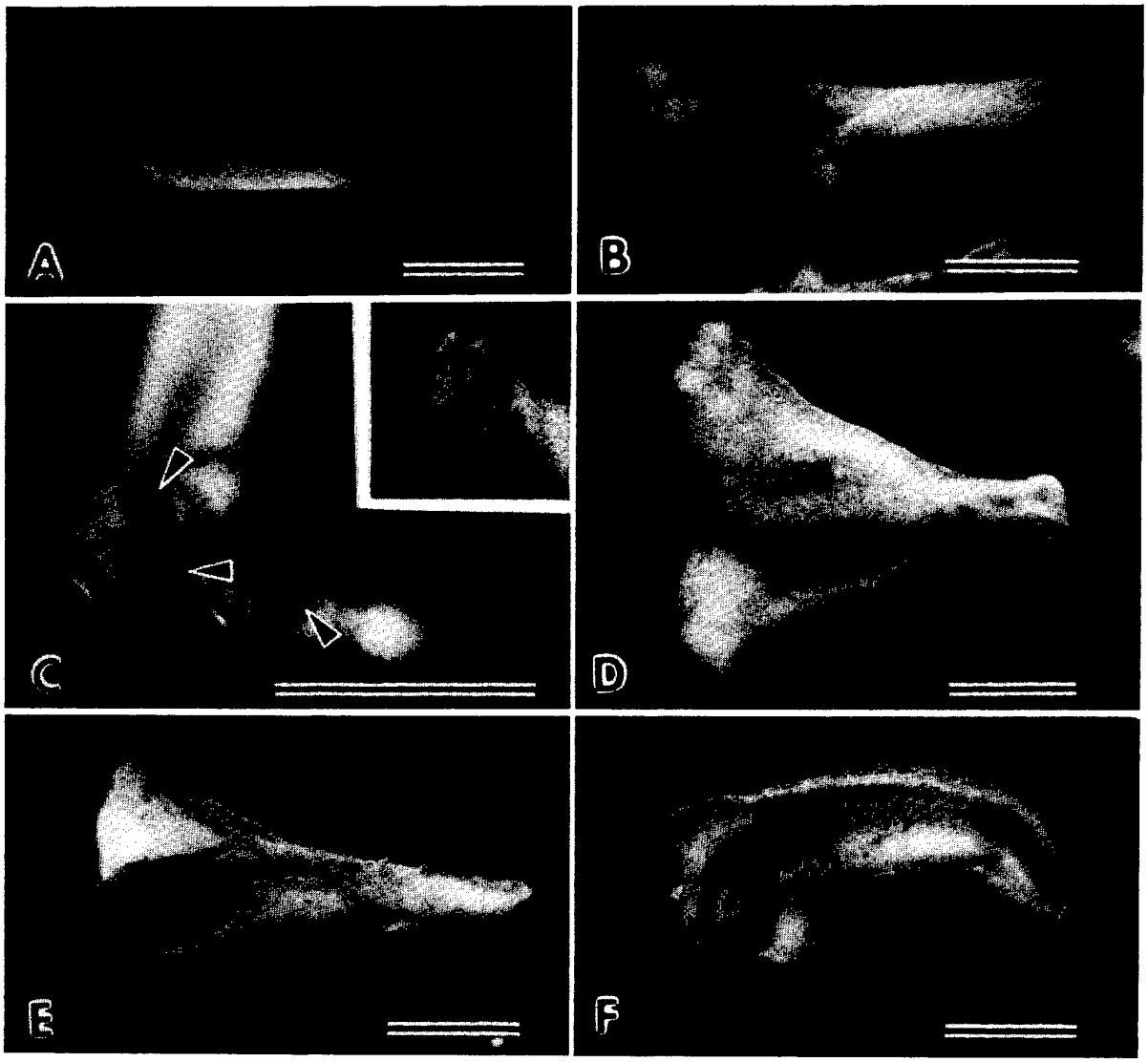


FIG. 1. Typical external views of the "twin embryos" developed from the eggs centrifuged before first cleavage, showing 6 types of twinning, which were divided tentatively based mainly upon the external morphological features. (A) Normal control embryo. Lateral view. (B), Type 1, almost normal but displaying a small protuberance. Lateral view. (C), Type 2, single-axis embryo but containing an apparent wide sucker (an arrowhead). Type 3, single-axis embryo but containing two distinct suckers (two arrowheads). Compare with normal control embryo (inset). Frontal view. (D) Type 4, double-axes embryo with no distinct sucker. Lateral view. (E), Type 5, double-axes embryo one of which contains a distinct sucker, but the other lacks a sucker. Dorsolateral view. (F), Type 6, double-axes embryo both of which show distinct suckers respectively. Lateral view. Bars: 1 mm.

almost normal to complete double-axes. We divided these embryos into the following 6 categories, based mainly upon the external morphological features (Fig. 1):

Type 1: almost normal (single-axis) embryos, but displaying a small protuberance similar to a secondary embryo (Fig. 1B).

Type 2: almost normal (single-axis) embryos, but containing a wide sucker (Fig. 1C).

Type 3: single-axis embryos with two distinct suckers (Fig. 1C).

Type 4: double-axes embryos with no distinct sucker (Fig. 1D).

Type 5: double-axes embryos, one of which contains a distinct sucker but the other lacks a sucker (Fig. 1E).

Type 6: double-axes embryos both of which show distinct suckers (Fig. 1F).

Of these 6 types, only Types 4, 5 and 6 showed complete duplication of external dorsal axial struc-

tures. Internal inspections revealed, however, similar indication of duplication of dorsal axial structures (such as somites) even in Types 1, 2 and 3 embryos. Thus, all embryos that showed the morphological features described above (Type 1–Type 6) were tentatively designated as "twin embryos".

Figure 2 shows typical internal structures of several twin embryos. In general, Type 6 twins (two axes–two suckers) contained two complete sets of dorsal axial structures in the half with the sucker and one incomplete set of the axial struc-

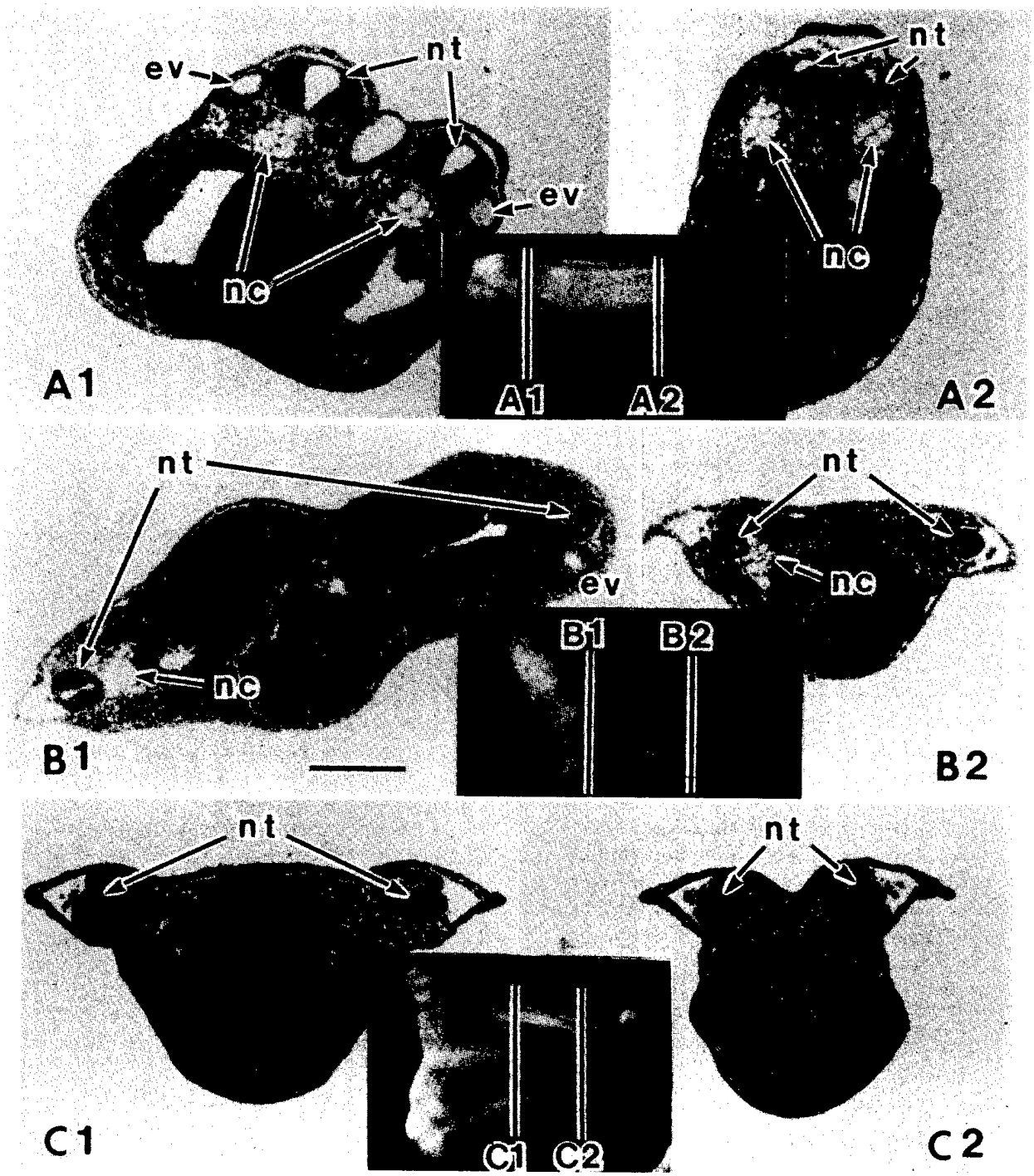


FIG. 2. Internal structures of twin embryos developed from the centrifuged eggs. The level of the sections was indicated in the inserted figures. (A), Type 6 twin (two axes—two suckers), showing two distinct neural tubes (nt) and notochords (nc). (B), Type 5 twin (two axes—one sucker), showing two neural tubes but one distinct notochord. (C), Type 4 twin (two axes—no sucker), showing two neural tubes but no distinct notochord. ev, ear vesicle. Bar: 100 $\mu$m.

tures in the other half. The notochord was underdeveloped in this half and so only one intact notochord was observed in the histological sections (Fig. 2B). Similarly, Type 4 twins (two axes-no sucker) showed two incomplete sets of dorsal axial structures: notochordal structures were underdeveloped, but neural tube-like structures were usually developed in both halves of the embryo (Fig. 2C).

Spatial relationships between the two axes were different among embryos regardless of the type of the twinning. Typically, twin embryos were divided into three groups; face-to-face (Fig. 2B), side-by-side (Fig. 2A) and in between (Fig. 2C). The spatial relationship depended upon the original location of the two dorsal lips of the blastopores, or the direction of the invaginations (Fig. 3). Face-to-face twins originated from the embryos which had shown the two dorsal lips just opposite to each other (Fig. 3C). Side-by-side twins were derived from the embryos which had had two distinct dorsal lips on one side of the embryo (Fig. 3B).

*Relationship between centrifugation conditions and frequency of twin formation*

Eggs were centrifuged at T=0.4–0.6 with two centrifugal forces (15 and 30×*g*) for different periods to establish optimal conditions of centrifugation for obtaining twin embryos (Table 1). The centrifugal force vector was at right angles to the animal-vegetal axis. The data in Table 1 represent 6 experiments using 3 different females. As controls, 384 eggs were embedded in gelatin but not centrifuged. They were freed from the gelatin after stage 4 and maintained in 10% Steinberg's solution thereafter in the same fashion as the centrifuged embryos. Approximately half of them were alive at stage 29. Only one embryo from 384 eggs (0.2% of eggs used or 0.5% of embryos alive at stage 29) twinned. In contrast, many twinned embryos developed from centrifuged eggs: 46 twins (13.6% of eggs centrifuged or 31.1% of the embryos alive at stage 29) developed from 339 eggs centrifuged at 30×*g* for 1 min. Although variations in survival and frequency of twin formation were considerably large from one experiment to another, it was clear that low speed centrifugation caused twin formation. When the eggs were spun at 15×*g* for 1 min or 5 min, a high frequency twinning occurred. At a higher centrifugal force (30×*g*) shorter periods of centrifugation (20 sec or 1 min) was sufficient for high frequency twinning.

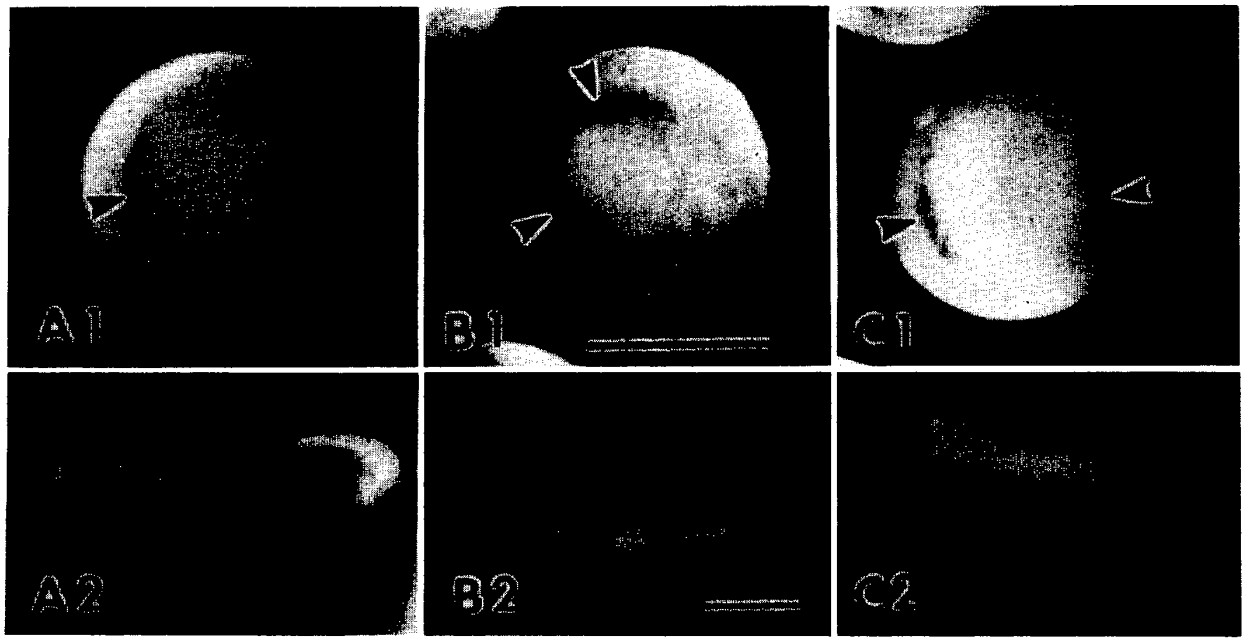


FIG. 3. Bottom views of the early gastrula embryos, showing the spatial relationship of two dorsal lips of the blastopore (upper, arrowheads) and the resulting tailbud embryos (bottom). (A), Normal control. (B), Typical "side-by-side" type twin. (C), Typical "face-to-face" type twin. Bars: 1 mm.

TABLE 1. Frequency of twin formation under various centrifugation conditions*

| Conditions of centrifugation | | No. of eggs centrifuged | No. of embryos at stage 29 alive | twinnned |
|---|---|---|---|---|
| 1×g | 0 min | 384 | 187 (48.7%) | 1 ( 0.5%)** |
| | 20 sec | 331 | 64 (19.3%) | 4 ( 6.3%) |
| 15×g | 1 min | 257 | 108 (42.0%) | 25 (23.1%) |
| | 5 min | 153 | 34 (22.2%) | 7 (19.3%) |
| | 20 sec | 335 | 88 (26.3%) | 17 (19.3%) |
| 30×g | 1 min | 339 | 148 (43.7%) | 46 (31.1%) |
| | 5 min | 161 | 9 ( 5.6%) | 1 (11.1%) |

* Fertilized *Xenopus* eggs from 6 different females were centrifuged at T=0.4−0.6, with a centrifugal force vector at right angles to the animal-vegetal axis.
** Percent live embryos at stage 29 which displayed twinning.

*Stage sensitivity of the egg to centrifugation*

In a previous study [14], the exact stage sensitivity of the egg to centrifugal effects on modification of the orientation of *Xenopus* embryonic axis was determined during first cell cycle, showing three different phases of the centrifugal effects; prior to T=0.4, during T=0.4–0.7 and after T=0.7. Thus, we attempted to determine the stage sensitivity of the egg to centrifugal effects on the twin formation. Eggs were centrifuged at 15×*g* for 5 min at various periods in the first cell cycle. The centrifugal force vector was at right angles to the animal-vegetal axis of the eggs. Figure 4 shows data summarized from 6 experiments using 6 different females. Although there were wide variations in the rate of twin formation among the experiments, it was clear that twin embryos developed largely from eggs centrifuged in the period of T=0.3–0.6. No twin embryos were observed from eggs centrifuged after T=0.7.

## DISCUSSION

It is clear that low speed centrifugation (15 or 30×*g*) at right angles to the egg's animal-vegetal axis causes twin formation in *Xenopus laevis* embryos. The results reported here are consistent with the recent studies by Black and Gerhart [9]. Thus, a fertilized *Xenopus* egg has an ability and probably materials to produce two complete sets of the body plan.

Since every twin embryo was always developed

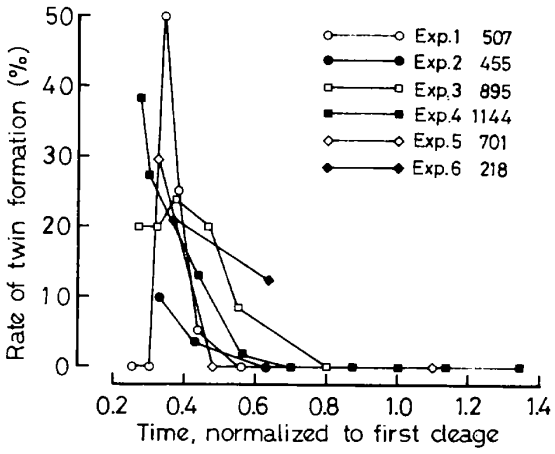


FIG. 4. Stage sensitivity of *Xenopus* eggs to centrifugation on the twin formation. Fertilized eggs from 6 different females were centrifuged (15×*g* for 5 min) at various periods in the first cleavage, with a centrifugal force vector at right angles to the animal-vegetal axis of the eggs. Number on right-top indicates the numbers of eggs centrifuged in each experiment. No twin embryos were observed from eggs centrifuged after T=0.7.

from the gastrula which showed two dorsal lips and furthermore the spatial relationship between the two axes of twin embryos depended upon the original location of the two blastoporal lips (Fig. 3), twin formation relates inevitably to the problems how and where the dorsal lip is specified, or more generally, of the establishment of the dorsal-ventral polarity during the early development of embryos. Because the place where cells of the blastopore start to invaginate is known to make the future dorsal side without exceptions [10], the location of the dorsal lip can serve as a manifestation of the ultimate dorsal-ventral polarity. In this respect, the dorsal-most vegetal cells of the 32- or 64-cell *Xenopus* embryo are reported to contain a set of determinants which enable them to induce neighboring cells to undertake dorsal axis formation [15, 16]. Furthermore, the dorsal-vegetal cells of the 8-cell embryo are known to receive already an ability to form dorsal axial structures [17, 18]. Provided that the set of "dorsal determinants", which are recently demonstrated to exist in fertilized *Xenopus* eggs by means of cytoplasmic withdrawal experiments [10], specifies the location of the dorsal lip, twin formation by centrifugation is considered to be involved in altered localization of the "dorsal determinants" after centrifugation of egg. That is, normal arrangements of the egg cytoplasm are modified by centrifugation or post-centrifugal rearrangements or the egg contents [14]. Presumably, a bifurcation of the "dorsal determinants" occurs. However, the mechanisms involved in the modification of the localization or partitioning of the "dorsal determinants" by centrifugation is complex and still remained to be clarified.

Recent studies have clearly demonstrated that centrifugations cause a stage-specific modification of the dorsal-ventral polarity: When centrifuged before T=0.4, the future dorsal side is specified in the centrifugal side of the eggs but in the centripetal side of the eggs after T=0.4 centrifugation, irrespectively of the sperm entry site [14]. Centrifugation between T=0.4–0.45 causes twin formation [9]. This stage-specific effect of centrifugation for obtaining twin embryos was confirmed in this work (Fig. 3). Presumably, the stage-specific effects of centrifugation on the modification of the future dorsal-ventral polarity are involved in the drastic changes in the cytoplasmic consistency during the first cell cycle of the fertilized eggs [19].

This is the first description of the morphological diversity of twin embryos developed from the centrifuged eggs. As illustrated in Figures 1 and 2 they showed a rather continuous spectrum in external and internal morphology from an almost normal to a complete double-axes embryos. This variability in twinning is probably due to the degree and localization to which bifurcation of the dorsal determinants after the centrifugation occurs: When the "dorsal determinants" are divided evenly into two groups of the vegetal blastomeres at 32- or 64-cell embryo [15, 16], complete double-axes embryos will result, whereas uneven partitioning of the "determinants" results in incomplete twin embryos. Spatial relationship between two axes was also shown to be continuous from face-to-face to side-by-side twin (Fig. 3). When the "determinants" are divided into just opposite to each other face-to-face twin will result, whereas partitioned into two distinct sites on one side of the egg results in side-by-side twin. Twin embryos developed from the centrifuged eggs were divided into 6 categories to allow further quantitative analyses of the centrifugal effects on twinning and the modification of the "dorsal determinants".

## ACKNOWLEDGMENTS

We are grateful to Prof. G. M. Malacinski (Indiana University, Bloomington, Indiana) for his critical reading of the manuscript and valuable discussions. Supported, in part, by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (60440100, 60540452).

## REFERENCES

1 Kageura, H. and Yamana, K. (1983) Pattern regulation in isolated halves and blastomeres of early *Xenopus laevis*. J. Embryol. Exp. Morphol., **76**: 221–234.

2 Witschi, E. (1952) Overripeness of the egg as a cause of twinning and teratogenesis: A review. Cancer Res., **12**: 763–786.

3 Kubota, T. (1967) A regional change in the rigidity of the cortex of the egg of *Rana nigromaculata* following extrusion of the second polar body. J.

Embryol. Exp. Morphol., **17**: 331–340.

4 Kirschner, M., Gerhart, J. C., Hara, K. and Ubbels, G. A. (1980) Initiation of the cell cycle and establishment of bilateral symmetry in *Xenopus* eggs. Symp. Soc. Dev. Biol., **38**: 187–215.

5 Gerhart, J., Ubbels, G. A., Black, S., Hara, K. and Kirschner, M. (1981) A reinvestigation of the role of the grey crescent in axis formation in *Xenopus laevis*. Nature, **292**: 511–516.

6 Cooke, J. (1986) Permanent distortion of positional system of *Xenopus* embryo by brief perturbation in gravity. Nature, **319**: 60–63.

7 Motomura, I. (1935) Determination of the embryonic axis in eggs of amphibia and echinoderms. Sci. Rep. Tohoku Univ. (Ser. 4), **10**: 211–245.

8 Young, R. S., Deal, P. H., Souza, K. A. and Whitfield, O. (1970) Altered gravitational field effects on the fertilized frog egg. Exp. Cell Res., **59**:267 –271.

9 Black, S. D. and Gerhart, J. C. (1986) High-frequency twinning of *Xenopus laevis* embryos from eggs centrifuged before first cleavage. Dev. Biol., **116**: 228–240.

10 Wakahara, M. (1986) Modification of dorsal-ventral polarity in *Xenopus laevis* embryos following withdrawal of egg contents before first cleavage. Dev. Growth Differ., **28**: 543–554.

11 Katagiri, Ch. (1978) *Xenopus laevis* as a model for the study of immunology. Dev. Comp. Immunol., **2**: 5–14.

12 Neff, A. W., Wakahara, M., Jurand, A. and Malacinski, G. M. (1984) Experimental analyses of cytoplasmic rearrangements which follow fertilization and accompany symmetrization of inverted *Xenopus* eggs. J. Embryol. Exp. Morphol., **80**: 197–224.

13 Nieuwkoop, P. D. and Faber, J. (1956) Normal Table of *Xenopus laevis* (Daudin), North-Holland Publ., Amsterdam.

14 Black, S. D. and Gerhart, J. C. (1985) Experimental control of the site of embryonic axis formation in *Xenopus laevis* eggs centrifuged before first cleavage. Dev. Biol., **108**: 310–324.

15 Gimlich, R. L. and Gerhart, J. C. (1984) Early cellular interactions promote embryonic axis formation in *Xenopus laevis*. Dev. Biol., **104**: 117–130.

16 Gimlich, R. L. (1985) Cytoplasmic localization and chordamesoderm induction in the frog embryo. J. Embryol. Exp. Morphol., **89**: Suppl., 98–111.

17 Kageura, H. and Yamana, K. (1984) Pattern regulation in defect embryos of *Xenopus laevis*. Dev. Biol., **101**: 410–415.

18 Kageura, H. and Yamana, K. (1986) Pattern formation in 8-cell composite embryos of *Xenopus laevis*. J. Embryol. Exp. Morphol., **91**: 79–100.

19 Elinson, R. P. (1983) Cytoplasmic phases in the first cell cycle of the activated frog egg. Dev. Biol., **100**: 440–451.