MATERIALS AND METHODS

1. Fly stocks

Oregon-R was used as a wild-type strain. gs(1)N26 and gs(1)N441 (abbreviated as N441 and N26, respectively) were originally isolated and characterized as grandchildless mutations (Niki and Okada, 1981). These mutations have been maintained heterozygous over the FM7 balancer chromosome. The paralog mutant (abbreviated as par) was isolated and named X^{1122} by Gans et al. (1975); its phenotype in blastoderm formation was described by Zalokar et al. (1975); its genetic analysis was made by Thierry-Mieg (1982). A y par v / FM3 stock was provided by Dr. M. Masson (CNRS). The above three mutations were confirmed to complement completely with each other (data not shown).

A mutant, $l(3)c21R^{RW630}$ (abbreviated as RW630), was isolated and characterized as a maternal-effect mutation causing uneven thickening of blastodermal layer by Cheney et al. (1984). RW630 red / TM3 stocks were provided by Dr. C. M. Cheney (Johns Hopkins University, Baltimore).

aurora and early D (abbreviated as aur and elD, respectively) were isolated by Dr. C. Nüsslein-Volhard and her collaborates (Max-Planck-Institute, Tübingen) through saturation mutagenesis experiments. These mutants had been characterized as maternal-effect early lethal mutations. The stocks of aur^{074-15} ru st e ca / TM3, aur^{175-5} ru st e ca / TM3, elD^{242-8} ru st e ca / TM3, and elD^{842-3} ru st e ca / TM3

were provided by Dr. R. Lehmann. Since both of two alleles showed respective similar phenotypes in these mutants, I abbreviate the name of the mutations merely as aur and elD in the text.

A tudor[1] (abbreviated as tud) was originally identified by Dr. E. Wieschaus and Dr. C. Nüsslein-Volhard (Max-Planck-Institute, Tübingen). A detailed characterization of tud was made by Boswell and Mahowald (1985). A tud bw sp / SM5 stock was provided by Dr. B. Mechler.

For cytological mapping of N26 and N441, the strains with deficiency around expected genetic loci as described in Lindsley and Zimm (1987) were used. The strains used in this work were Df(1)RA2, Df(1)KA14, Df(1)C52, Df(1)v^{-L15}, Df(1)ras-v-17Cc8, Df(1)RA37, Df(1)N71, Df(1)HA85, Df(1)KA6, Df(1)KA10, Df(1)N105, Df(1)JA26, Df(1)HF368 and Df(1)C246. They were provided by Pasadena Stock Center, California State University, National Institute of Genetics (Mishima) and Dr. Y. Hotta (University of Tokyo).

A haplo-insufficient female sterile^{EH326} mutant (abbreviated as hfs^{EH326}) used for the complementation test with N26 was provided by Dr. D. F. Eberl (University of Guelph, Canada).

All stocks except aur, elD and tud were maintained at 25±1°C on a dead yeast-corn meal-glucose-agar or dead yeast-corn meal-sucrose-agar medium. aur, elD and tud stocks were maintained at 18.0±1.0°C on a dead yeast-corn meal-glucose-agar medium.

2. Egg collection

In N26, N441 and par, newly emerged female flies were fed for 4-5 days at 25.5±1.0°C (restrictive temperature), or for 8-10 days at 18.0±1.0°C (permissive temperature) before being allowed to lay eggs. Since all these mutations have temperature-sensitive periods during oogenesis (Niki and Okada, 1981; Thierry-Mieg, 1982; Maruo and Okada, 1984), eggs collected from these females are ensured to be produced entirely under the restrictive condition or under the permissive condition.

For the analysis of nuclear migration along the anteroposterior axis throughout the cleavage stages, eggs were collected for 90 min at 25.5±1.0°C for the restrictive condition, or for 3 hours at 18.0±1.0°C for the permissive condition. For the detailed analysis of nuclear migration between cycle 1 and 2 in the wild type, N441 and N26, eggs were collected every 20 min at 25.5±1.0°C.

In RW630, newly emerged female flies were fed for 6 days at 18.0±1.0°C (permissive temperature) and for additional 36 hours at 27.0±1.0°C (restrictive temperature) according to Cheney et al. (1984, 1986) to observe its maternal-effect phenotype. After 36 hours, eggs were collected every 90 min at 27.0±1.0°C.

In aur and elD, newly emerged females were fed for 4-5 days at 25°C and eggs were collected every 90 min. To observe syncytial blastoderm embryos, collected eggs were

allowed to develop for further 60 min at 25°C prior to fixation.

In tud, newly emerged females were fed for 4-5 days at 25°C and eggs were collected every 60 min. Collected eggs were allowed to develop up to a required stage at 25°C before fixation.

To collect wild-type eggs to be subjected to an inhibitor treatment, female flies were allowed to lay eggs for 20 min at 25°C after a first 90-min egg collection was discarded.

3. Nuclear staining

Nuclear staining in whole mount embryos was performed as described in Hatanaka and Okada (1991a), which was a slight modification of the methods by Zalokar and Erk (1977). Embryos were manually dechorionated, fixed, hydrolysed in 3N HCl for 12 min at 55°C, and stained with basic fuchsin. The embryos were then rapidly washed on a depression slide, which was coated with a Teflon spray (IUCHI) in advance, transferred to 70% ethanol and dehydrated through an ethanol series (70-100%), and infiltrated with n-butanol and xylene for 5 min each. For the analysis of nuclear migration throughout the cleavage stage, stained embryos were whole-mounted in Eukitt (O. Kindler, Germany). For a detailed analysis of cycle-2 embryos, stained embryos were whole-mounted in glycerol with two pieces of glass fiber (250 μm in diameter) and the cover slip was sealed with nail enamel.

Although the latter preparation allowed to observe nuclei only for several days, distortion of embryos was minimized in this method. Mounting in Spurr's resin with spacers was not available, because reduction in volume of the resin during polymerization caused squash of mounted embryos. Approximately 200 embryos from each strain were mounted and cycle-2 embryos were chosen and examined.

In some cases, nuclei were stained with DAPI according to Warn and Magrath (1983) and observed with an epifluorescence microscope (Nikon).

4. Staging of embryos

According to Foe and Alberts (1983), the stages of embryos were defined corresponding to the order of nuclear cycle: cycle 1 was defined to start at the beginning of interphase just after karyogamy and to terminate at the end of the first mitotic telophase; cycle 2 was defined to start at the beginning of interphase in 2-nucleus stage and to terminate at the end of the second mitotic telophase, and so on. Thus the staging of an embryo was performed by counting the number of nuclei in the embryo during cleavage stages (up to 256-nucleus stage, cycle 9).

During syncytial blastoderm stages, a density of cortical nuclei was used as an index of the order of nuclear cycle, since I showed that cortical nuclear density was approximately 1, 2, 4, 8 and 16 nuclei / 400 μm^2 at cycle 10,

11, 12, 13 and 14, respectively, in histological sections of wild-type embryos.

5. Time-lapse video analysis of live embryos

Cleavage embryos were collected and allowed to develop in Voltareff's fluorocarbon oil in a narrow slit (the width was approximately 300 μm) between two cover slips, which were fixed on a glass slide. To prevent the embryos from drying, this glass slide was kept all through the observation in a small moist chamber set on the microscope stage. The embryos were observed with an inverted microscope (Nikon) and their development was recorded with a time-lapse video (Panasonic).

6. Histology

For histological analysis, embryos were processed according to Zalokar and Erk (1977). Briefly, fixed embryos were temporally stained with 1% eosin in 70% ethanol, dehydrated through ethanol series, infiltrated with n-butanol and n-butanol-paraffin (1:1), and mounted in paraffin. Serial sections of 3 μm thick were made, stained in Heidenhain's iron-hematoxylin, mounted in Eukitt, and observed under a compound microscope (Nikon).

7. Electron Microscopy

To observe embryos with a transmission electron microscope, embryos were fixed and stained basically according to Zalokar and Erk (1977). To obtain high contrast images of

plasma membrane and polar granules to distinguish them from mitochondria, tannic acid was added to the fixatives at a final concentration of 2 mg/ml. Fixed embryos were washed in 0.1 M PBS (pH 6.8) with at least six changes (10 min each) at 5°C, then in 10 mM cacodylate buffer (pH 7.2) with at least six changes (10 min each). Subsequently, the embryos were postfixed with osmic acid and were embedded in Spurr's resin. Ultrathin sections of embedded embryos were made using a Porter Blum MT1 ultramicrotome and glass knives, poststained with uranyl acetate and lead citrate, and observed with a transmission electron microscope (JEM-100C, JEOL) at an accelerating voltage of 80 kV.

8. Cuticle preparations

The cuticles of first instar larvae were prepared according to van der Meer (1977) and observed under a compound microscope (Nikon).

9. Application of inhibitors

Appropriately staged wild-type embryos were manually dechorionated, transferred on a siliconized depression slide, permeabilized with octane for 20 sec and incubated in a drop of an incubation medium containing a given concentration (0 to 10 µg/ml) of cytoskeletal inhibitors for 5 min according to Limbourg and Zalokar (1973). After the medium was removed, the embryos were covered with a drop of paraffin oil. The slide was kept in moist air at 25°C to allow the

embryos developing up to a required stage. After the incubation, the embryos were briefly washed with heptane for three times and were fixed and stained according to Zalokar and Erk (1977), and whole-mounted in Eukitt. Because it was difficult to remove the vitelline membrane from colchicine-treated embryos by the above methods, those embryos were fixed with 8% paraformaldehyde in 10 mM PBS (pH 7.2) for 5 min and vitelline membrane was removed in the same fixative on double-stick tape (Scotch), followed by postfixation in the same fixative for 30 min and staining with 0.5 µg/ml DAPI for 10 min. The embryos were washed, whole-mounted in glycerol and observed with an epifluorescence microscope (Nikon).

Cytochalasin B, D, colchicine and lumicolchicine were purchased from Sigma. Cytochalasin was solubilized in dimethylsulfoxide (DMSO) by 1 mg/ml. Final concentration of DMSO in the medium was less than 1% at which embryos developed normally (Zalokar and Erk, 1976; Hatanaka and Okada, 1991a).

10. Determination of three-dimensional nuclear position in embryos at cycle 1 and 2

Whole mount preparations stained with basic fuchsin were observed under a compound microscope (Nikon) according to Hatanaka and Okada (1991b) to record the position of nuclei with the help of two ocular micrometers (100-division linear and 20x20 grating type) and of the micrometer on the fine

focal adjustment knob of the microscope, the scale of which was calibrated in advance. To minimize the effect of shrinkage or swelling of embryos during preparation, each value was normalized by the egg length (EL). To locate the nuclei in cycle-2 embryos, it was presumed that the anteroposterior axis of embryos on the glass slide is set precisely vertical to the light axis of the microscope and that polar body nuclei are on the dorsal midline of embryos.

I examined anteroposterior nuclear migration between cycle 1 and 2 and three-dimensional distance between two nuclei at cycle 2 (detailed methods are described in RESULTS). In addition, three-dimensional nuclear positions at cycle 2 were determined with respect to the anteroposterior and dorsoventral axes of the embryo (also see RESULTS), which may represent the correlation between nuclear positioning and the cortex.

11. Double fluorescent staining of nuclei and F-actin

Manually dechorionated embryos were fixed with 8% paraformaldehyde in 10 mM PBS (pH 7.2) according to Warn and Magrath (1983). Embryos were punctured at the dorsal side in the fixative solution with a fine glass needle on a micromanipulator (Narishige) to facilitate the fixative to enter into the embryos. Double-staining with rhodaminyl phalloidin (Molecular Probes Inc.) and DAPI (Sigma) was performed according to Warn et al. (1984). Because F-actin

was found to become extremely sensitive to fixation if embryos were overdesiccated, embryos were kept in moist air using an ultrasonic humidifier during dechorionation of approximately 10 min. It was also necessary to determine an optimum desiccation time for each strain to prevent cytoplasm leaking out through the puncture. The optimum desiccation time was varied from 1.5 min (par) to 3.5 min (wild-type strain).

For frozen sections, embryos were fixed, stained and washed, then were infiltrated with 50% Tissue-Tek II (MILES) for 5 min, embedded in Tissue-Tek II, oriented with a hand-driven centrifuge, and frozen in isopropanol cooled with liquid nitrogen. Serial frozen sections of 5 μm thick were made, rapidly dried on a glass slide, and mounted in Tissue-Tek II just before observation.

As a control, embryos were stained only with DAPI, or were pretreated with 1 mg/ml phalloidin (Sigma) in 10 mM PBS (pH 7.2) for 15 min to block the specific binding of rhodaminyl phalloidin to elucidate whether autofluorescence or non-specific binding of the fluorescent dye was detectable or not.

All preparations were observed with an epifluorescence microscope (Nikon or Olympus) or a laser scanning confocal fluorescence microscope, MRC-500 (Bio-Rad Inc.), and photographed on Panatomic-X, Ektachrome 100 or Ektachrome 400 film (Kodak).

12. Two-dimensional gel electrophoresis

For analysis of proteins in N441 and N26 ovaries and embryos, a non-equilibrium micro-two-dimensional gel electrophoresis system was used, which had been established by Dr. F. Maruo in our laboratory. 20 ovaries or 30 dechorionated embryos were homogenized with 60 µl or 7 µl of a lysis buffer, respectively, and the homogenate was loaded on 1% agarose gel (0.7 mm in diameter and 6 cm in length) filled in a plain hematocrit glass tube (TERUMO) for non-equilibrium gel electrophoresis. For the second dimension, SDS-polyacrylamide gel electrophoresis was performed on a 10% polyacrylamide gel (5 cm in length, 9 cm in width and 0.5 mm in thickness). Subsequently, the gel was fixed and stained with Coomassie brilliant blue.