DISCUSSION

A major conclusion obtained from the present study is that there are two groups of maternal-effect genes that regulate organization of F-actin during the early development of Drosophila. One group of genes including N441$^+$, N26$^+$ and par$^+$ is required for cleavage-stage-specific organization of F-actin that has an important role in normal egg contraction and nuclear migration. The other group of genes including aur$^+$ and elD$^+$ is required for syncytial-blastoderm-specific organization of F-actin that is required for keeping cortical nuclei separate from each other during a series of synchronous mitoses. Although it has been shown that the distribution pattern of F-actin is characteristic of the stage and the region of embryos during early development (Warn and Magrath, 1983; Warn et al. 1984; Warn et al. 1985; Warn, 1986; Karr and Alberts, 1986), this is the first report about maternal genes that regulate the organization of F-actin. Identification of these genes and characterization of their roles may be an important step to elucidate molecular mechanisms underlying developmental processes in the stages from cleavage to blastoderm formation. In addition, the present study has shed light on the mode of contribution of major cytoskeletal systems, i.e., the microfilament and microtubule systems, to nuclear migration, and further on the importance of the normal process of nuclear migration for the normal pattern formation.

1. Mutants affecting cleavage-stage-specific organization of F-actin

Here I showed that three X-linked maternal-effect mutations, N441, N26 and par, caused a similar disorder in F-actin reorganization in cleavage embryos. Cytological loci of N441 and N26 were verified to be separated from any of the six reported actin loci (Tobin et al. 1980; Fyrberg et al. 1980) and also from the so-far known genetic loci of other cytoskeletal proteins (see Fyrberg, 1989 for review). The cytological locus of par has been located at 3B3 (Thierry-Mieg, 1982), at which no cytoskeletal protein has been mapped either. In addition, homozygous N441 and N26 were comparable to wild-type strains in the relative amount of actin in an ovarian egg and in a deriving embryo, as revealed with two dimensional gel electrophoresis. Thus the abnormal F-actin reorganization in N441 and N26 embryos can not be ascribed to abnormal structure or inadequate amount of actin molecules, but presumably to molecules other than actin.

The cortical F-actin layer, which is smooth in the wild type, is rough in mutant embryos due to many irregular aggregates scattered on its inner surface. However, the layer itself is not fragmented as it is in the cytochalasin-induced phenocopy. Such a rough surfaced F-actin layer was never induced by cytochalasins under any conditions that I examined. These results suggest that the abnormal

reorganization of cortical F-actin caused by these mutations does not result from simple disruption of actin filaments.

It is very probable that both failure in egg contraction and retardation in nuclear migration are attributed to a common genetically caused flaw in the pathway required for F-actin reorganization at the cleavage stage. Cytologically, however, there is no discernible difference in the cortical F-actin layer between cleavage embryos and mature oocytes in the wild type, although no electron microscope level scrutiny has been extended to cytoskeletal structures in the cortical cytoplasm of oocytes and early embryos. Only ultrastructural changes reported in the cortex occurring after fertilization or artificial activation of oocytes are increases in the number of multivesicular bodies and some other cytoplasmic organelles (Mahowald et al. 1981; Mahowald et al. 1983). At this moment, it is rather premature to draw a conclusion, but I am inclined to presume that a wild-type function of the genes in this group is essential for reorganization of cortical F-actin triggered to be initiated at fertilization, and this reorganization enables embryos to contract, and is probably essential for normal nuclear migration (see below).

1.1. Requirement of F-actin reorganization for nuclear migration and synchronous nuclear penetration of the whole periplasm

The developmental changes in distribution pattern of F-actin and nuclei were highly reproducible in mutant embryos

as in the wild type during cleavage stages (cf. Fig. 28). In many insect groups, nuclei are distributed more or less spherically within the yolk mass in embryos at the mid-cleavage stage (Counce, 1973). In Drosophila, this type of nuclear distribution is distinct only during cycle 2 through 4 (Zalokar and Erk, 1976; the present observation), and this is the very period when the manifest central domain of F-actin is present. At cycle 5, the spatial distribution of nuclei has already been elongated anteroposteriorly (Zalokar and Erk, 1976; the present observation). This change in the distribution of nuclei accompanies the transition of F-actin distribution from the central to the energid domain via a transient state of elongated distribution in the central region. Afterwards, nuclei gradually approach the cortex and they are distributed equidistantly from the cortex as well as from each other. On the other hand, both in the mutants and the phenocopies, their retarded nuclear migration toward the poles was always accompanied by a delay in the development of the central domain of F-actin and its transition to the energid domain. These results suggest that F-actin in the inner cytoplasm is involved in the mechanism of nuclear migration in Drosophila cleavage embryos.

In the mutant and phenocopied embryos, nuclei remain in the anterior region retaining their spherical distribution for much longer period of time than in the wild type. The sphere carrying nuclei continues growing and contact the cortex where premature nuclear penetration occurs. In

contrast, in the wild type, nuclei are inhibited from reaching the cortex before cycle 10 except in the posterior pole region. In addition, the distance between each nucleus and its next neighbors is shorter in the mutants than in the wild type during late cleavage stages. Probably the function of the wild-type F-actin organization is necessary to keep each nucleus apart from its next neighbors and also from the cortex beyond a certain distance during the cleavage stages. Although I have obtained no clue to explain how F-actin organization works in cleavage embryos, it may, by itself or in collaboration with other cytoskeletal elements, stiffen the cytoplasm enough to act as a mechanical barrier. My assumption is that there is a genetical regulation to maintain this barrier through the cleavage stage and then to make it abruptly disappear to ensure a simultaneous nuclear penetration of periplasm in the somatic regions. The results from colchicine treatment of wild-type embryos and from the analysis of RW630 phenotype suggest that microtubules give the final thrust to nuclei to penetrate the periplasm under the genetical control of the $RW630^{+}$ gene. Results of a detailed analysis of nuclear migration between cycles 1 and 2 may support the above idea (see below).

1.2. Positioning of the two nuclei at cycle 2

I quantitatively showed that two maternal-effect Drosophila mutants, N441 and N26, in which F-actin reorganization is defective, differed in the mode of nuclear

migration from the wild type even at a very early cleavage stage. The measurement of the position of nuclei was carried out on the assumption that the anteroposterior axes of whole-mounted embryos are perpendicular to the light axis of the microscope. Inaccuracy caused by this assumption can be negligible, because the both poles of an embryo were practically on the same focal plane in most preparations in the present work. The error included in the measurements based on this assumption was estimated to be less than 5 degrees. In addition, since there was no significant difference between d_x and d_z in each strain, the embryos should not be deformed during preparation.

A precise comparison of the nuclear position between embryos at cycle 1 and 2 revealed that wild-type and mutant embryos were different in the following characteristics. First, nuclear migration toward the posterior was detectable at the first cleavage in the wild type, but not in the mutants. Accumulation of this small difference at every cleavage could result in the mutant phenotype at late cleavage stages, in which nuclei remain in an anterior region of embryos assembling in a compact "nuclear sphere" (a virtual sphere within which nuclei are distributed). Second, nuclei were less-separated from each other in the mutants than in the wild type. This also explains the compact nuclear distribution at late cleavage stages in the mutants. Third, in the wild type, two sister nuclei were oriented more frequently anteroposteriorly than expected from random

distribution. However, with respect to the dorsoventral axis, their orientation was random. In contrast to the wild type, the orientation of two sister nuclei in cycle-2 mutant embryos was three-dimensionally random.

The hypothesis proposed above can explain the tendency of frequent anteroposterior orientation of two sister nuclei in cycle-2 wild-type embryos. Two nuclei are separated from each other and from the egg cortex farther in wild type, so that they are rejected to approach cortex. With respect to the dorsoventral axis and the right-left axis, however, the egg shape is nearly symmetrical, and it is no wonder that the orientation of two nuclei is dorsoventrally random. Two nuclei were oriented perpendicular to the anteroposterior axis in rare cases. I have noticed that in those cases the distance between two nuclei was relatively short or nuclei were located posterior to those in other embryos.

A random orientation of two nuclei in the mutants may be caused by a relatively small size of the nuclear sphere and by a defect in the separation of nuclei from the egg cortex. In the mutants at late cleavage stages, defects in F-actin reorganization may allow premature arrival of nuclei in the anterior lateral cortex. Thus the differences in nuclear distribution between wild-type and mutant embryos throughout the cleavage stage can be explained only by hypothesizing that these genes are required for keeping nuclei away from each other and from the cortex as early as the first cleavage.

The hypothesis can also explain a mechanism of nuclear migration after cycle 2. In wild-type embryos, the mode of nuclear migration during the cleavage stage includes three characteristic phases, (1) total posteriorward migration of the "nuclear sphere" in stages from cycle 1 to 4, (2) a change in the range of nuclear distribution from the initial spherical to the ellipsoidal one at cycle 5, and (3) final enlargement of the range of nuclear distribution while maintaining distances between neighboring nuclei as well as between nuclei and the egg cortex at stages from cycle 6 to 9 (Zalokar and Erk, 1976; Hatanaka and Okada, 1991a). These three phases of nuclear migration in wild-type embryos could also be explained by the above mentioned "keeping separation" hypothesis.

From cycle 1 to 4, the nuclear sphere is small enough to stay in the anterior region of an embryo where the space is narrower than in the middle of the embryo. At these early cleavage stages, the central domain of F-actin may be needed to keep the spherical distribution of nuclei. At cycle 5, an enlarged nuclear sphere can no longer be accommodated in the narrow anterior region. Thus the nuclear sphere is elongated, accompanying the transition of F-actin distribution from the central domain to the energid domains. Probably the role of the central domain of F-actin has been accomplished at cycle 5. After cycle 5, nuclei have lost attraction of the center of the nuclear sphere. Thus, henceforth, nuclei are forced to separate them from their neighbors and from the

cortex. The present observation of the distribution of F-actin may suggest the forces generated by F-actin in the energid and in the cortex.

It has been reported that orientation of the spindle at the first mitotic metaphase in wild-type embryos is random (Parks, 1936). Although his histological study was neither quantitative nor statistical, his conclusion can not necessarily be in contradiction with the present results. I recorded the position of nuclei, while he did the direction of spindles. Thus non-random distribution of two nuclei in wild-type cycle 2 embryos might arise from a randomly oriented spindle. On the other hand, it was quite unexpected that the anterior nucleus at cycle 2 in wild-type embryos had a tendency to localize in the right hemisphere. Although this result was statistically significant, I think that the conclusion is premature to be drawn.

1.3. Nuclear migration in other insects

It has been reported that cytochalasin B does not inhibit the nuclear migration in the cleavage embryo of a gall midge Wachtliella (Wolf, 1978). Although the cause of the difference in the sensitivity to cytochalasins between the gall midge and Drosophila is unknown, the following explanation is possible. In Drosophila, F-actin keeps cleavage nuclei a certain distance away from the periplasm during the cleavage stage, and this causes a change of the initial spherical distribution of nuclei into the final

elongated ellipsoid distribution lining the periplasm. In Wachtliella, however, the egg is small and extremely elongated, and has only space for energids in a single file in the early cleavage stage (Wolf, 1969), and so the embryo does not have any stage when nuclei are distributed spherically as in Drosophila. Thus it is probable that an F-actin-dependent nuclear migration mechanism has not developed or secondarily disappeared in Wachtliella. In many insect species, nuclei penetrate the periplasm almost synchronously in all egg regions in spite of the initial spherical nuclear distribution and the varieties in egg shapes. Thus I presume that a gene like $N441^{+}$ is conserved in various insect species to function during the cleavage stage.

1.4. Genetical analysis

The present genetical analysis showed that both N441 and par are amorphic under the restrictive condition that I used, although it has been reported that par behaves like an antimorphic allele (Thierry-Mieg, 1982). In addition to the maternal effects I described, N26 and par have a zygotic effect. Both N26/Y and par/Y male flies showed a low lethality at the pupal stage. Moreover, all five strains with the double mutation of N26 and par showed a high recessive zygotic lethality at the pupal stage regardless of sex (Hatanaka, unpublished data). This high lethality can not be explained as a simple additional effect of the mutations, since there was no detectable lethality in

N26/N26, N26/Df(1)RA37, par/par and par/Df(1)w^{258-45} females. Albeit it is still unknown whether their pupal lethality is caused by defective F-actin reorganization or not, N26 and par may have some defects in a closely related mechanism operating in pupal development. Among the three mutants I tested, only N441 behaved as a simple recessive maternal-effect mutant. Thus I am inclined to regard $N441^{+}$ as a candidate for a gene that has a key role in a mechanism underlying cleavage-stage-specific F-actin reorganization.

1.5. Defects in segmentation in the mutants

The mutants, N441, N26 and par, showed a maternal-effect disorder in the segment pattern. The defects include fusion and deletion of segments all along the body, most frequently in the mid-abdominal region. Although the cause of the defects has not been elucidated, the segment phenotype would most probably be a result of asynchronous nuclear arrival in the cortex, but not of the primary effect of the mutations. In mutant embryos, the nuclei prematurely penetrating the periplasm in an anterior region (a zone along the altitude at 70% egg length) immediately initiate their syncytial blastodermal mitosis. From this stage, intermittent streaming of cortical cytoplasm and nuclei toward the non-nucleated posterior region is generated in the periplasm coordinating to the rhythm of the division of nuclei in the anterior cortex. The cortical streaming apparently causes the blastoderm nuclei drifting posteriorward from their

original positions. It is possible that this drift exposes the nuclei to an ectopic cytoplasm, which can disorganize the regulation of the expression of zygotic segmentation genes that are responsible for determination of segment or parasegment boundaries (Nüsslein-Volhard and Wieshaus, 1980). Alternatively, the streaming can disrupt the original distribution of maternal gene products that give signals for the anteroposterior polarity (Nüsslein-Volhard *et al.* 1987). On the other hand, there are pieces of evidence suggesting interaction of cytoskeletal components with polarity gene products (Wharton and Struhl, 1989). Furthermore, in a maternal-effect mutant, *swallow*, abnormal nuclear migration and defective cellularization of the blastoderm have been reported (Zalokar *et al.* 1975) in addition to failure in the anterior localization of the anterior determinant, *bicoid*$^{+}$ mRNA (Berleth *et al.* 1988; Stephenson *et al.* 1988; Johnston *et al.* 1989). Thus there is another possibility that disorder in the localization of any one of the polarity gene products by malfunction of microfilament system may result in the segment phenotype of *N441*, *N26* and *par*.

2. Mutants affecting F-actin organization in the syncytial blastoderm stage

In the present study, I showed that two maternal-effect mutants on the third chromosome, *aur* and *elD*, had a complementary phenotype to that of *N441*, *N26* and *par*. Although this group of mutations could not be genetically

analysed, the phenotype observable in live embryos of the mutants is very similar to that reported in mat(3)1 (Rice and Garen, 1975), in which pole cell formation is normal, whereas cellularization of blastoderm is totally inhibited. Thus mat(3)1 might be another member of this group of mutants, or another allele of any one of aur or elD.

In contrast to the wild type, aur and elD embryos showed no change in the cortical F-actin layer after nuclear penetration of the cortex. Consequently, cortical nuclei collide with their neighbors during mitoses, just like in a wild-type embryo that treated with cytochalasins at a late cleavage stage. Although the phenotype of the mutants is quite different from the first group of mutants in which abnormality in F-actin organization is limited to the cleavage stages, a resulting phenotype has a common characteristic; that is, separation between neighbor nuclei is incomplete in both group of mutants. Thus a basic function of F-actin to separate nuclei from each other may be required also in the syncytial blastoderm. However, reorganization of F-actin for accomplishing this function is regulated by different genes from those in embryos at the cleavage stages.

Here I propose a scheme about the functions of F-actin and their control by maternal genes as follows. At the beginning of development, fertilization may trigger the first reorganization of F-actin by functions of N441^{+}, N26^{+} and par^{+} gene products to separate the cleavage nuclei from each

other and from the cortex, and consequently, synchronized nuclear penetration of the cortex occurs. Then, nuclear penetration of the cortex may trigger the second reorganization of F-actin by functions of *aur*$^+$ and *elD*$^+$ genes to separate the cortical nuclei with each other. When four cycles of cortical nuclear divisions have been accomplished, a local nuclear density (or nucleo-cytoplasmic ratio as reported by Edgar *et al.* 1985) exceeds a certain value (10 nuclei / 400 μm^2). This is used as a signal to trigger the initiation of the cellularization of the blastoderm. The developmental changes in the reorganization of F-actin, which I revealed in this thesis, may be important to enable *Drosophila* embryos to undergo a series of cytological events in temporarily stable manner and highly reproducibly.

3. Regulation of cytoskeletal elements during pole cell formation

It is unknown how a few nuclei arrive in the posterior pole cortex at cycle 9 prior to the somatic regions, where nuclear arrival occurs at cycle 10, in wild-type embryos. Since the posterior pole region is known to include a defined cytoplasm called polar plasm, involvement of the polar plasm in the primary arrival of nuclei could be speculated. However, in *tud* embryos, in which pole cell formation is deficient and abdominal segments are reduced or deleted, arrival of nuclei in the posterior polar cortex normally occurred at cycle 9. Thus an unknown mechanism, that

regulates cytoskeletal machinery to pull a few nuclei into the posterior polar periplasm prior to the somatic regions, may exist independent of the activity to form pole cells. I could not find any special organization of F-actin around the posterior pole in cycle-9 wild-type embryos. In addition, the results from experiments involving colchicine treatment suggest that nuclear penetration of the cortex may be controlled by microtubules as mentioned above. At present, there is no clue to explain the mechanism for the primary nuclear penetration at the posterior polar region. Studies on the organization of microtubules in the posterior region should be required to solve the problem.

A role of microtubules may be also important for the formation of pole buds and subsequent pole cells. I preliminary observed that pole buds were very sensitive to low temperature. When a pole-bud stage embryo was transferred into an ice-cold chamber, pole buds rapidly reduced in size and resulting small pole buds were occupied almost only by nuclei. The cold-sensitivity is a characteristic of microtubules. Although I here showed a difference in the organization of cortical F-actin between pole buds of the wild type and of tud, probably continuous protrusion of pole buds in wild-type embryos requires not only a defined organization of cortical F-actin but also a specific organization of microtubules in the pole buds. Recently, it was demonstrated that after treatment of cycle-7 to -8 wild-type embryos with aphidicolin, DNA synthesis and

nuclear migration toward the cortex was completely inhibited, while centrosomes were separated from nuclei and migrated to penetrate the cortex, and "pole cells" without nuclei were formed (Raff and Glover, 1989). These results suggest that centrosomes may organize microtubules to initiate pole cell formation, although it is still unknown why centrosomes initiate cytokinesis only at the posterior pole region. To elucidate a detailed cytological mechanism of pole cell formation, more informations about the organization of cytoskeletal elements during pole cell formation and overall analysis of the posterior group mutations should be required.