Development 111, 379-391 (1991)
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Independent roles of centrosomes and DNA in organizing the *Drosophila* cytoskeleton

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Summary

The early embryonic divisions of *Drosophila melanogaster* are characterized by rapid, synchronized changes of the nuclei and surrounding cytoskeleton. We report evidence that these changes are carried out by two separately organized systems. DNA was sufficient to cause assembly of nuclear lamina and the formation of nuclear membrane with pore structures. Free centrosomes were correlated with the formation of microtubule, microfilament and spectrin networks in the absence of nuclei. In addition, we found that the morphology of the cytoskeleton associated with the free centrosomes cycled in response to the embryonic cell cycle cues. These observations suggest that the centrosomes may be responsible for the organization of this extensive cytoskeleton. The early divisions may therefore result from the independent cycling of two systems, the nucleus and the surrounding cytoskeleton, that respond separately to the mitotic cues in the embryo and function together to give the synchronized early divisions. The *Drosophila* embryo has an 'intermediate' mitotic system in which the nuclear membrane does not break down completely during mitosis. We speculate that the principles of cytoskeleton organization in this system may be different from those of the *Xenopus* 'open' mitotic system.

Key words: cytoskeleton, *Drosophila* embryo, centrosomes, nuclear membrane.

Introduction

The early embryonic development of *Drosophila melanogaster* has been well documented (Rabinowitz, 1941; Sonnenblick, 1950; Turner and Mahowald, 1976; Zalokar and Erk, 1976; Foe and Alberts, 1983). The first ten divisions are characterized by rapid, synchronous mitotic cycles without cytokinesis. The first seven divisions are within the interior of the embryo. During the eighth and ninth divisions, most of the nuclei migrate to the periphery of the embryo. The peripheral nuclei divide metasynchronously four more times generating a monolayer of approximately 6000 nuclei at the periphery of the embryo. The nuclei that remain in the interior become the vitellophages and divide twice more before undergoing two rounds of endoreplication. The embryo remains a syncytium until a metasynchronous wave of cellularization following the 13th mitotic division results in the formation of the cellular blastoderm.

During the nuclear cycle there is an orchestrated change in cytoskeletal and nuclear organization. The embryonic nucleus undergoes an 'intermediate' form of mitosis in which the nuclear membrane is only broken down at the poles and new membrane is generated by the addition of a second membrane layer onto the 'spindle' membrane (Stafstrom and Staehelin, 1984). High concentrations of microtubules (Warn and Warn, 1986; Warn *et al.* 1987; Karr and Alberts, 1986; Kellogg *et al.* 1988), microfilaments (Warn *et al.* 1984, 1985; Karr and Alberts, 1986; Kellogg *et al.* 1988), intermediate filaments (Walter and Alberts, 1984) and spectrin (Pesacreta *et al.* 1989) are detected in the cytoplasmic islands or 'energids' surrounding each nucleus and are believed to be responsible for the visible cytoplasmic clearing. Studies have demonstrated that the integrity of the cytoskeleton is required for the rapid mitoses, nuclear migration and regulated cellularization seen in the early embryo (Zalokar and Erk, 1976; Foe and Alberts, 1983).

The organizing principles of the cytoskeleton have been investigated in a number of organisms (Byers *et al.* 1978; Hyams and Borisy, 1978; Picard *et al.* 1988). It has been addressed in *Xenopus* by a combination of *in vivo* and *in vitro* methods (Forbes *et al.* 1983; Karsenti *et al.* 1984; Newport, 1987). *Xenopus* undergoes 'open' mitoses in which the entire nuclear membrane breaks down and membranes are reformed by the assembly of membrane vesicles onto the chromosomal template (Newport, 1987). Plasmid or phage DNA injected into the *Xenopus* egg or developing embryo is sufficient to organize a complete surrounding nuclear membrane.

Injected DNA also causes formation of a diffuse anastral microtubule array in metaphase-arrested eggs. Centrosomes isolated from a variety of sources can organize cytoskeletal elements both *in vitro* and *in vivo*. They are not, however, sufficient to cause aster formation in unactivated eggs, which are arrested at metaphase of meiosis II, or in embryos arrested in mitotic metaphase with cytostatic factor. Karsenti *et al.* (1984) concluded that centrosomes are required simply to add polarity to the DNA-organized spindle during metaphase. The organization of other cytoskeletal elements was not investigated in these studies.

To investigate the organizing principle for the cytoskeleton during the rapid mitotic cycles in the *Drosophila* embryo, we used two strategies: (1) the ability of DNA to organize the cytoskeleton was studied by injection of plasmid DNA into the developing embryo; (2) UV-irradiation of early embryos caused not only a continued replication of the centrosomes in the absence of nuclear replication but also a physical separation of the centrosomes from the nuclei. We were therefore able to monitor the effects of free centrosomes on the organization of the cytoskeleton. The cytoskeletal organization in response to either DNA or centrosomes was observed by scoring for the presence of a number of cytoskeletal elements.

The results show that the presence of injected plasmid DNA in the embryo was sufficient to cause the organization of a nuclear membrane including nuclear lamins. The centrosomes were correlated with the organization of microtubule asters through all phases of the mitotic cycle. The organization of cytoskeletal elements around the centrosomes was not limited to microtubules but also included microfilaments and spectrin. Furthermore, we found that the morphology of this centrosome-associated cytoskeleton changed in response to the mitotic cues in the embryo.

Materials and methods

Stocks, egg collections, staging

Eggs from 3–7 day old 'Sevelen' wild-type female flies were precollected for one hour on fresh media and then for 30, 15 and 15 min on 5% agar plates supplemented with acetic acid and yeast. All precollections were done at 25°C. Following the precollections, eggs were collected for 10 min at 25°C. Subsequent development was at room temperature (21–23°C). Staging of the embryos was done visually using phase contrast optics.

Reagents

Centrosomes were detected using Rb188 (at 1:400), a polyclonal rabbit serum provided by W. Whitfield (Whitfield *et al.* 1988). Microtubules were detected using an anti-chick brain α-tubulin monoclonal antibody (at 1:800, Amersham). Filamentous actin was detected using rhodamine-conjugated phalloidin obtained from Molecular Probes. 354 (at 1:1000), a rabbit polyclonal serum directed against *Drosophila* α-spectrin was provided by D. Branton and is described in Byers *et al.* (1987) and Pesacreta *et al.* (1989). Nuclear lamins were detected using two antibodies to *Drosophila* nuclear lamin: (1) 614F10H10 (1:10), a mouse monoclonal antibody provided by D. Kellogg which recognizes 2 bands on a Western blot, one at 53K and one at 75K, corresponding to the molecular weights of nuclear lamin proteins (D. Kellogg, personal communication); (2) T40 (1:100), a mouse monoclonal antibody produced and characterized in the Saumweber lab (Risau *et al.* 1981; Frasch *et al.* 1988) which was provided by J. Sedat. Secondary antibodies (at 1:200) were either goat anti-mouse or goat anti-rabbit affinity-purified antibodies conjugated to fluorescein (FITC), rhodamine (TRITC), or Texas Red (Cappel).

DNA Injection

The injections were performed using a Leitz micromanipulator and a Wild compound microscope, according to Foe and Alberts (1983). Eggs were hand dechorionated on double stick tape and lined up on a coverslip on a strip of double stick tape glue dissolved with heptane. Drawn out 50 μl Drummond capillary pipettes with the broken tips were used as injection needles. Injection and fix times were varied in order to observe the progression of changes to the DNA.

In preliminary experiments, different types and sizes of DNA (lambda≈56 kb; whole Sevelen *Drosophila* genomic ~50 kb; sheared Sevelen *Drosophila* ~40 kb; cosmid RK12 ~40 kb; pSH ~17.5 kb) were injected at a concentration of 0.5 $\mu g\,\mu l^{-1}$. No significant differences were seen either in the behavior of the DNA or in the response of the embryonic nuclei. For convenience, pSH, a pBR322 plasmid (provided by S. Henikoff) was chosen for subsequent experiments. This plasmid contains the *leu2* gene and regions of the 2μ plasmid from *S. cerevisiae* as well as the *Drosophila gart* locus. The DNA was suspended in injection buffer and a dilution factor of fifty fold upon injection was assumed (Foe and Alberts, 1983).

For some of the histological preparations, the DNA was biotinylated using the Photoprobe kit from Vector Labs. The DNA was prepared according to the kit directions with the omission of carrier DNA.

UV irradiation

The irradiations were done using the TM36 Transilluminator from UVP Inc. This unit produces a maximal output at ~320 nm. Irradiation was done at a dose rate of 13.5 $J\,m^{-2}\,s^{-1}$ (determined with a Ysi-Kettering Model 65 Radiometer) for 12–13 s for a total dose of ~170 $J\,m^{-2}$. This dose produces a high frequency of developing embryos in which there is localized termination of mitoses. Embryos were hand dechorionated on double stick tape and arranged on parafilm to expose primarily the anterior tip of the embryo to UV irradiation. In all cases, irradiation was done at 75±5 min AED (after egg deposition) and embryos were fixed at 140±5 min AED.

Antibody detection

DNA-injected embryos stained for lamins were fixed in equal volumes of 4% formaldehyde in PBS (130 mM NaCl, 10 mM $NaPO_4$, pH 7.0) and heptane (Limbourg and Zalokar, 1973) for 30 min. Those stained for other cytoskeletal elements were fixed in a bilayer of 4% formaldehyde in PEM (0.1 M Pipes, 20 mM EGTA, 1 mM $MgSO_4$, pH 6.9) and heptane for 30 min. All injected embryos were then hand devitellinized using tungsten needles and transferred to PBS containing 10% fetal calf serum. This solution was used for all subsequent rinses.

The fixation conditions used for the UV-irradiated embryos also differed for the detection of different elements. The above 4% formaldehyde/PBS fix conditions were employed for the detection of nuclear lamins. The embryos stained for spectrin were initially placed in heptane for 30 s then fixed in a

bilayer of heptane and a solution of 33% formaldehyde and 50 mM EGTA for 5 min. These embryos were devitellinized by agitation in a bilayer of heptane and methanol. Embryos stained for filamentous actin were fixed under the same conditions as those stained for spectrin but then hand devitellinized. Embryos to be stained for microtubules were fixed and devitellinized in a bilayer of heptane and a solution of 97% methanol and 3% 0.5 M EGTA as desribed in Kellogg *et al.* (1988). Subsequent rinses for all UV irradiated embryos were carried out in a solution of PBS with 0.1% bovine serum albumin and 0.1% Triton X-100.

The staining protocol followed was the same for both classes of embryos. Non-specific staining was blocked by incubation of the embryos in a rinse solution containing 5% normal goat serum. Incubation with primary and secondary antibodies were either overnight at 4°C or for 2–4 h at room temperature. Rinses between incubations were for 1–2 h at room temperature with numerous washes. For double staining, antibody staining was processed in succession rather than simultaneously. Staining of actin with phalloidin was done as in Wieschaus and Nüsslein-Volhard (1986). Following antibody or phalloidin staining, the embryos were stained with DAPI at 1 $\mu g\,ml^{-1}$ in PBS for 4 min. Embryos were mounted in 1:1 glycerol: 50 mM Tris–HCl, pH 8.8, 1 $mg\,ml^{-1}$ *p*-phenylenediamine (added as an anti-fading agent) under a coverslip with pieces of tape used as spacers. Embryos were viewed with a Nikon Microphot fluorescent microscope or a Bio-Rad scanning confocal microscope. For serial optical sectioning with the confocal microscope, 1 μm steps were taken through the material and the image was then reconstructed using the confocal software package.

Electron microscopy

The method used for the preparation of material for electron microscopy is derived from a number of protocols (Turner and Mahowald, 1975; Newman and Schubiger, 1980; Staffstrom and Staehelin, 1984; and Tomlinson and Ready, 1987). Embryos injected with biotinylated DNA were fixed for 2 to 4 min in a biphasic solution of 4% EM grade paraformaldehyde, 0.1% EM grade glutaraldehyde in PBS and an equivalent of heptane. Although a higher concentration is optimal for ideal fixation, less glutaraldehyde had to be used in order to visualize the biotinylated DNA. After the fix and heptane were removed, the embryos were placed in fresh fix for 30 min on a rototorque at 4°C. They were then hand-devitellinized in fresh fix solution and fixed for an additional 1 to 2 h at 4°C. The DNA was then visualized using the biotin detection method from Vector as in Zusman and Wieschaus (1987). The embryos were postfixed in 2% EM grade glutaraldehyde, 2% EM grade paraformaldehyde in PBS. They were then osmicated for 2 h at room temperature in 2% osmium followed by dehydration through an alcohol series. Penetration of Epon into the embryos was accomplished with a stepwise introduction of the Epon diluted in propylene oxide. Embryos were embedded individually and left at 60°C overnight to allow the Epon to polymerize. Following polymerization, the blocks were mounted then cut in 1 μm sections using glass knives on a Sorvall JB-4 microtome. These thick sections were periodically screened during the cutting to determine the location of the injected DNA within the embryo. When the appropriate depth had been reached, the blocks were thin sectioned (50 to 60 nm) on a Sorvall MT2 Ultra microtome, using a diamond knife, then mounted on metal grids. Before viewing on a Philips EM300 electron microscope, the grids were stained in a saturated uranyl acetate solution followed by lead citrate staining (Venable and Coggeshall, 1965).

Results

I. DNA injection: 'nuclei' in the absence of centrosomes

Plasmid DNA injected into a developing *Drosophila* embryo underwent a process of structural reorganization. Embryos in nuclear cycles 4 or 5 were injected with DNA at concentrations of 1 $\mu g\,\mu l^{-1}$, 5 $\mu g\,\mu l^{-1}$, and 10 $\mu g\,\mu l^{-1}$, so that the amount injected was equivalent to the amount of nuclear DNA present in uninjected embryos at cycles 11, 13, or 14–15, respectively. After injection, the DNA was distributed as a broad extended streak through the embryo. 20 minutes later, the DNA had condensed into a narrow streak (Fig. 1A). By 70 min after injection, vesicles of DNA appeared. These apparently pinched off from the condensed DNA streak (Fig. 1B). The clusters of DNA vesicles often appeared as conglomerate spheres. The vesicles and clusters of vesicles ranged in length from 3 mm to

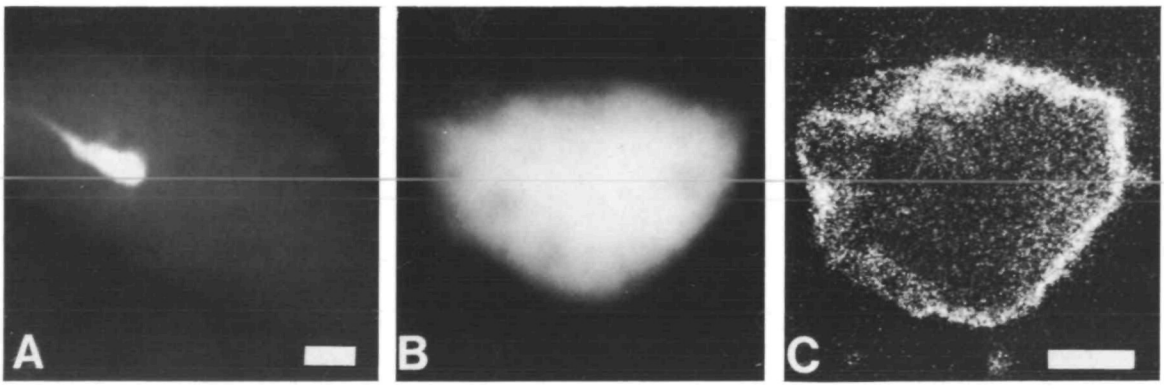

Fig. 1. Injected plasmid DNA is vesiculated and then surrounded by nuclear lamins. (A) 20 min after injection, the injected DNA was seen as an extended streak when stained with DAPI, bar=50 μm. The DNA underwent further changes in condensation so that 120 min after injection, vesicles of injected DNA had formed. (B) A DAPI-stained image of a large vesicle. (C) These vesicles were surrounded by nuclear lamins. In this confocal section, the nuclear lamins were visualized with T40 (anti-nuclear lamin) antibody. Bar=10 μm.

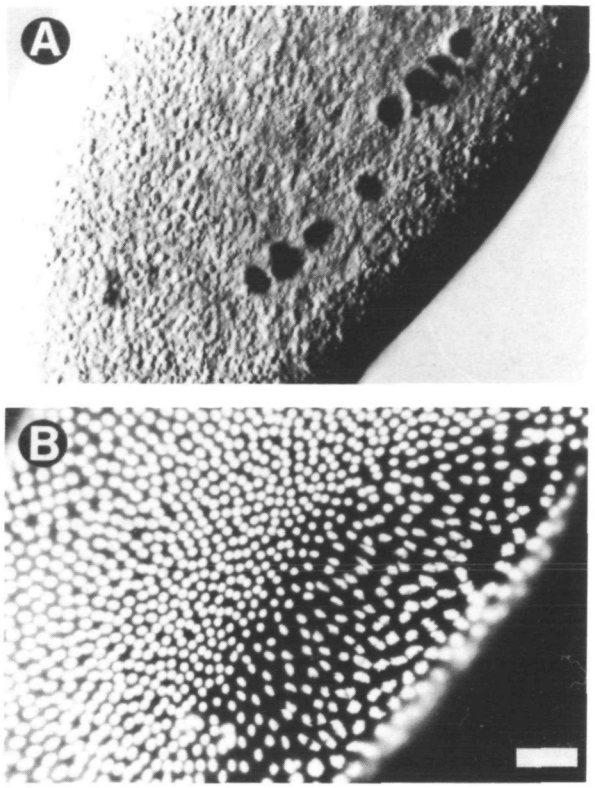

Fig. 2. Evidence for the loss of embryonic nuclei in the vicinity of the injected DNA. Plasmid DNA was labelled with biotin and injected into cycle 6 embryos. The embryos were fixed in cycle 14, 180 min later, and stained to visualize the injected DNA (A) and DAPI to visualize the embryonic nuclei (B). The DIC image (A) shows the injected DNA has generated a discontinuous streak of vesicles in the interior of the embryo. In the surface DAPI image (B), there is an obvious decrease in nuclear density of the peripheral nuclei in the region overlying the injected DNA vesicles. Bar=50 μm.

169 mm (n=113). These observations are similar to those reported by Steller and Pirrotta (1985).

In normal development, the majority of embryonic nuclei migrate to the periphery during telophase/interphase of cycles 8 and 9. Although the great majority of the injected DNA vesicles remained in the interior of the embryo, vesicles of injected DNA that were the same size or smaller than endogenous nuclei were occasionally found out at the periphery.

The injection of DNA was also associated with the loss of host nuclei from the dividing population, leading to an uneven distribution of nuclei, with a lower nuclear density in the vicinity of the injected DNA. The vesiculation of injected DNA and the resulting lowered nuclear density are shown in Fig. 2. The extent of nuclear loss was DNA concentration dependent: at the highest concentration, 10 μg ml^{-1}, 66% (n=56) of the embryos were abnormal with respect to nuclear density and at the lowest concentration, 1 μg ml^{-1}, 32% (n=81) were abnormal. The loss was also evidenced by the presence of heteropycnotic embryonic nuclei, which usually were adjacent to the regions of lowered nuclear density. These affected regions often contained centrosomes that were not associated with nuclei. Time-lapse video analysis revealed that embryos with a region of lowered nuclear density underwent a compensatory extra division and could develop into a normal blastoderm and larva.

DNA was surrounded by nuclear lamins

In the *Xenopus* system, Forbes and coworkers (1983) observed that injected phage DNA organizes a complete nuclear membrane around the DNA. Since the DNA injected into *Drosophila* appeared to be vesiculated, we examined its organization using 2 different antibodies to nuclear lamin (Risau *et al.* 1981; D. Kellogg, personal communication). No antibody staining was present around the injected DNA 10 min after injection. 70 min after injection, weak antibody staining was observed. By two hours after injection, all the DNA was vesiculated and staining was strong around all the DNA vesicles although it was still weaker than that around the embryonic nuclei. The conglomerate spheres conformation, which could be detected with the DNA staining, was more pronounced with the nuclear lamin staining. The presence of nuclear lamins surrounding the injected DNA vesicle was confirmed by reconstruction of serial thin optical sections generated by confocal microscopy (example section shown in Fig. 1C). The staining around the injected DNA, however, was uneven and most comparable to the pattern of staining around metaphase nuclei. The basic result that injected DNA could organize nuclear lamins was obtained with two different lamin antibodies and in the absence of protein synthesis (data not shown).

We observed that the lamin antibody staining of the embryonic nuclei and of the yolk nuclei was weaker in mitosis than in interphase. Additionally, the spherical shape of the lamin antibody staining pattern was less defined during mitosis than in interphase. These observations are documented in Fig. 3. The lamin morphology of the peripheral embryonic nuclei in cycle 11 (Fig. 3A) was different for the different phases of the mitotic cycle, as evidenced by the DAPI staining of DNA (Fig. 3B). Internally, the nuclear lamin morphology (Fig. 3C) and chromosome condensation (Fig. 3D) of the yolk nuclei changed in correspondence with the changes in the peripheral nuclei. At higher magnification (Fig. 3E and F), it is evident that the 'metaphase-like' lamin morphology around the injected DNA differed from the interphase lamin morphology around the yolk nuclei. We observed this same lamin morphology around injected DNA in embryos in which the yolk nuclei had a metaphase lamin configuration. Thus, the nuclear lamins around the injected DNA vesicles did not show any evidence of the cycle-dependent changes observed for the peripheral and yolk nuclei.

DNA organized nuclear membranes

Since the nuclear lamin antibodies demonstrated the presence of lamins around the injected DNA, we used electron microscopy to examine the fine structure of the

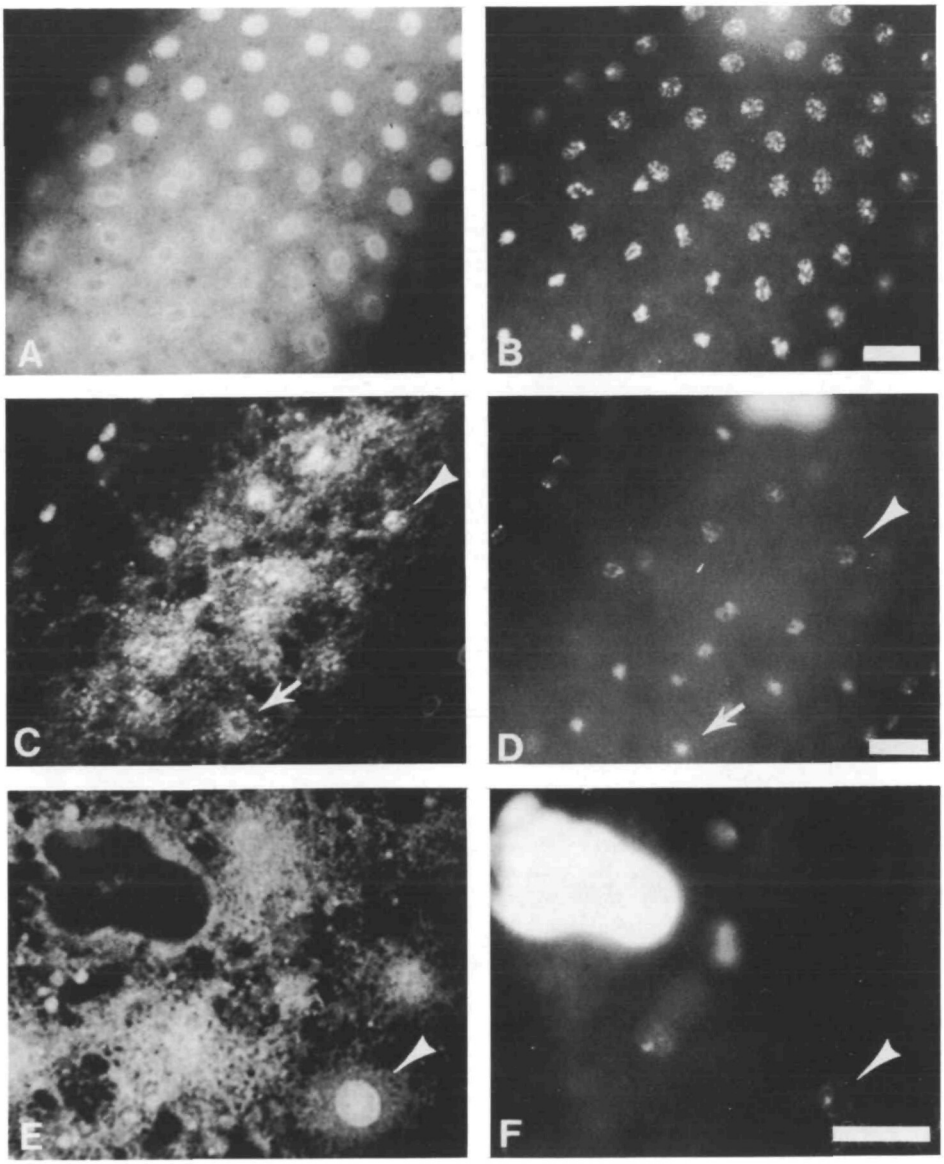

Fig. 3. Nuclear lamins organized by injected plasmid DNA do not cycle. Embryos were injected during cycle 3, fixed during cycle 11 and stained with anti-nuclear lamin antibody (A, C, and E) and DAPI (B, D, and F). The lamin antibody images are optical thin sections generated by confocal microscopy. The nuclei on the surface are undergoing a mitotic wave as evidenced by the cycle-dependent changes in nuclear lamins (A) and chromosomal condensation (B). A medial section of the same embryo shows the cycling of the yolk nuclei in parallel with the surface nuclei (C and D). Yolk nuclei in prophase (arrowhead) and metaphase (arrow) are observed in the same section. The bright DAPI staining at the top of the image (B and D) is the injected DNA. In E and F the lamin and nuclear staining around the injected DNA in this embryo is shown at higher magnification. In contrast to the prophase lamin configuration around the yolk nuclei (arrowhead), the lamin associated with the injected DNA has a 'metaphase-like' morphology. Bar=25 μm.

vesicles. We were able to demonstrate the formation of a double membrane with pores encompassing portions of the injected DNA. Embryos were injected with DNA 30 min after egg deposition (cycle 3) and then fixed for EM analysis 225 min after egg deposition (late cycle 14). The injected DNA was biotinylated and labeled histochemically so that it could be differentiated from the embryonic nuclei. This DNA staining was easily seen in thick sections (Fig. 4A) and allowed for the identification and orientation of the relevant thin sections. Since the DNA labelling was clearer when large amounts of label were present, our electron microscope observations were limited to the examination of membrane organization around large vesicles of DNA. Fig. 4B is a representative thin section electron micrograph of the embryo shown in Fig. 4A. The stained DNA could still be differentiated from the endogenous yolk nuclei by its electron denser appearance. Nuclear membrane was clearly evident around the injected DNA. In the same sections, the vitellophage nuclear membrane provided a control for comparison. In Fig. 4B, the DNA-organized nuclear membrane can be seen to differ from the vitellophage nuclear membrane in two aspects: (1) the DNA was not completely surrounded by membrane; and (2) the nuclear pore complexes were much less regularly distributed in the membrane surrounding the injected DNA than in the vitellophage nuclear membrane. At higher magnification, nuclear pore complexes could be found in the DNA-organized membranes (Fig. 4C). Comparison to the vitellophage nuclear pores (Fig. 4D) revealed differences in the electron density and apparent structural complexity of the organized pore structures.

The DNA-organized nuclear membranes were often associated with additional layers of nuclear membrane. These additional layers may be analogous to the annulate lamellae, membrane stacks that are found around the peripheral embryonic nuclei and the vitellophages (Stafstrom and Staehelin, 1984).

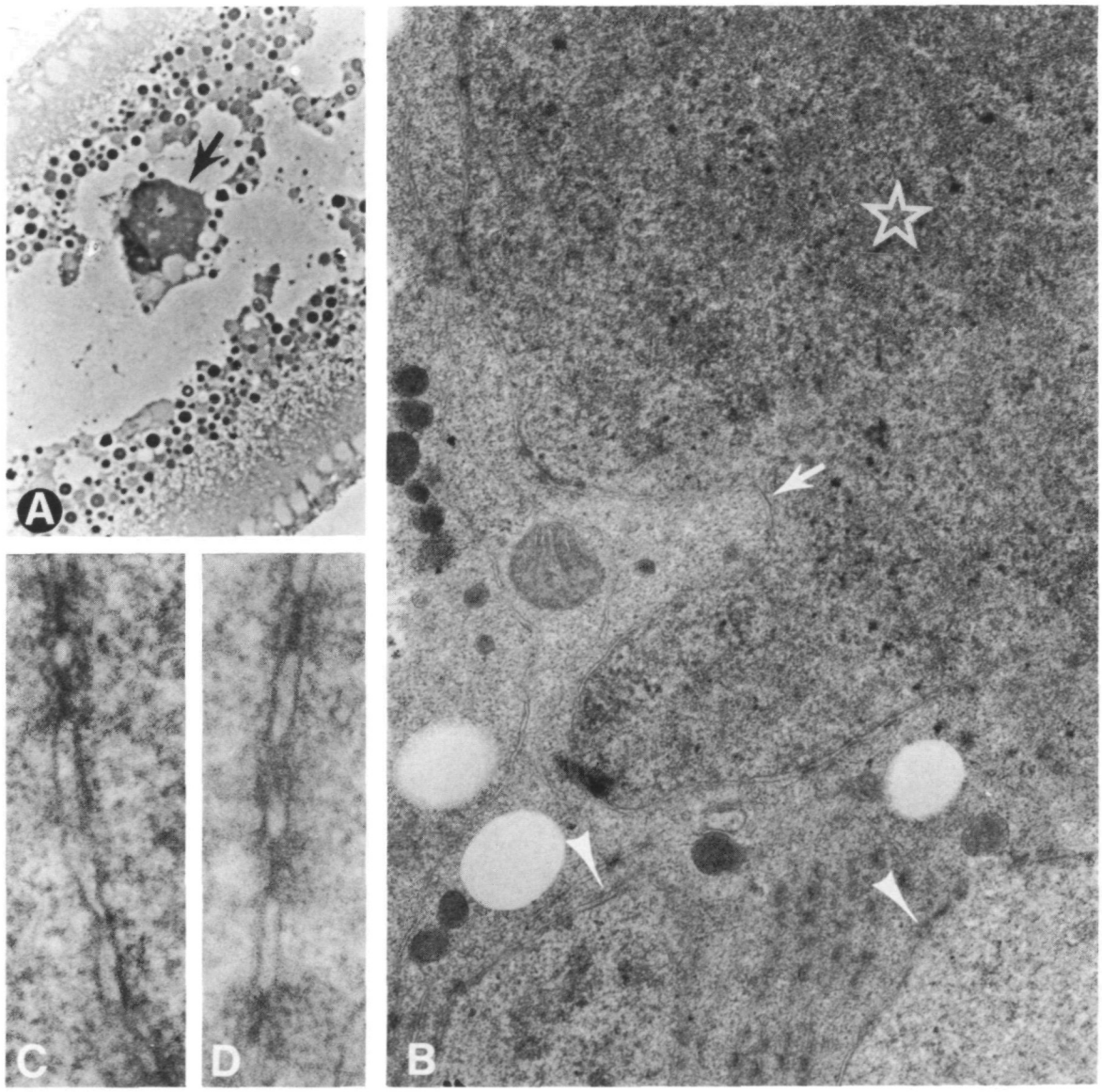

Fig. 4. Injected plasmid DNA organizes nuclear membrane containing nuclear pore structure. In thick sections (A), the labelled, injected DNA (arrow) is visible within the embryo. In thin section (B), the injected DNA (region denoted by star) is surrounded by a bilayered nuclear membrane (arrow). The nuclear membrane around two yolk nuclei are also evident in the same section (arrowheads). The nuclear pore structures in the DNA organized membrane (C) do not show the extended physical dimensions or structural complexity of the pore complexes in the yolk nuclei (D). Magnification for A) ×150, B) ×26 600, C and D) ×110 000.

DNA does not organize other cytoskeletal elements

In *Xenopus*, injected DNA is sufficient to organize microtubule arrays in metaphase cytoplasm in the absence of centrosomes (Karsenti *et al.* 1984). Using specific reagents for different cytoskeleton elements, we were unable to detect any consistent organized assembly of microtubules, microfilaments, or centrosomes around the DNA vesicles in embryos in which we could clearly detect cytoskeletal organization around yolk nuclei (data not shown).

II. UV irradiation: centrosomes in the absence of nuclei

Togashi and Okada (1984) reported that UV irradiation (300 nm) of *Drosophila* embryos prior to nuclear migration blocked mitoses in the nuclei underlying the irradiated region. We confirmed this result and also found that after embryos were irradiated at one pole, the embryo could continue to develop into a normal larva (Yasuda *et al.* in preparation). UV-irradiation of the anterior pole of the embryo resulted in regions deficient in nuclei at the anterior surface following migration (Fig. 5A). Using an antibody against a centrosomal antigen (Whitfield *et al.* 1988), we detected centrosomes throughout the nuclear-free regions (Fig. 5B). The presence of free centrosomes in the absence of nuclei resulted in part from the continuation of centrosome replication in the absence of nuclear

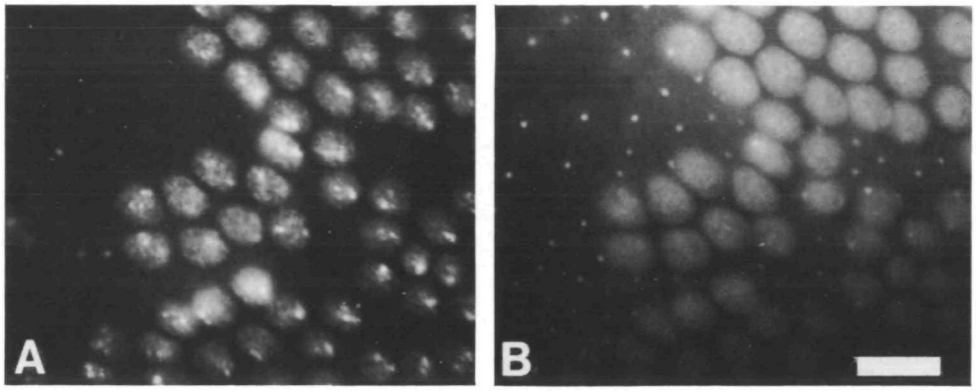

Fig. 5. Evidence for additional cytoskeletal organization around free centrosomes in regions devoid of nuclei. In the anterior UV-irradiated embryos, the arrest of mitoses in a subset of nuclei results in a localized deficit of nuclei at the anterior surface, as shown by DAPI staining (A) of this cycle 12 embryo. In these nuclear-free regions, centrosomal antibody staining reveals the presence of centrosomes (bright dots) surrounded by haloed staining, which was similar to, although less intense than, the staining seen around the neighboring embryonic nuclei (B). Bar=40 μm.

replication as determined by analysis of fixed preparations of embryos at 3 time points after UV-irradiation (data not shown). In the UV-irradiated embryos, the replicated centrosomes initially remained associated with the nuclei, but then appeared at the periphery at about the time when nuclear migration would occur in control embryos. Similarly, Raff and Glover (1988) showed that after blocking DNA synthesis in *Drosophila*, replication and migration of centrosomes continued.

After nuclear migration, the staining pattern of the centrosomal antibody changed so that there was diffuse haloed staining of the entire interphase nucleus in addition to the tight centrosome staining (Whitfield *et al.* 1988). Interestingly, this haloed staining pattern was also observed around the free centrosomes (Fig. 5B). Confocal scanning microscopy revealed staining of a spherical structure which was similar to that observed for complete nuclei (data not shown). The haloed pattern suggested a further cytoplasmic organization around the centrosomes in the nuclear free regions. Therefore, we monitored the distribution of a number of cytoskeletal elements in the irradiated embryos.

Microtubule asters were correlated with centrosomes during all phases of the cell cycle

Centrosomes, centrioles or spindle pole bodies have been demonstrated to organize the formation of microtubule asters in a number of organisms, including *Drosophila* (Byers *et al.* 1978; Hyams and Borisy, 1978; Mitchison and Kirschner, 1984; Picard *et al.* 1988; Karsenti *et al.* 1984; Raff and Glover, 1988). We checked for this ability in irradiated embryos by double staining with the antibodies to centrosomes and α-tubulin. Comparison of the centrosome staining to the tubulin staining revealed a complete coincidence of centrosomes with microtubule asters. This result was obtained whether or not there was DNA associated with the centrosome and aster. The free centrosomes were associated with asters in embryos in which the embryonic nuclei were in all phases of the mitotic cycle (Fig. 6). The staining of the aster associated with the free centrosome in metaphase appeared to be less intense, but it was similar to the asters found at each pole of the mitotic spindle formed by the embryonic nuclei (Fig. 6D). The centrosomes were therefore correlated with microtubules in metaphase as well as interphase cytoplasm, but were not correlated with the formation of chromosomal spindles. Spindles formed only when both centrosomes and chromosomes were present. Spindles formed even if the associated nuclei were pycnotic.

Microfilament networks with cycle-dependent morphology were correlated with centrosomes

The microfilament network in the irradiated embryos was determined by double staining with the centrosomal antibody and phalloidin, a toxin which binds filamentous actin. Once again, the presence of centrosomes correlated with the organization of microfilaments. However, the actin network associated with the free centrosomes displayed morphological variations which mimic the changes seen in the actin network surrounding embryonic nuclei during the mitotic cycle (Warn *et al.* 1984; Karr and Alberts, 1986; Kellogg *et al.* 1988). Comparison of the actin networks associated with the free centrosomes alone to the mitotic phase of the embryonic nuclei revealed that the changes in the actin network were mitotic cycle dependent. This change in morphology was clearest when comparing actin conformation in early interphase and in metaphase. In interphase cytoplasm, the actin was organized as a small cap above the nuclei or as a small cap around the free centrosomes (Fig. 7A,B,C). In metaphase cytoplasm, the actin cap enlarged and spread below the surface actin layer to surround the mitotic spindle (Fig. 7D,E,F) of the complete nucleus. In the absence of nuclei, the actin cap surrounded the centrosome to form pseudo-cleavage furrows. In addition, a 'contractile ring-like' accumulation of actin (arrow in Fig. 6D) was centered above the metaphase plate of the nuclei. This structure was not detected either above single

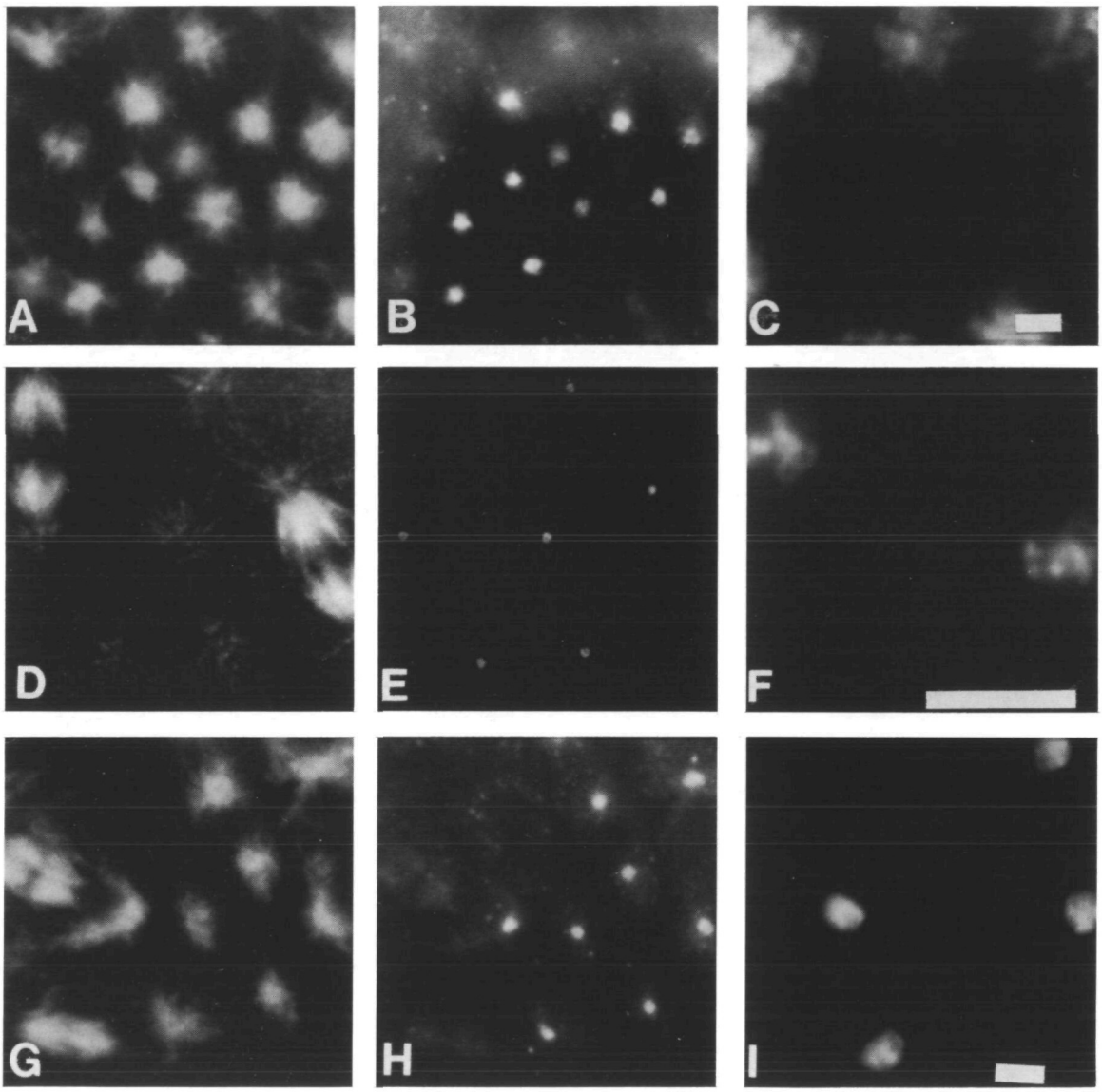

Fig. 6. Microtubules are associated with centrosomes in all phases of the cell cycle even in the nuclear-free regions. Anterior irradiated cycle 12 embryos were stained with anti-α-tubulin antibody (A, D, and G), anti-centrosomal antibody (B, E, and H), and DAPI (C, F, and I). Centrosomal organization of microtubule asters is shown in interphase (A, B, and C), metaphase (D, E, and F), and telophase (G, H, and I) cytoplasms. In metaphase cytoplasm, the centrosome organized asters are less distinct and show weaker antibody staining than in other phases of the cycle but are very similar to the asters at each pole of the mitotic spindle (D). Bar=10 μm.

centrosomes or between adjacent free centrosomes. The actin network associated with the free centrosomes appeared to respond to the temporally regulated mitotic cues in the embryo.

Spectrin networks were correlated with centrosomes

To analyze the morphology of spectrin which may serve as a stabilizing cross-link between a number of cytoskeletal systems, we double stained irradiated embryos with the α-tubulin antibody and the spectrin antibody (Dubreuil *et al.* 1987; Byers *et al.* 1987). We were unable to use the centrosomal antibody double staining because the spectrin antibody had also been generated in a rabbit. Since we had found that centrosomes were correlated with α-tubulin, the α-tubulin staining served as a marker for the presence of centrosomes. We found there was also a complete coincidence between the presence of centrosomes and the organization of spectrin (Fig. 8). The spectrin organization around free centrosomes closely resembled that found around complete nuclei. As was reported for spectrin organization around normal nuclei by Pesacreta *et al.* (1989), the spectrin structure around the centrosome paralleled the f-actin structure around the centrosomes.

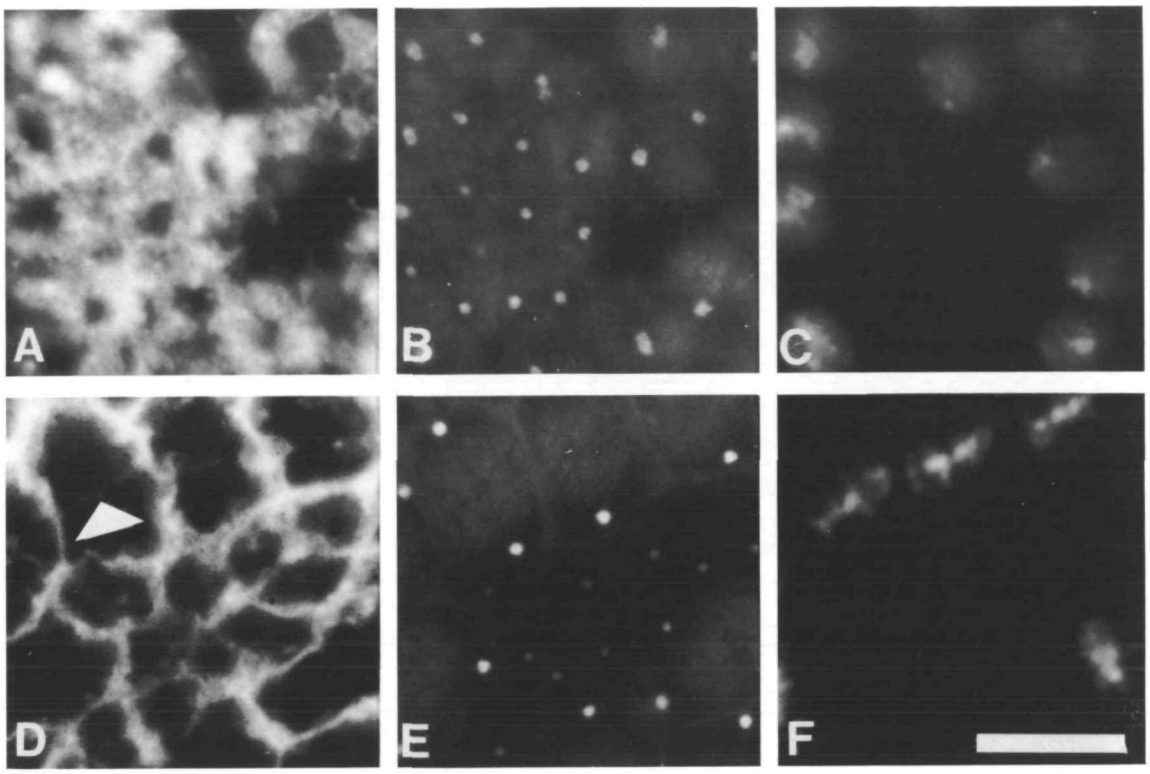

Fig. 7. Free centrosomes are correlated with a dynamic microfilament network which changes in response to the embryonic cell cycle cues. Anterior irradiated cycle 12 embryos were stained with phalloidin (A and D), anti-centrosomal antibody (B and E), and DAPI (C and F). The microfilament organization is shown in two phases of the cell cycle. In interphase cytoplasm, the centrosomes organize an actin cap similar to that seen above the embryonic nuclei (A, B, and C). In metaphase cytoplasm, the centrosomes are surrounded by an expanded actin cap which has spread below the surface layer (D, E, and F). This is similar to the actin network around the embryonic nuclei but the nuclei have an additional 'contractile-ring-like' staining pattern (arrowhead in D). Bar=10 μm.

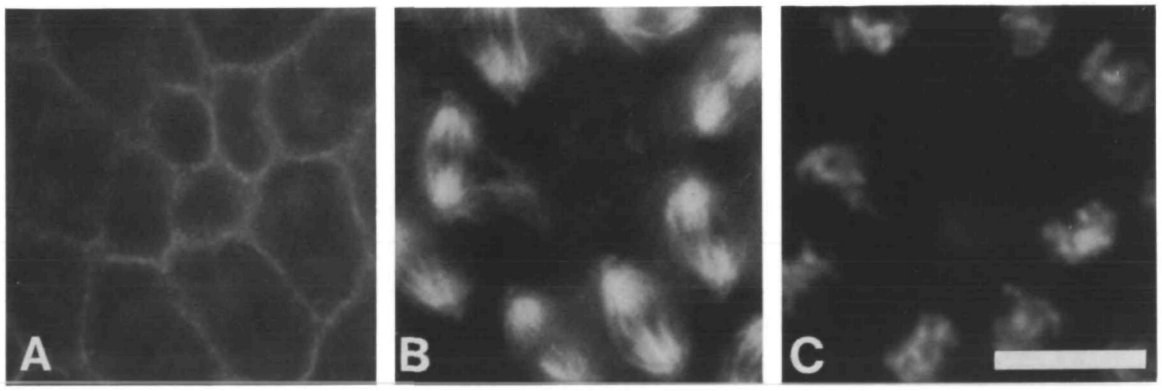

Fig. 8. Centrosomes are correlated with the organization of spectrin. Anterior irradiated cycle 12 embryos were stained to visualize spectrin. The series shows a region in metaphase stained with anti-spectrin antibody (A), anti-α-tubulin antibody (B), and DAPI (C). The anti-tubulin staining marks the positions of the normal spindles. The clear area in the middle contains four free centrosomes (faint aster staining) each of which has organized a surrounding ring of spectrin. Bar=10 μm.

Organization of nuclear lamins was not correlated with centrosomes

The ability of the centrosomes alone to organize the accumulation of nuclear lamins was investigated by double staining irradiated embryos with the centrosomal antibody and with either of the two nuclear lamin antibodies. We were unable to detect lamin organization associated with free centrosomes with either of

the two lamin antibodies. Lamin organization was only found around centrosomes associated with nuclei (data not shown).

Discussion

The organization of cytoskeletal elements in response to either plasmid DNA or free centrosomes indicates the presence of two basic organizing principles to generate the cytoarchitecture of the early syncytial embryo. Injected DNA organized nuclear envelopes as evidenced both by the nucleation of nuclear lamins and by the presence of extended patches of bilayered nuclear membrane around the DNA. Free centrosomes separate from nuclei were correlated with the organization of the surrounding cytoplasm. When present in the cortical cytoplasm, they were associated with the formation of microtubule, microfilament and spectrin networks. We interpret these results as evidence that the centrosomes serve as an organizing center for an extensive cytoskeleton. However, we have not tested whether this association is the result of a direct interaction of the individual cytoskeletal elements with the centrosome or the result of indirect interactions between cytoskeletal elements.

Nuclear assembly by plasmid DNA

The result that plasmid DNA was sufficient to organize nuclear envelope elements in *Drosophila* embryos was also found in *Xenopus* eggs and cell-free extracts. The initial progression of changes in DNA condensation that we have observed in *Drosophila* was morphologically similar to those described in *Xenopus*. This suggests that the DNA injected into *Drosophila* underwent the same protein modifications required for scaffold assembly and nuclear membrane organization that occur in *Xenopus* (Newport and Kirschner, 1982; Forbes *et al.* 1983; Newport, 1987). The DNA vesicles in *Drosophila*, however, differed from the 'synthetic' nuclei in *Xenopus* in two basic ways: (1) the DNA vesicles did not show any signs of mitotic cycling; (2) the DNA vesicle nuclear membranes were not completely normal. Specifically, they did not completely surround the DNA and had an abnormal distibution of structurally abnormal nuclear pores. The absence of cycling and the incomplete membrane organization observed may result from the rapid mitotic cycles in the *Drosophila* embryo or may indicate that the three hours between injection of DNA and fixation of the embryos was not sufficient to form a complete nuclear membrane around the injected DNA. A more interesting alternative is that the formation of incomplete membrane may reflect the difference between the mitotic process and the organization of the cytoskeleton in *Drosophila* and *Xenopus* embryogenesis. The mitoses in *Xenopus* are referred to as 'open' because the nuclear membrane breaks down completely during mitosis. After each mitosis, the membrane is reassembled onto chromatin templates from components stored in vesicles. At the other end of the spectrum, yeast has 'closed mitoses' in which the entire mitotic cycle occurs within an intact nuclear membrane (reviewed in Heath, 1980). *Drosophila* has an intermediate form of mitosis in which the nuclear membrane breaks down only at the poles (Stafstrom and Staehelin, 1984). The addition of membrane components at the end of mitosis involves the acquisition of precursors in a second closely adherent membrane cisternae. Since this mechanism does not apparently require *de novo* organization of nuclear membranes following mitosis, it could lead to a requirement for specific nuclear factors in order to get complete nuclear assembly.

Support for the proposal that additional chromosomal or nuclear elements are required for cycling comes from observations on chromosomes lost during the embryonic divisions. The paternal effect mutation *pal* (paternal loss) results in the loss of paternally derived chromosomes in the early embryo (Baker, 1975). These lost chromosomes are not associated with centrosomes as judged by centrosomal antibody staining. However, the lost chromosomes are in the same condensation state as the surrounding embryonic nuclei (J. Tomkiel, personal communication). Specifically, they appear as micronuclei when the embryonic nuclei are in interphase or prophase, and as condensed chromosomes when the embryonic nuclei are in metaphase, anaphase, or telophase. Chromosome-organized micronuclei therefore appear to respond to the mitotic cues in the cytoplasm in the absence of centrosomes.

The DNA injections also led to the loss of endogenous embryonic nuclei from the dividing population. Associated with these regions of lowered nuclear density were heteropycnotic nuclei which appeared to be degrading. The frequency of injected embryos showing an abnormal nuclear distribution or morphology was correlated with the amount of injected DNA. Because the loss of host nuclei was coupled to the appearance of injected DNA vesicles, this negative effect on the endogenous nuclei may result from the titration of required nuclear components by the injected DNA. The ability of the injected DNA to accumulate nuclear components was directly demonstrated for nuclear lamins and nuclear membrane materials and may be implied for other components (such as histones and topoisomerase II) from the similarity of the DNA condensation process in *Drosophila* to that in *Xenopus*. If this general titration occurs, the injection of an additional cycle 11 to cycle 14+ equivalence of DNA may remove enough material from the embryo to drastically effect the population of embryonic nuclei.

Centrosome organization of microtubules may reveal a difference in spindle organization between mitotic systems

The free centrosomes produced following UV irradiation or DNA injection were correlated with the formation of tubulin asters in all phases of the mitotic cycle. While we could not quantitate tubulin nucleation, the staining with the tubulin antibody appeared to be less intense in metaphase than in

interphase. Since the aster organized by the free centrosomes in metaphase was similar to the asters observed at the poles of the mitotic spindles, the difference in staining intensity may indicate a cycle dependency. Alternatively, the difference may reflect the loss of tubulin availability because of competition from the normal spindles. The ability of centrosomes to organize tubulin was expected because it had been demonstrated previously in a number of organisms (Byers *et al.* 1978; Hyams and Borisy, 1978; Mitchison and Kirschner, 1984; Picard *et al.* 1988; Karsenti *et al.* 1984; Raff and Glover, 1988). In three of these systems, the cycle dependency of tubulin organization by the centrosome was investigated. In *S. cerevisiae*, Hyams and Borisy (1978) were unable to detect a difference in nucleation ability between newly replicated spindle pole bodies (interphase) and the majority species of spindle pole body (metaphase) from an exponentially growing culture. In the starfishes *Marthasterias glacialis* and *Astropecten aranciacus*, Picard *et al.* (1988) reported the continuation of centrosome duplication and periodic changes in aster morphology in enucleated embryos.

In *Xenopus*, Karsenti *et al.* (1984) reported that they were unable to detect tubulin organization around isolated centrosomes injected into oocytes arrested in metaphase of meiosis II or injected into activated eggs arrested in metaphase with cytostatic factor (CSF). There is good evidence that these *in vivo* results reflect the microtubule behavior during the mitotic divisions. *In vitro* studies have confirmed that there is a cycle-dependent difference in the extent of tubulin organization around isolated centrosomes between interphase and mitotic extracts (Belmont *et al.* 1990). In addition, XMAP, the dominant microtubule polymerization-promoting factor in the egg, is regulated in the same manner through the meiotic and mitotic divisions (Gard and Kirschner, 1987). The *in vitro* studies do, however, differ from the original *in vivo* studies in that small asters are organized by centrosomes in the metaphase extracts. It is not clear whether this difference results from changes in cytoplasm that may have occurred during the preparation or the experimental manipulation of the *in vitro* extracts or from the increased ease of detection of asters in the *in vitro* system. In the absence of a definitive answer, it may be most prudent to assume that free centrosomes organize microtubules in mitotic metaphase and that the characterisitics of tubulin organization during the mitotic cycle will therefore be very similar in *Xenopus* and *Drosophila*.

We failed to detect organization of microtubules around the vesicles of injected DNA. While the centrosomes were always examined at the surface, the DNA usually remained in the interior of the embryo among the vitellophages. The vitellophages are associated with microtubules but they do not organize as extensive a microtubule array as the nuclei at the periphery. Although we did not detect microtubules around DNA in the same embryos in which we detected microtubule organization around vitellophages, it is formally possible that the inability to detect organization may result from this difference in environment. However, it should be noted that the paternal effect mutation *pal* can result in free chromosomes at the surface. These chromosomes do not obviously organize microtubules even though they do undergo cyclical changes in condensation (J. Tomkiel, personal communication). Thus, the presence of complete chromosomes at the surface is apparently insufficient for the organization of tubulin. The absence of microtubule organization may therefore result from the inability of DNA or chromosomes to act as an organizing center for microtubules rather than from the effects of the embryonic environment.

Taken together with the observations on the association of centrosomes with tubulin, the apparent inability of DNA injected into *Drosophila* to organize tubulin may be interpreted as reflecting a difference in the mechanism for spindle organization in this 'semi-closed' mitotic system. Karsenti *et al.* (1984) reported that DNA injected into *Xenopus* embryos is able to concentrate tubulin during metaphase and an anastral spindle is organized. It is the combination of centrosomes and DNA that leads to the formation of a normal looking spindle. From these results, they proposed that in this 'open' mitotic system the spindle is formed because of a local increase in concentration of tubulin around the DNA which allows for polymerization from the neighboring centrosomes. In contrast to this scenario, in the *Drosophila* 'semi-closed' system normal spindle formation required both the presence of centrosomes and chromosomal material, but this requirement for chromosomes did not apparently result from a general DNA-mediated concentration of tubulin as seen in *Xenopus*. Instead, the chromosomes may only be required for the stability of the centrosomal tubulin array. This function could be supplied by the kinetochores which have been inferred to stabilize microtubules (Salmon and Begg, 1980; Rieder, 1981; Mitchison *et al.* 1986). Our experiments, however, did not allow us to distinguish whether any DNA or specifically chromosomal material, which possibly contains kinetochore activity, was required for organization of a spindle.

The cytoskeleton associated with the centrosomes is stable through multiple cycles but responds to the embryonic cell cycle cues

In contrast to the subtle changes in tubulin organization around the free centrosomes during the mitotic cycle, the centrosome-associated microfilament networks underwent drastic changes in morphology. These changes closely mimic the cycle-dependent changes in microfilament networks around the neighboring complete nuclei. These results therefore indicate that free centrosomes may serve as an organizing center for microfilament arrays that are not only normal in appearance but also normal in their response to mitotic cues in the embryonic cytoplasm. The importance of the centrosome in microfilament organization had been proposed by Karr and Alberts (1986) because of the centrosome's position at the apparent nucleation point

for the developing actin networks following nuclear migration. The correlation between centrosomes and actin organization was reported by Raff and Glover (1989) in their studies on DNA-synthesis-inhibited embryos. Mitosis is blocked in these embryos but somatic budding continued with an abnormal cycle timing (Raff and Glover, 1988). This budding was associated with changes in the size of the actin caps. Although this experimental system did not apparently generate normal actin morphology or allow for direct comparison to normal nuclei, it could be inferred from this observation that cyclical changes in centrosome-associated microfilament network were also occurring in the DNA-synthesis-arrested embryos.

While the cycle-dependent changes in microfilaments demonstrate the dynamic nature of the centrosome-associated cytoskeleton, the presence of spectrin may provide evidence for the stability of the centrosome-associated cytoskeleton, a property required for its maintenance throughout the mitotic cycle. Spectrin is a major contributor to the cortical cytoskeleton, which gives the mammalian erythrocyte its shape and stability (Elgsaeter *et al.* 1986). In non-erythroid cells, the available evidence points to a role for the multivalent protein in the organization of membrane domains (reviewed in Morrow, 1989). Spectrin has been reported to interact with microfilaments (Glenney *et al.* 1982), microtubules (Sobue *et al.* 1987) and intermediate filaments (Langley and Cohen, 1987) as well as with the cytoplasmic surface of the plasma membrane (Bennett, 1985). In *Drosophila*, the spatial and temporal changes in distribution indicate that spectrin has a role in stabilizing rather than initiating changes in structural organization during embryogenesis (Pesacreta *et al.* 1989).

Our interpretation that the centrosomes organize cytoskeletal elements is further supported by our observations on DNA-injected embryos. As mentioned, free centrosomes were observed in the regions of lowered nuclear density. In these same regions, we observed the organization of microtubules and microfilaments (data not shown). Taken together with the observations in aphidicolin-injected embryos (Raff and Glover, 1988, 1989), it is clear that the correlation between organization of cytoskeletal elements and the presence of centrosomes is not dependent upon the manner in which centrosomes were separated from nuclei. Therefore, the organization of cytoskeletal elements is likely to be a property of the centrosomes.

A functionally autonomous cytoskeleton organized by the centrosome

The complex of associated structural elements that make up the nucleus and surrounding cytoskeleton may be thought of as two separately organized systems which act in concert to generate the rapid early mitotic divisions. The ability of the cytoskeleton associated with the centrosome to respond to a central, common mitotic regulator was clearly demonstrated by the dynamic microfilament network which changed in parallel with the microfilament networks around the embryonic nuclei. Great progress has recently been made toward understanding the molecular basis of the embryonic mitotic regulator (reviewed in Murray and Kirschner, 1989). These experiments have defined this simple oscillator as being driven by the continuous translation and cyclic destruction of cyclin proteins. While the synchrony of the nuclear and centrosomal cycles is normally complete, recent experiments have called into question the absolute dependence of centrosomal replication on the defined mitotic regulator. These experiments show that centrosomal replication continues in *Xenopus* and in sea urchin in the absence of protein synthesis (Gard *et al.* 1990; Sluder *et al.* 1990). The centrosomal replication cycles in these embryos were variable and differed from control embryos in their average cycle time. Together with our observations, these finding indicate that the centrosome may be able to replicate and organize an extensive cytoskeleton autonomously of the nucleus or the translation-dependent mitotic oscillator.

We would like to acknowledge Dan Branton, Doug Kellogg, Jon Sedat, and Wil Whitfield for generously providing antibodies. Doug Kellogg and John Tomkiel for communicating results prior to publication. Clyde Lulham and Paul Goodwin for assistance with confocal microscopy. Steve Brocco, Loretta Goetsch and Breck Byers for assistance with electron microscopy. Barbara Wakimoto, Breck Byers, Margrit Schubiger, William Sullivan, Lauren Yasuda, and the entire Schubiger lab for their commments on this manuscript. This work was supported by an NSF predoctoral fellowship to G.K.Y. and NIH Research grant GM33656 to G.S.

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(*Accepted 19 October 1990*)