Live Analysis of Free Centrosomes in Normal and Aphidicolin-treated *Drosophila* Embryos

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Abstract. In a number of embryonic systems, centrosomes that have lost their association with the nuclear envelope and spindle maintain their ability to duplicate and induce astral microtubules. To identify additional activities of free centrosomes, we monitored astral microtubule dynamics by injecting living syncytial *Drosophila* embryos with fluorescently labeled tubulin. Our recordings follow multiple rounds of free centrosome duplication and separation during the cortical divisions. The rate and distance of free sister centrosome separation corresponds well with the initial phase of associated centrosome separation. However, the later phase of separation observed for centrosomes associated with a spindle (anaphase B) does not occur. Free centrosome separation regularly occurs on a plane parallel to the plasma membrane. While previous work demonstrated that centrosomes influence cytoskeletal dynamics, this observation suggests that the cortical cytoskeleton regulates the orientation of centrosome separation. Although free centrosomes do not form spindles, they display relatively normal cell cycle–dependent modulations of their astral microtubules. In addition, free centrosome duplication, separation, and modulation of microtubule dynamics often occur in synchrony with neighboring associated centrosomes. These observations suggest that free centrosomes respond normally to local nuclear division signals. Disruption of the cortical nuclear divisions with aphidicolin supports this conclusion; large numbers of abnormal nuclei recede into the interior while their centrosomes remain on the cortex. Following individual free centrosomes through multiple focal planes for 45 min after the injection of aphidicolin reveals that they do not undergo normal modulation of their astral dynamics nor do they undergo multiple rounds of duplication and separation. We conclude that in the absence of normally dividing cortical nuclei many centrosome activities are disrupted and centrosome duplication is extensively delayed. This indicates the presence of a feedback mechanism that creates a dependency relationship between the cortical nuclear cycles and the centrosome cycles.

The centrosome plays a fundamental role in the organization of eukaryotic cells. This organelle regulates the number, distribution, and dynamics of microtubules within the cell, and orchestrates the generation and orientation of the bipolar mitotic spindle. In most higher eukaryotes, each centrosome is a complex and amorphous mass of material encompassing a pair of microtubule-based structures called centrioles (for reviews see Kalt and Schliwa, 1993; Kellogg et al., 1994). Recent studies have identified γ-tubulin as a key centrosomal protein responsible for microtubule nucleation (Oakley et al., 1990; Moritz et al., 1995; Zheng et al., 1995). Among cytoplasmic components, the centrosomes are distinct because they are precisely duplicated once each division cycle. Despite decades of research on the centrosome, much remains unknown about its duplication, movement, modulation of microtubule dynamics, and molecular composition.

Studies involving the initial embryonic divisions in amphibians, marine invertebrates, and insects have provided much of our knowledge about the centrosome. The initial divisions in these organisms lack many of the well established cell cycle checkpoints and consequently it has been possible to uncouple the nuclear and centrosome cycles (Hartwell and Weinert, 1989). For example, centrosome duplication continues in sea urchin and starfish embryos with arrested nuclear cycles (Nagano et al., 1981; Sluder and Lewis, 1987). Enucleated sea urchin embryos are capable of undergoing multiple rounds of centrosome dupli-

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cation and separation (Sluder et al., 1986). These free centrosomes also undergo normal cyclic modulation of their microtubule asters. In addition to demonstrating a lack of dependence of the centrosome cycle on the nuclear cycle, they also show that centrosome duplication and separation in sea urchin embryos does not rely on a physical proximity to the nuclear envelope. Analysis of *Xenopus* and sea urchin embryos injected with protein synthesis inhibitors demonstrates that centrosome duplication can occur in the absence of a detectable cell cycle (Gard et al., 1990; Sluder et al., 1990).

Genetic, cellular, and biochemical studies have demonstrated that the *Drosophila* embryo is also a valuable system for studying the centrosome. The initial nuclear divisions in *Drosophila* are rapid, synchronous, and occur without accompanying cytokinesis (Rabinowitz, 1941; Sonnenblick, 1950; Turner and Mahowald, 1976; Zalokar and Erk, 1976; Foe and Alberts, 1983; Stafstrom and Staehelin, 1984; Minden et al., 1989). During nuclear cycles 9 and 10, the majority of the nuclei migrate to the periphery where they undergo four more rounds of synchronous divisions and cellularize during interphase of nuclear cycle 14. These syncytial divisions alternate between M and S with no obvious G1 and G2 phases (Foe et al., 1993).

Analysis of mutations disrupting the initial divisions of the *Drosophila* embryo has provided a number of insights concerning centrosome behavior and function. While the initial nuclear divisions are disrupted in embryos derived from the maternal-effect mutation *gnu*, centrosome duplication continues (Freeman et al., 1986; Freeman and Glover, 1987). These free centrosomes migrate to the cortex, form astral microtubules, and induce cytoskeletal rearrangements. Free centrosomes have also been detected in the maternal-effect mutations *asp* (Gonzalez et al., 1990) and *abc* (Vessey et al., 1991). Another mutation, *dal*, disrupts centrosome separation during the cortical divisions (Sullivan et al., 1990, 1993*a*). Analysis of this mutation indicates that proper cortical cytoskeletal dynamics depend on regular centrosome spacing. In a number of mutations and chromosomal rearrangements, the products of abnormal nuclear divisions sink into the interior of the embryo while their associated centrosomes remain on the surface (Sullivan et al., 1993*b*). This indicates that the centrosome is closely associated with the cortical cytoskeleton and that the nucleus may interact with the cortical cytoskeleton via the centrosome.

A number of issues concerning the behavior of free centrosomes in *Drosophila* embryos remain unresolved. Although there is evidence for free centrosome duplication, it is not clear whether it is occurring in an unregulated fashion or if the centrosomes are still responding to normal division signals. It is not known how many cycles of free centrosome duplication occur nor whether they maintain their ability to normally separate from one another. The extent to which free centrosomes maintain their ability to modulate the nucleation of microtubules in a cell cycle–dependent fashion also has not been thoroughly examined. During the cortical divisions, sister centrosomes separate so that they lie on a plane parallel to the plasma membrane. It is not known whether free centrosomes are capable of maintaining this orientation.

The dependency relationship between the centrosome cycle and the nuclear cycle also requires further examination. Previous studies examined the response of the nuclear and centrosome cycles in syncytial *Drosophila* embryos to aphidicolin, an inhibitor of DNA synthesis (Raff and Glover, 1988, 1989). As fixed analysis was used, it was not technically feasible to monitor the migration and duplication patterns of individual centrosomes after aphidicolin injection.

We directly address these issues by examining centrosomes, both free and nuclear associated, in living syncytial *Drosophila* embryos. This is accomplished by injecting embryos with fluorescently labeled tubulin (Kellogg et al., 1988) and fluorescently labeled histones (Minden et al., 1989). Our confocal recordings demonstrate that free centrosomes maintain a surprising repertoire of activities and that these activities occur in synchrony with the normal division cycle. In addition, we demonstrate that in aphidicolin-treated embryos large numbers of nuclei recede into the interior while their centrosomes remain on the cortex. Our recordings also demonstrate an extensive delay in the duplication cycle of free centrosomes in aphidicolin-treated embryos. This suggests the presence of a feedback mechanism which establishes a dependency relationship between the centrosome and nuclear cycles. These results are discussed in the context of previous studies performed in *Drosophila* and other embryonic systems.

Materials and Methods

Drosophila Stocks

All of the experiments relied on the wild-type Oregon-R stock (Lindsley and Grell, 1968). The stock was maintained on a standard corn meal/molasses media at 25°C.

Fixation and Immunofluorescence

Embryos were fixed using formaldehyde by a modification of the Mitchison and Sedat procedure (1983). This method is described in detail elsewhere (Theurkauf, 1992). Immunofluorescence analysis was performed as described by Karr and Alberts (1986). Centrosomes and nuclei were stained with the Rb188 anti-centrosomal antibody (Whitfield et al., 1988) and propidium iodide (Fogarty et al., 1994), respectively. The microtubules were stained with an anti–α-tubulin antibody. The embryos were extensively rinsed in PBS and mounted in a 50% glycerol, PBS solution containing 1 mg/ml *N-N*-1-4-phenylenediamine.

Microscopy was performed using an inverted microscope (IMT2; Olympus Corp., Precision Instrument Division, Lake Success, NY) equipped with a laser confocal imaging system (600, Bio-Rad Laboratories, Hercules, CA). The lenses used included the Olympus S Plan Apo 60, Oil and the Olympus D Plan Apo 20, UV, Oil. The nuclear cycle of the cortical divisions was determined by using the Bio-Rad imaging software to estimate nuclear densities.

In Vivo Fluorescence Analysis

The in *vivo* analysis of nuclear and centrosome behavior was accomplished by microinjecting fluorescently labeled histones and tubulin into embryos during the syncytial cortical divisions (Kellogg et al., 1988; Minden et al., 1989). The embryos were prepared for microinjection by hand dechorionation and mounting on a coverslip with a thin film of glue (Minden et al., 1989). Observations and time-lapse recordings were made on an Olympus IMT2 microscope equipped with a Bio-Rad MRC 600 confocal imaging system.

A 100-μg/ml solution of aphidicolin dissolved in a 0.5% DMSO, 5 mM KCl, 0.1 mM sodium phosphate (pH 6.8) solution was used to inhibit DNA synthesis. 1-h collections of embryos aged for 30 min were injected with either rhodamine-labeled histones or rhodamine-labeled tubulin.

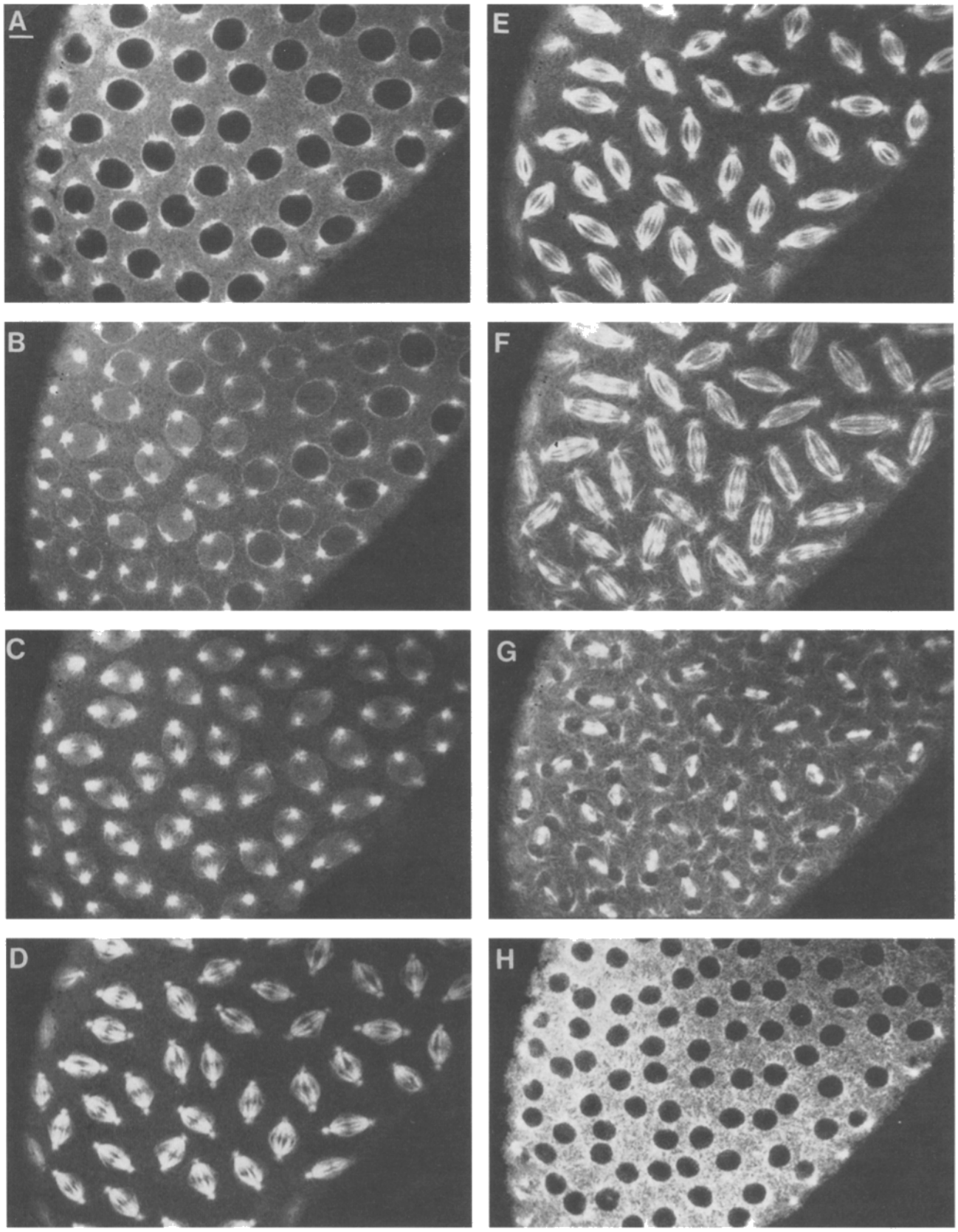

Figure 1. Confocal images of a living embryo injected with rhodamine-labeled tubulin. The recording follows an embryo from prophase of nuclear cycle 11 to interphase of nuclear cycle 12. The stage and the total elapsed time are as follows (min : s): (*A*) cycle 11 prophase, 0:00; (*B*) late prophase, 2:30; (*C*) early metaphase, 3:00; (*D*) metaphase, 4:30; (*E*) late metaphase, 6:00; (*F*) anaphase, 6:30; (*G*) telophase 7:30; (*H*) cycle 12 interphase, 9:30. Bar, 5 μm.

Once the nuclei migrated to the cortex, these embryos were injected with 100 μg/ml aphidicolin. The microtubule and nuclear dynamics in these embryos were observed for up to 1 h after the injection.

Fixed Analysis of Aphidicolin-injected Embryos

Nuclear cycle 9 embryos, identified by pole bud formation, were allowed to develop another 10 min and injected with 100 μg/ml aphidicolin. These embryos, covered with Halocarbon oil were either fixed immediately or allowed to develop another 45 min (at 25°C in a moist chamber) before formaldehyde fixing. After fixation, the embryos were hand devitellinized and double stained for their centrosomes and nuclei.

Results

Normal Centrosome Behavior in Syncytial Drosophila Embryos

We followed the microtubule and centrosome dynamics during the cortical divisions by injecting embryos with fluorescently labeled tubulin and recording a confocal image every 30–60 s. These images extend previous studies of *Drosophila* embryonic microtubule dynamics (Karr and Alberts, 1986; Warn and Warn, 1986; Warn et al., 1987; Kellogg et al., 1988). The divisions remain synchronous and few errors are observed. Fig. 1 follows microtubule dynamics from prophase of nuclear cycle 11 to interphase of cycle 12. The centrosomes are detected easily by their extensive nucleation of microtubules. During prophase, the centrosome-induced asters are observed at opposite sides of each nucleus (*A*). The bright background is produced by unincorporated fluorescently labeled tubulin dispersed throughout the cytoplasm. The nuclei appear as dark spheres because the intact nuclear envelope prevents the entry of labeled tubulin. Nuclear envelope breakdown during prophase results in an influx of labeled tubulin into the nuclear space (*B*). In this panel, the mitotic wave is preceded by a wave of nuclear envelope breakdown. One minute later, spindles are observed forming between sister centrosomes (*C* and *D*). As the spindles mature, the density and length of the astral microtubules dramatically increase (*E*) and reach a maximum during anaphase (*F*). The nuclear envelope reforms during early telophase excluding the labeled tubulin (*G*). Also during telophase, the microtubules reorganize and form distinct midbodies between sister nuclei (*G*). By late telophase, the duplicated centrosome pairs have separated and display a flattened configuration (*G*). As the nuclei enter the next interphase, the centrosomes no longer lie in the focal plane (*H*). This is a consequence of either nuclear rotation or centrosome migration so that the centrosome pairs lie between the nuclear envelope and the plasma membrane. These images highlight the tight linkage of the centrosomes to the nuclear envelope.

Free Centrosome Behavior in Drosophila Embryos

Previous studies of free centrosomes in *Drosophila* have relied on fixed analysis of embryos in which global defects were produced through the use of drugs, UV irradiation, or mutations (Freeman et al., 1986; Raff and Glover, 1988; Yasuda et al., 1991). To examine the behavior of free centrosomes in a normal embryo, we have relied on the observation that the products of occasional spontaneous nuclear division errors recede into the interior of the embryo while their centrosomes remain on the cortex (Minden et al., 1989; Sullivan et al., 1990, 1993*b*).

Free centrosomes are readily detected by their associated microtubule asters. To demonstrate this, we induced large numbers of free centrosomes by heat shocking embryos. These embryos were formaldehyde fixed and stained both for microtubules and centrosomes. We used the well characterized anti-centrosome antibody Rb188 (Whitfield et al., 1988). Immunofluorescent analysis demonstrates that both the free and spindle-associated centrosomes are encompassed by distinct astral microtubule arrays (data not shown). This is true for all free asters. Thus the asters serve as a reliable indicator of centrosome position and activity.

Rhodamine-labeled tubulin injections provide a means of following free centrosome behavior in undisturbed living embryos. As free centrosomes are rare, only 8 of 21 recordings of syncytial embryos exhibited free centrosomes. In total, of 2,038 centrosomes observed, 43 were free (Table I). The images depicted in Fig. 2 follow a free centrosome in a normal embryo from metaphase of nuclear cycle 11 to prophase of nuclear cycle 13. As this embryo progresses from metaphase to telophase of nuclear cycle 11, the asters of both the associated and free centrosomes become more extensive (compare *A* and *B*). By late telophase, the centrosomes associated with the reformed nuclear envelope have clearly duplicated (*C*). The arrow in panel C highlights a free centrosome which has also duplicated. During interphase of nuclear cycle 12, both the associated and the free sister centrosomes separate from one another (see arrows, *D–H*). Upon entering metaphase, diminished astral microtubule arrays are observed for both the associated and free centrosomes (*H*). *I–L* follow the embryo as it progresses through anaphase, telophase and into interphase of nuclear cycle 13. These images demonstrate that each of the sister products of the original free centrosome undergoes another round of duplication and separation (see arrows, *H–L*). Each of these centrosomes

Table I. Summary of the Live Analysis of Free Centrosome Behavior during the Cortical Divisions of Normal Drosphila Embryos

Summary of live analysis
21 tubulin movies (21 separate embryos examined)
43 of 2,038 centrosomes examined were free
Duplication of free centrosomes
34 duplicated
6 did not
3 could not determine
Synchrony of free centrosome separation
25 duplicated and separated in synchrony with neighboring associated centrosomes
9 separated with >3-min delay
Plane of free centrosome separation
26 separated in a plane parallel to the plasma membrane
2 separated in a plane not parallel to the plasma membrane
6 could not determine separation plane
Distance of free centrosome separation
At nuclear envelope breakdown, free centrosomes separated 80% of the distance observed for associated centrosomes (11 centrosome pairs followed)
By late anaphase, the same centrosome pairs separated only 60% of the distance observed for associated centrosomes

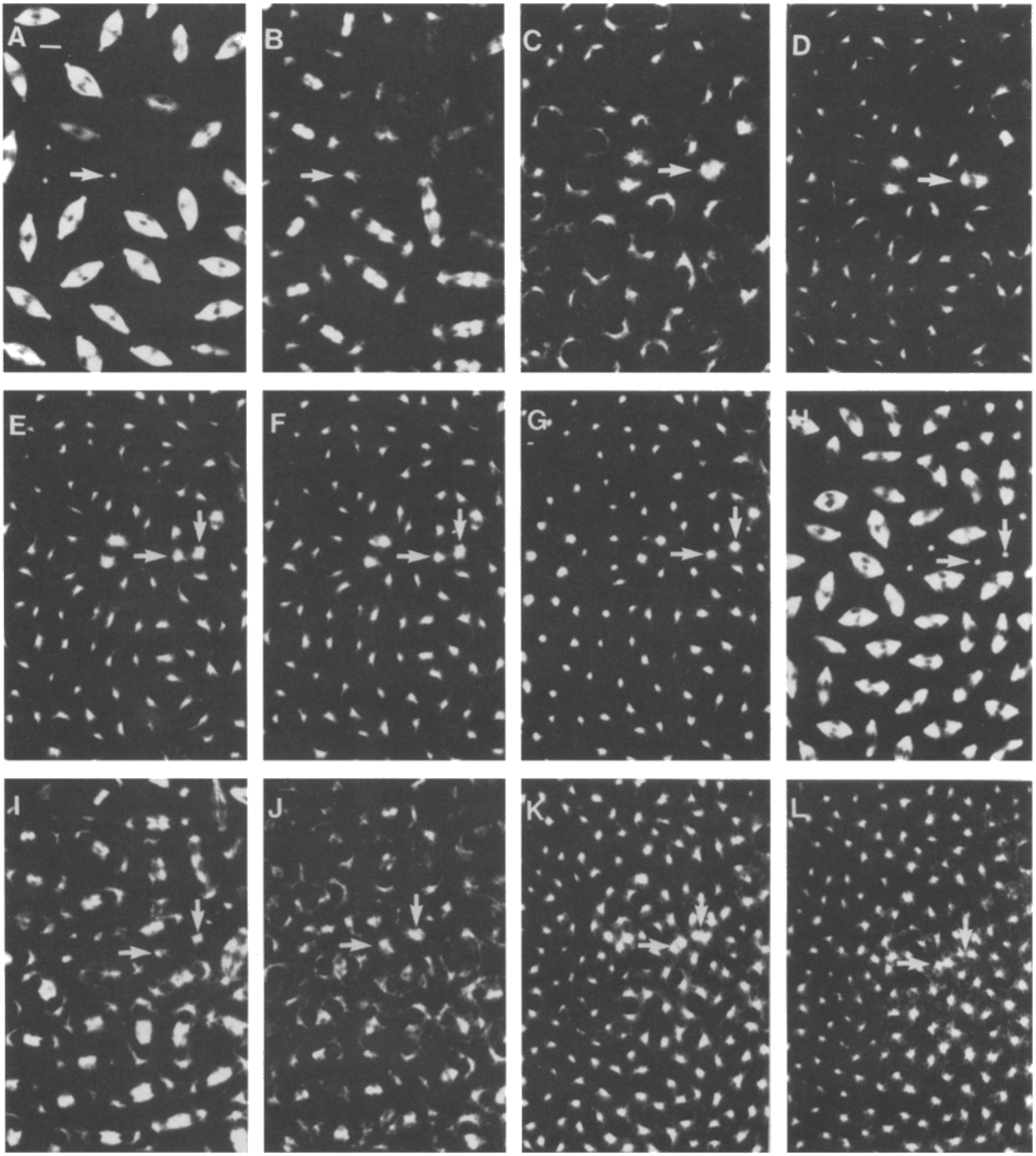

Figure 2. Confocal images of a living *Drosophila* embryo injected with rhodamine-labeled tubulin. Duplication of a single centrosome-induced aster leads to four free asters (see arrows). The stage and total elapsed time are as follows : (*A*) cycle 11 metaphase, 0:00; (*B*) anaphase, 1:30; (*C*) telophase, 4:00; (*D*) cycle 12 interphase, 4:30; (*E*) early prophase, 7:00; (*F*) prophase, 8:00; (*G*) late prophase, 10:00; (*H*) metaphase, 11:30; (*I*) telophase, 15:30; (*J*) late telophase, 16:30; (*K*) cycle 13 interphase, 20:30; (*L*) prophase, 21:30. Bar, 5 μm.

separate, but the distance in this second round of separation is significantly less than that observed for normal centrosomes. The second round of centrosome duplication and separation also occurs in synchrony with associated centrosomes.

We observed that free centrosomes do not always duplicate and separate. Of 43 free centrosomes, 34 duplicated and separated from one another, 6 did not, and for 3 centrosomes it was not possible to determine (Table I). In Fig. 2 *D*, it is evident that while the nuclear-associated cen-

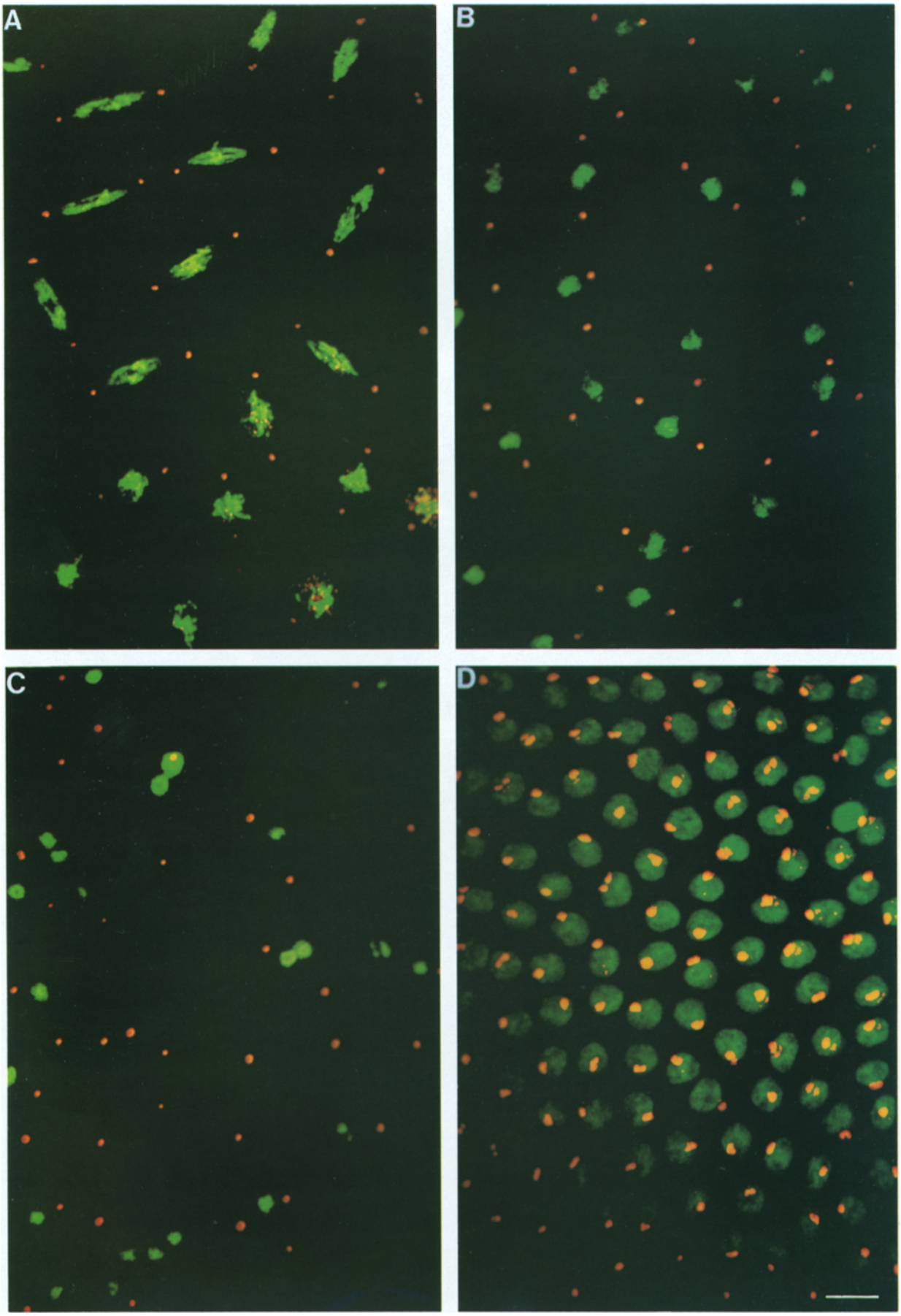
A
B
C
D

Table II. Nuclear and Centrosome Density Counts in Normal and Aphidicolin-injected Embryos

	Embryo	Number of nuclei/ 6,500 um^2	Number of centrosomes/ 6,500 um^2	Centrosomes/ nuclei
Uninjected nuclear cycle 10 embryos	1	13	29	2.2
	2	15	26	1.7
	3	24	46	1.9
	4	15	35	2.3
	5	30	57	1.9
	6	14	27	1.9
	7	13	22	1.7
	Average	**18**	**35**	**2.0**
Cycle 10 aphidicolin-injected fixed immediately	1	26	44	1.7
	2	14	28	2.0
	3	20	56	2.8
	4	30	64	2.1
	5	20	63	3.1
	6	20	50	2.5
	Average	**22**	**51**	**2.3**
Cycle 10 aphidicolin-injected fixed after 45 min	1	—	37	—
	2	—	48	—
	3	—	79	—
	4	—	62	—
	5	—	82	—
	6	—	55	—
	Average		**61**	
Cycle 10 buffer-injected fixed after 45 min	1	128	157	1.2
	2	114	167	1.5
	3	145	203	1.4
	4	112	165	1.5
	5	125	178	1.4
	6	86	168	2.0
	Average	**118**	**173**	**1.5**

Nuclear and centrosome density counts in uninjected nuclear cycle 10 embryos, embryos fixed immediately after aphidicolin injection at nuclear cycle 10, embryos fixed 45 min after injection of aphidicolin at nuclear cycle 10, and embryos fixed 45 min after the injection of buffer at nuclear cycle 10.

trosomes and free centrosomes marked by the arrow have duplicated, the unmarked neighboring free centrosomes did not. The centrosomes that fail to duplicate retain their ability to modulate astral microtubule dynamics. In general, when free centrosomes duplicate and separate, they do so in synchrony with neighboring associated centrosomes. Of the 34 free centrosomes in which this could be unambiguously determined, 25 duplicated and separated in synchrony with neighboring associated centrosomes. The remaining nine centrosomes separated after a greater than 3-min delay (Table I).

Of 34 pairs of separating free centrosomes, 26 pairs separated on a plane parallel to the plasma membrane; the separating sister centrosomes remained on a single focal plane. Two pairs did not separate on a plane parallel to the plasma membrane. Of the six remaining pairs, it was not possible to determine the plane of separation. The initial rate of free and associated centrosome separation is approximately equal: 1.5 μm/min (average of 4) and 2.0 μm/min (average of 4), respectively. However, the separation of the free centrosomes stops prematurely. At nuclear envelope breakdown (late prophase) the free centrosomes separated on average 80% (11 centrosome pairs followed) of the distance observed for associated centrosomes. By late anaphase these free centrosomes are separated by only 60% (same 11 centrosome pairs followed) of the distance observed for the associated centrosomes. The reduced percentage reflects the fact the free centrosomes do not undergo the second phase of centrosome separation (anaphase B) that occurs for associated centrosomes. These results are summarized in Table I.

Fixed Analysis of Centrosome Behavior in Aphidicolin-injected Embryos

Fixed analysis was performed by injecting embryos with aphidicolin at nuclear cycle 10. The initiation of pole cell formation enabled us to identify nuclear cycle 9 embryos. 10 min after these embryos were identified, they were injected with a 100-μg/ml aphidicolin solution and either fixed immediately or fixed 45 min after the injection. These embryos were hand devitellinized and double stained with the DNA stain propidium iodide and the anticentrosomal antibody Rb188. With the nuclei and centrosomes depicted in green and red, respectively, Fig. 3 presents merged images of these double-stained embryos.

Figure 3. Merged images of nuclei (*green*) and centrosomes (*red*) in normal cycle 10 embryos (*A*), and embryos fixed immediately (*B*) or 45 min (*C*) after injection with aphidicolin at nuclear cycle 10. *D* depicts an embryo fixed 45 min after injection of buffer at nuclear cycle 10. Bar, 10 μm.

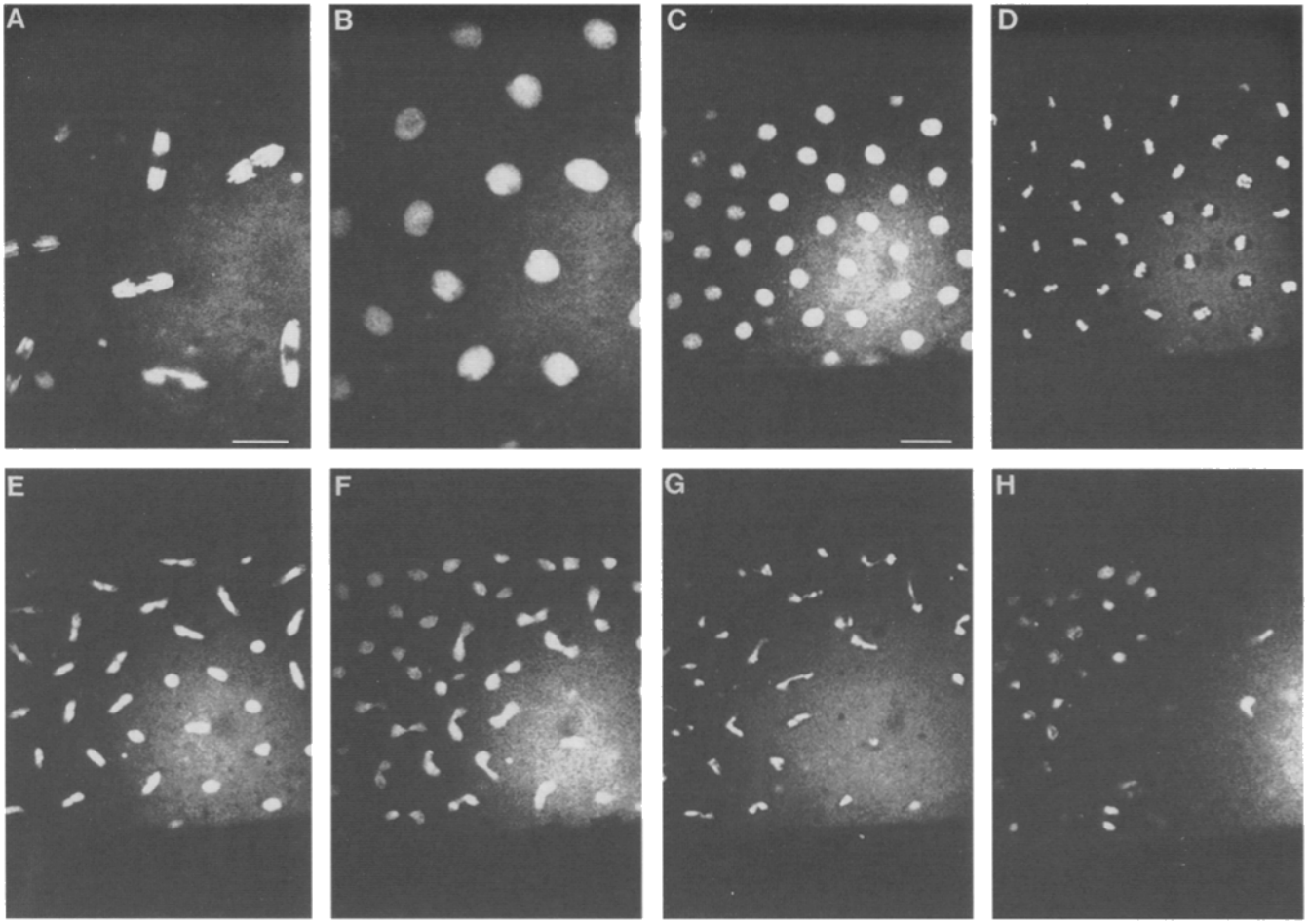

Figure 4. Confocal images of a living *Drosophila* embryo injected with rhodamine-labeled histone followed by a second injection of 100 μg/ml aphidicolin. *A* (0:00) and *B* (4:41) represent images just before and after injection of the aphidicolin. *C–H*, with total elapsed time of 5:35, 8:35, 12:20, 16:50, 25:18, and 33:35, respectively, follow the embryo as it progresses through the next anaphase. *A* and *B*: Bar, 5 μm; *C–H*: Bar, 10 μm.

A depicts an uninjected nuclear cycle 10 embryo. *B* and *C* depict embryos injected with aphidicolin at nuclear cycle 10 and fixed immediately and after 45 min, respectively. *D* depicts an embryo fixed 45 min after injection with buffer at nuclear cycle 10. Fixing immediately after injection of aphidicolin (*B*) demonstrates that incubating for 10 min after pole bud formation is a reliable means of injecting cycle 10 embryos. Seven uninjected nuclear cycle 10 embryos yield an average of 18 nuclei/6,500 μm^2, while six embryos fixed immediately after injection yield an average of 22 nuclei/6,500 μm^2 (Table II). The nuclear density in embryos fixed 45 min after injection of buffer were, as expected, significantly increased to an average of 118 nuclei/6,500 μm^2 (*D* and Table II). However the nuclear density of embryos fixed 45 min after injection of aphidicolin appeared lower than that found in normal cycle 10 embryos (*C*). Precise density counts were not feasible, because the size, shape, and spacing of the nuclei were irregular. In addition, the nuclei were no longer distributed in a monolayer. These observations suggested that the aphidicolin-treated nuclei eventually recede into the interior of the embryo (see below).

The average centrosome densities of embryos fixed immediately and 45 min after aphidicolin injection at nuclear cycle 10 are 51 centrosomes/6,500 μm^2 and 61 centrosomes/6,500 μm^2, respectively (*B* and *C*, Table II). This increase is probably the result of occasional splitting of sister centrosomes, but it is clear that the centrosomes are not undergoing multiple rounds of duplication in the aphidicolin-treated embryos. The centrosome density in embryos fixed 45 min after buffer injection at nuclear cycle 10 is dramatically increased (173 centrosomes/6,500 μm^2) (*D*, Table II). This value demonstrates that the injection and incubation techniques do not disrupt centrosome duplication.

Live Analysis of Centrosome Behavior in Aphidicolin-injected Embryos

We also followed nuclear and centrosome behavior in living embryos injected with aphidicolin. Injection of fluorescently labeled histones enabled us to follow the nuclear divisions (Minden et al., 1989).

Fig. 4 *A* depicts a histone injected embryo in anaphase of nuclear cycle 10. Immediately after this image was recorded, the embryo was then injected with 100 μg/ml aphid-

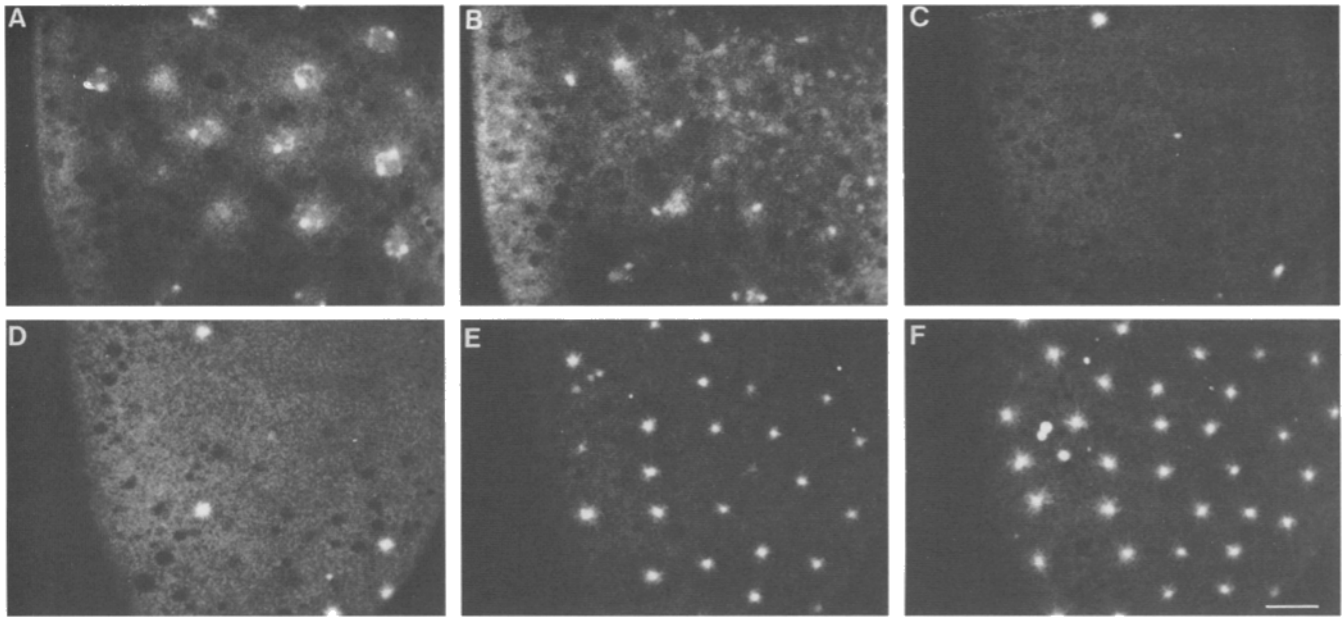

Figure 5. Confocal images of a living *Drosophila* embryo injected with rhodamine-labeled tubulin followed by a second injection of 100 μg/ml aphidicolin. *A–F* are representative images at 5:00, 11:15, 27:20, 39:21, 44:51, and 57:22 after aphidicolin injection, respectively. Bar, 10 μm.

icolin. *B* depicts the embryo in interphase of nuclear cycle 11 directly after injection (4 min 41 s elapsed between the images recorded on *A* and *B*). Lower power images demonstrate that the embryo progresses into metaphase normally (*C* and *D*), but the initiation of anaphase is disrupted (*E*). Many of the failed and abnormal anaphase products recede into the interior of the embryo (*F–H*). Because so many nuclei are lost from the cortex, nuclear density counts are not a reliable indicator of the cortical nuclear cycle.

Double injections of syncytial embryos with rhodamine-labeled tubulin and aphidicolin enabled us to follow in real time centrosome behavior in the absence of DNA replication. Fig. 5 *A* depicts a nuclear cycle 10 embryo 5 min after injection of the aphidicolin. 28 centrosome-induced asters are visible on opposite poles of the nuclei. Over the next 30 min, the centrosomes lose their association with their nuclei, drop a few microns, and then return to the surface (*B–E*). Almost an hour after the aphidicolin injection, 35 centrosome-induced asters are visible (*F*). Examination of a series of focal planes indicates that all the asters reside on a single plane parallel to the cortex. An equivalent analysis on another aphidicolin-injected embryo followed for 29 min produced only a slight increase in centrosome number (from 40 to 50). These results are in accord with the fixed data.

We also used the fluorescently labeled tubulin to continuously follow individual centrosomes in three dimensions in aphidicolin-injected embryos. Fig. 6 *A* depicts an embryo ~2 min after a double injection of fluorescently labeled tubulin and 100 μg/ml aphidicolin. At 7 and 8 min postinjection most of the centrosomes have split or duplicated (*B* and *C*). The arrows in *C–H* follow two pairs of centrosomes through 49 min postinjection of aphidicolin. These centrosomes do not undergo additional rounds of splitting or duplication. Table III summarizes the data from a series of recordings in which individual free centrosomes were followed. For the occasional free centrosomes found in control embryos, ~60% (12/20) underwent two rounds of duplication (or splitting). In the aphidicolin-treated embryos, none (0/44) underwent two rounds of duplication (or splitting). These results indicate that multiple rounds of free centrosome duplication are extensively delayed in aphidicolin-treated embryos.

Discussion

Previous work demonstrated that in the syncytial *Drosophila* embryo, centrosomes unassociated with a nucleus maintain a number of activities including the induction of pole cell formation and cytoskeletal rearrangements (Freeman et al., 1986; Raff and Glover, 1988; Yasuda et al., 1991). We have extended these studies by examining the behavior of free centrosomes in living embryos. Injection of fluorescently labeled tubulin highlights the microtubule-based asters surrounding each centrosome. We have also taken advantage of the observation that the products of an abnormal cortical nuclear division sink into the interior of the embryo while their centrosomes remain on the cortex (Sullivan et al., 1993*b*). Thus we were able to follow the behavior of a few free centrosomes in otherwise normally developing syncytial embryos.

Through live analysis of astral microtubule dynamics, our work provides the first direct demonstration of free centrosome duplication in *Drosophila.* In a number of instances, we were able to follow a single aster through two rounds of division to yield four asters. This observation may be the result of either two complete rounds of centriole duplication or one round of centriole duplication followed by a splitting of mother–daughter centrioles (Sluder and Rieder, 1985). We could no longer follow the progeny of a single aster after it had divided to produce four asters

Table III. Free Centrosome Duplication in Aphidicolin- and Control-injected Embryos

	Control injected			
Embryos				
1	3	0	0	1
2	0	1	0	3
3	1	1	0	2
4	0	2	0	0
5	0	0	1	5
Totals	4	4	1	11
	Aphidicolin injected			
Embryos				
1	0	10	0	0
2	0	9	0	0
3	0	10	0	0
4	3	10	0	0
5	1	1	0	0
Totals	4	40	0	0

Individual free centrosomes were identified and continuously followed in control and in aphidicolin-injected embryos. Those free centrosomes that separated were followed for at least another 20 min in control-injected embryos and another 30 min in aphidicolin-injected embryos. Each centrosome was classified into one of the division categories depicted. While none of the free centrosomes underwent multiple rounds of duplication in the aphidicolin-treated embryos, ~60% of the free centrosomes underwent multiple rounds of duplication in the control embryos.

and therefore do not know whether the free centrosomes undergo another round of duplication.

6 of the 43 free centrosomes observed did not duplicate. Analysis of centrosome duplication in sea urchins provides an explanation for this variability in free centrosome duplication. Slowing down the sea urchin embryonic division cycle with mercaptoethanol results in a tetrapolar mitotic spindle with a single centriole at each pole (Sluder and Reider, 1985). This leads to four cells each with a single centrosome bearing a single centriole. Centrosome duplication does not occur until a daughter centriole is created. In *Drosophila*, the free centrosomes that duplicate may consist of two centrioles and those that do not may consist of only a single centriole. Whether a free centrosome consists of one or two centrioles may depend on when in the division cycle it disassociates from the nucleus (Calliani and Riparbelli, 1992). Alternatively, some free centrosomes may not duplicate because they are unable to respond to division signals generated within the embryo.

During interphase and prophase in the cortical divisions of the *Drosophila* embryo, sister centrosomes migrate in a precise manner along the envelope of each nucleus to establish the poles of the mitotic spindle. The mechanisms generating the force and controlling the orientation of the separating centrosomes are not known. Our recordings of free centrosomes demonstrate that the separation does not depend on the presence of a nuclear envelope. The rate and distance of free centrosome separation approximates the initial interphase and prophase separation observed for associated centrosomes. This is in accord with other studies suggesting that separation of the sister centrosomes may depend on cytoskeletal elements other than microtubules (Callaini and Riparbelli, 1990; Waters et al., 1993).

The free centrosomes rarely exhibit the second, higher rate of separation observed during anaphase B spindle elongation. These results indicate that centrosomes without a spindle are not competent to undergo anaphase B separation. Overlapping interzone microtubules and forces intrinsic to each aster both contribute to anaphase B separation (Nislow et al., 1992; Aist et al., 1993; Waters et al., 1993). Our results indicate that the integrity of the spindle is essential to anaphase B centrosome separation.

Previous work demonstrated that centrosomes influence cortical cytoskeletal dynamics (Raff and Glover, 1989; Sullivan et al., 1990; Yasuda et al., 1991). Our studies suggest that the converse is also true; the cortical cytoskeleton influences the behavior of the centrosomes. We find that the separation of free centrosomes usually occurs in a plane parallel to the plasma membrane. This suggests that the orientation of centrosome separation is at least partially determined by the cortical cytoskeleton. In addition, the observation that when abnormal nuclei retreat into the interior of the embryo their centrosomes remain on the surface suggests that the centrosomes are intimately associated with the cortical cytoskeleton (Sullivan et al., 1990, 1993*b*). Callaini and Riparbelli (1992) demonstrated that disruption of microfilaments in the syncytial *Drosophila* embryo prevents prophase separation of sister centrosomes. Studies in other organisms also demonstrate that centrosome positioning and migration rely on an intact actin cytoskeleton (Euteneuer and Schliwa, 1985; Schatten et al., 1988; Buendia et al., 1990; Palmer et al., 1992). In *S. cerevisiae*, proper spindle pole body positioning and migration rely on interactions between the actin cytoskeleton and astral microtubules (Palmer et al., 1992). Although no proteins have been identified that mediate interactions between the centrosomes and the cytoskeleton, likely candidates exist among the many *Drosophila* actin- and tubulin-binding proteins that localize to the cortex (Kellogg et al., 1989; Miller et al., 1989).

In addition to duplication, free centrosomes maintain their ability to modulate microtubule dynamics. Fig. 2 demonstrates that the aster morphology of the free centrosome undergoes nuclear cycle–dependent variations equivalent to those observed in associated centrosomes. For instance, during anaphase the length of the microtubules increases dramatically both in the free and associated centrosomes. This suggests that the regulation of aster morphology is independent of the association of the centrosome with the nuclear envelope. However, the free centrosomes never form a metaphase spindle, indicating that the formation of this structure requires chromatin.

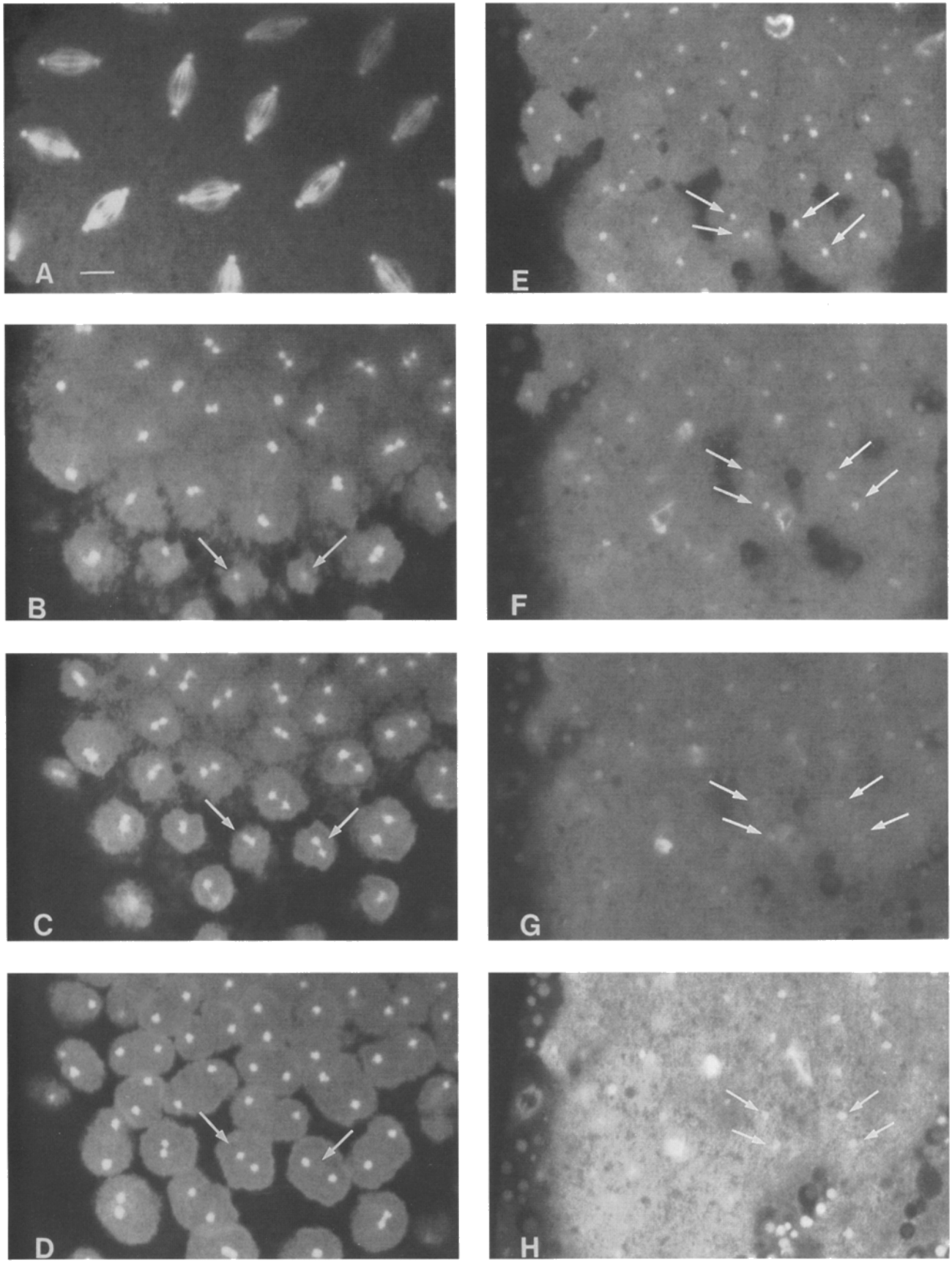

Figure 6. Individual centrosomes are followed through multiple focal planes in embryos doubly injected with 100 μg/ml aphidicolin and rhodamine-labeled tubulin. *A–H* depict the embryo 2:00, 7:00, 8:00, 14:00, 22:30, 29:45, 41:42, and 48:55 after the injection of aphidicolin, respectively. At 7 min postinjection, all of the free centrosomes separate (*B*). Arrows in *C–H* follow two sets of free centrosomes for about 49 min postinjection. Bar, 5 μm.

This observation is in accord with previous studies in *Xenopus* demonstrating that during mitosis, the centrosome acts as a mitotic organizing center only in the proximity of nuclei or chromatin (Karsenti et al., 1984).

Free centrosomes that do not duplicate nevertheless exhibit normal astral microtubule dynamics. Therefore, the cycle of astral microtubule dynamics is independent of the centrosome duplication cycle. Free centrosomes usually duplicate and separate in synchrony with neighboring nuclear-associated centrosomes. In addition, the modulation of microtubule dynamics in free centrosomes occurs synchronously with that of normal centrosomes. Free centrosomes appear capable of receiving and responding appropriately to the embryonic division signals. This conclusion is in accord with studies demonstrating that key cell cycle regulatory proteins directly modulate the phosphorylation of centrosomal proteins (Kuriyama, 1989; Messinger and Albertini, 1991; Ohta et al., 1993; Rose et al., 1993). In the *Drosophila* embryo, as well as cell culture, cyclin B localizes to the centrosome, also suggesting that it is interacting directly with this organelle (Bailly et al., 1992; Debec and Montmory, 1992; Maldonado-Codina and Glover, 1992).

Aphidicolin, an inhibitor of DNA synthesis, was used to determine whether the centrosome cycle depends on a proper nuclear cycle. The live recordings demonstrate that after one round of centrosome duplication or splitting in aphidicolin-treated embryos, subsequent rounds are extensively delayed. This is most dramatically illustrated in the aphidicolin-treated embryo shown in Fig. 6. The majority of free centrosomes split, but they do not undergo a second round of division during the 49 min in which they were observed. That is, aphidicolin significantly delays the centrosome cycle. This conclusion is confirmed by fixed analysis of centrosome behavior in aphidicolin-injected embryos.

Previous studies indicated that multiple rounds of centrosome duplication occur in the absence of DNA replication (Raff and Glover, 1988). These results are not incompatible with our findings. Raff and Glover, using fixed analysis, examined embryos 45 and 90 min after injection. In our live analysis, it was not possible to follow aphidicolin injected embryos for greater than 50 min because of deteriorating image quality. Free centrosomes in aphidicolin-treated embryos may be duplicating at such a dramatically reduced rate that additional rounds of duplication may not be observed until well after our 50-min time point. In fact, when the receding of abnormal nuclei from the cortex is taken into account, results from the 45-min time point in the Raff and Glover analysis are in accord with our results of a single round of centrosome splitting.

A maternal effect mutation has been identified in which division stops at nuclear cycle 12. In addition, incorporation of labeled histones specifically does not occur once the embryos reach nuclear cycle 12. The majority of nuclei recede into the interior while their centrosomes remain on the cortex. Live analysis demonstrates that these centrosomes do not undergo multiple rounds of duplication (Theurkauf, W., personal communication).

The *Drosophila* maternal-effect mutations *gnu*, *pan-gu*, and *plutonium* disrupt the initial embryonic divisions (Freeman et al., 1986; Shamanski and Orr-Weaver, 1991). In embryos derived from these mutations, the DNA continues to replicate, but nuclear division does not occur and they arrest with a few large polyploid nuclei. In spite of nuclear division failure, in each of these mutations the centrosomes continue duplicating. In light of our findings that disruption of the later cortical divisions with aphidicolin greatly reduces the rate of centrosome duplication, it would be interesting to determine the rate of centrosome duplication in these mutant embryos. Studies by Dasso and Newport (1990) demonstrate that as nuclear density increases during the initial divisions of *Xenopus* embryos, dependency relationships are added to the division cycle. It may be that in the *Drosophila* embryo, centrosome duplication becomes dependent on proper nuclear division only during the later syncytial cycles. Alternatively, centrosome duplication may be strictly dependent on a proper S-phase rather than a proper nuclear cycle.

Many cell cycle dependency relationships are relaxed during the initial divisions in *Xenopus*. Mitosis is not dependent on complete DNA replication or undamaged DNA and the initiation of anaphase is not dependent on proper spindle assembly (Hara et al., 1980; Kimmelman et al., 1987). In addition, centrosome duplication occurs in the absence of protein synthesis (Gard et al., 1990). In contrast, the initial syncytial divisions of *Drosophila* maintain a number of dependency relationships; disrupting the spindle or chromosome structure delays initiation of anaphase (Zalokar and Erk, 1976; Sullivan et al., 1993*b*). It is likely that feedback mechanisms operating during the syncytial divisions are responsible for these dependency relationships. The studies presented here demonstrate another dependency relationship that is also likely to be a consequence of feedback controls operating during the cortical syncytial divisions: that of centrosome duplication on proper DNA synthesis. In contrast to other embryonic systems, the early *Drosophila* embryo may rely heavily on feedback mechanisms to maintain the integrity of the syncytial divisions.

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