CENTROSOMES AND THE CELL CYCLE IN EARLY *DROSOPHILA* EMBRYOS

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ABSTRACT

The experiments described in this thesis examine aspects of cell cycle regulation and cytoskeletal organisation in the early Drosophila embryo. By injecting aphidicolin into early embryos, I have shown that cycles of centrosome replication, cortical budding, chromosome condensation, and nuclear envelope breakdown all continue when DNA synthesis is inhibited. Aphidicolin treatment, leads to the dissociation of replicating centrosomes from non-replicating nuclei. I used this to examine the relative role of these organelles in the processes of nuclear migration and cortical cytoskeletal re-arrangement. I found that centrosomes alone can migrate to the cortex leaving the nuclei behind, suggesting that nuclear migration is mediated via forces acting on the centrosome. Once at the surface, the centrosomes can organise much of the cortical cytoskeleton, including the process of pole cell formation, in the absence of nuclei. Finally, I examined the mechanisms responsible for localising the mRNA for cyclin B in the early Drosophila embryo. I found that the mRNA is closely associated with nuclei, and this leads to a cortical accumulation of the mRNA as the nuclei migrate to the cortex. The mRNA is also accumulated at the posterior pole of the embryo, and I have shown that this localisation probably depends on the binding of the mRNA to polar granules.

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LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
cdc	Cell Division Cycle
cDNA	copy DNA (synthesised from mRNA)
Ci	Curie
DEPC	Diethylpyrocarbonate
cpm	Radioactive counts per minute
DIC	Differential Interference Microscopy
DMSO	Dimethyl Sulphoxide
DNAse	Dioxyribonuclease
DTT	Dithiothreitol
EDTA	Diaminoethanetetra-acetic Acid
EGTA	Ethyleneglycol-bis-N,N,N,N'-tetraacetic acid
FCS	Foetal Calf Serum
kB	Kilobase: 1000 Nucleotides or Nuceotide Pairs of Nucleic Acid
kD	Kilodalton: 1000 Daltons
M	Molar
mCi	Millicurie
mM	Millimolar
MPF	Maturation-Promoting Factor
mRNA	Messenger RNA
MTOC	Microtubule Organising Centre
PBS	Phosphate Buffered Saline
PP	4% Paraformaldehyde in PBS
pers. comm.	Personal Communication

RNAse	Ribonuclease
SDS	Sodium Dodecyl Sulphate
SSC	Saline Sodium Citrate
TCA	Trichloro-acetic acid
TE	Tris-EDTA
Tris	Tris(hydroxymethyl)-aminoethane
tRNA	Transfer RNA

CHAPTER 1

Introduction

1.1 The Cell Cycle

A fundamental challenge in cell biology is to understand how the eukaryotic cell cycle is controlled. In most eukaryotes, the cell cycle can be divided into two main phases, an S phase where the genome of the cell is replicated, and an M phase where the nucleus divides and is usually separated into two daughter cells. These two phases are usually separated by "gap" periods, G1 between M and S phase, and G2 between S and M phase. The study of the molecular basis of cell cycle control has largely relied on two approaches, one biochemical, involving studies on either cells in culture or the eggs and early embryos of amphibians and marine invertebrates, and the other genetic, mainly involving studies on the budding yeast Saccharomyces cerevisiae or the fission yeast Schizosaccharomyces pombe. A combination of these approaches has led to the identification of some of the key molecules involved in controlling the initiation of mitosis, and recently it has become apparent that many of these molecules have been highly conserved through evolution.

Drosophila is an attractive system in which to study the cell cycle as it allows both a cell-biological, and a genetic approach to be used (Smith et al., 1985; Glover, 1989). In this thesis, I will describe experiments designed to examine the cell cycle and the behaviour of the centrosome in the early Drosophila embryo. Before introducing this work, however, I shall review the recent advances in our

understanding of the molecular basis of eukaryotic cell cycle control.

1.2 Biochemical Studies of the Cell Cycle

Important insights into how the cell cycle is regulated were gained from the experiments of Rao and Johnson (Rao and Johnson, 1970; Johnson and Rao, 1970). By fusing cells that were in different phases of the cell cycle they were able to identify three important cytoplasmic activities that govern the physiological state of the cell. The first is involved in the G1/S transition and can be termed an "S-phase activator", present in all S-phase cells. When a cell in G1 is fused with a cell in S-phase, the cell in G1 is induced to enter S phase prematurely. If, however, an S-phase cell is fused with a cell in G2, the G2 cell cannot be driven to enter S-phase again; evidently the newly replicated DNA is in some way marked so that it cannot be replicated again until it has passed through some critical aspect of mitosis. The second activity is an "M-phase Promoting Factor" (MPF), which promotes the G2/M transition. Once this activity is produced, it is dominant over all others: any cell in G1, G2, or S-phase, will precociously be driven into M-phase by fusion with an M-phase cell. The third activity can be described as an "M-phase delaying activity". It enables cells that have been blocked in DNA synthesis, or have had their DNA damaged in some way, to delay the activation of MPF until DNA synthesis is complete or the damage to the DNA has

been repaired. While this activity can delay the activation of MPF, it cannot switch off active MPF.

How a cell decides to begin DNA synthesis is poorly understood. Studies on cells in culture have revealed the existence of a "restriction point" in the cell cycle that occurs late in G1 (Pardee, 1974). Once a cell has passed through this point it will often continue through the cell cycle, even if deprived of growth factors or if protein synthesis is blocked, suggesting that the cell has made an irreversible commitment to enter S-phase. Genetic studies have revealed a similar point in the yeast cell cycle called "start". As will be described later, some of the molecules required to traverse start also have a role in the G2/M transition.

The recent development of extracts (made from Xenopus eggs) that are capable of initiating and completing one (or more) round(s) of DNA synthesis in vitro, has allowed progress to be made in understanding how DNA replication is controlled such that the entire genome is replicated once and only once per cell cycle. Exogenous sperm nuclei added to these extracts become decondensed and surrounded by a nuclear membrane (Lohka and Masui, 1983). Once this has occurred, DNA synthesis can begin, but only one round of semi-conservative replication occurs (Blow and Laskey, 1986; Newport, 1987; Hutchison et al., 1987). In some of these extracts, however, the nuclei can proceed through mitosis and

then undergo a second round of replication (Hutchison et al., 1988). Recent studies have suggested that the only aspect of mitosis that is essential to allow a second round of replication is the permeabilisation of the nuclear membrane which normally occurs when the nuclear envelope breaks down on entry into mitosis (Blow and Laskey, 1988). On the basis of this observation, these investigators have proposed a simple model to explain how the re-replication of DNA in a single cell cycle is prevented. They suggest that DNA synthesis requires the presence of a "licensing factor" bound to DNA. This factor is destroyed by the passage of the replication fork, so that this DNA is no longer competent to be replicated. New licensing factor can only be bound to the DNA after membrane permeabilisation allows in fresh licensing factor that is stored outside the nuclear envelope. In this way, replicated DNA cannot undergo further replication until the nuclear envelope has broken down.

Although our understanding of the control of the G1/S transition is not as advanced as that of the G2/M transition (discussed below), a combination of biochemical and genetic approaches will allow this control point to be studied at the molecular level. It is precisely this combination of approaches that has led to a more detailed understanding of how the regulation of the activity of MPF is crucial in controlling the G2/M transition.

Maturation-promoting factor was originally identified as an

activity in unfertilised eggs of Xenopus laevis. The Xenopus oocyte is arrested in a G2 like state at the first meiotic prophase. It can be induced to mature by progesterone which causes the eggs to undergo germinal vesicle (nuclear) breakdown, complete the first meiotic division, and then arrest at the second meiotic metaphase, awaiting fertilisation (Kirschner et al., 1985). When cytoplasm from mature eggs is injected into oocytes, the oocytes are induced to mature (Masui and Markert, 1971; Smith and Ecker, 1971). The activity that caused maturation was initially termed Maturation-promoting factor, but subsequent studies revealed that this activity is present in the M-phase cytoplasm of cycling Xenopus embryos (Wasserman and Smith, 1978; Gerhart et al., 1984), and in a wide variety of mitotic cells (e.g. Sunkura et al., 1979; Kishimoto et al., 1982; Weintraub et al., 1982; Adlakha et al., 1988). It was later shown that this activity is not only temporally correlated with M-phase, but it can promote nuclear envelope breakdown, chromosome condensation and spindle formation in cell-free extracts of interphase-arrested Xenopus eggs (Miake-Lye et al., 1983; Miake-Lye and Kirschner., 1985; Lohka and Maller, 1985). Thus, this activity is now commonly termed M-phase Promoting Factor.

MPF proved to be extremely difficult to purify, partly because of the insensitivity of the oocyte injection assay and partly because of the apparent instability of active MPF. By using cell-free systems to assay MPF activity (Lohka and

Masui, 1983, 1984; Lohka and Maller, 1985), Lohka et al., (1988) have recently purified MPF from Xenopus eggs by approximately 3000 fold. An M-phase specific Histone H1 kinase activity has also recently been purified from starfish embryos, and this is now thought to be the same entity as MPF (Labbe et al., 1988a; Arion et al., 1988).

All of the above preparations of MPF contain a 32-34kD protein that is part of a higher molecular weight complex. The size and subunit composition of MPF differ in all of these reports; the significance of these differences is not clear. All of the preparations of MPF have protein kinase activity and can phosphorylate Histone H1 and a number of other substrates, including (at least in one preparation) MPF itself. As will be discussed later, the 32-34kD component of MPF is now known to be homologous to the 34kD product of the cdc2 gene of S. pombe, and many of the studies that I will mention refer to this component as $p34^{cdc2}$.

The finding that purified MPF has protein kinase activity, and may well be phosphorylated itself, is consistent with earlier studies suggesting that protein phosphorylation was likely to play an important role in regulating MPF activity (Cyert and Kirschner 1988; Labbe et al., 1988b; Cyert et al., 1988; Meijer and Pondaven, 1988). Recently it has been shown that in Xenopus (Dunphy and Newport, 1989), and also human (Dreatta et al., 1988) and mouse (Morla et al., 1989) cells, the protein kinase activity of MPF is closely correlated with

its phosphorylation state and this varies in a cell-cycle dependent manner.

As well as regulating the activity of MPF, protein phosphorylation seems to play a major role in the events downstream from MPF activation that lead to entry into mitosis. When cells enter M-phase, there is a dramatic increase in total protein phosphorylation (e.g. Maller et al., 1977; Doree et al., 1983; Lohka et al., 1987; Karsenti et al., 1987). Some of these proteins are presumably phosphorylated by MPF itself. For example, Histone H1 is phosphorylated by MPF _in vitro_, and this protein also becomes hyperphosphorylated on entry into mitosis _in vivo_; this may play a role in promoting chromosome condensation (Mueller et al., 1985). Other proteins that are phosphorylated on entry into mitosis are probably not phosphorylated directly by MPF. For example, the lamins become hyperphosphorylated on entry into mitosis (perhaps playing a role in nuclear envelope breakdown - Gerace, 1986), but they are probably not phosphorylated directly by MPF. Presumably these proteins are phosphorylated by other kinases that are activated by MPF. One such kinase may be the $pp60^{c\text{-}src}$ protein kinase. This protein can be phosphorylated by MPF (Shenoy et al., 1989; Morgan et al., 1989), and MPF itself can be phosphorylated on a specific tyrosine residue by $pp60^{c\text{-}src}$ _in vitro_ (Dreatta et al., 1988). This raises the possibility that a feedback mechanism involving tyrosine-phosphorylation may control MPF activity, and it has recently been shown that

certain tyrosine residues of MPF must be de-phosphorylated to activate MPF (Morla et al., 1989; Dunphy and Newport, 1989).

Although it is clear that MPF plays a central role in the G2/M transition, it appears that the amount of MPF present in a cell does not vary through the cell cycle (Cyert and Kirschner, 1988; Dunphy and Newport, 1988; Labbe et al., 1988b), suggesting that it cannot be de novo synthesis of MPF that drives the G2/M transition. Protein synthesis is, however, normally required for entry into M-phase (Wasserman and Smith, 1978; Gerhart et al., 1984). In the eggs and early embryos of a number of organisms, a family of proteins called cyclins have been found to undergo a periodic synthesis and degradation in time with the cell cycle (Rosenthal et al., 1980; Evans et al., 1983; Standart et al., 1987). In clams (where the cyclins were first described), the cyclins accumulate continuously during S-phase and are then rapidly degraded at about the time of the metaphase-anaphase transition (Evans et al., 1983), suggesting that the cyclins may be involved in triggering entry into mitosis.

The first direct evidence that cyclins are involved in the activation of MPF came from experiments showing that clam cyclins can drive Xenopus oocytes into meiosis (Swenson et al., 1986; Pines and Hunt, 1987). More recently, in Xenopus cell-free extracts which require protein synthesis to enter mitosis and undergo more than one round of DNA synthesis, it has been demonstrated that the translation of cyclin mRNA is

necessary for entry into M-phase (Minshull et al., 1989). Most impressively, it has been shown that if such a system is depleted of endogenous mRNA, the addition of clam cyclin B mRNA reconstitutes both the characteristic periodic synthesis and destruction of the cyclin B protein and the normal cycling of MPF activity (Murray and Kirschner, 1989a). If, on the other hand, the added cyclin B mRNA contains a deletion that abolishes the ability of the protein to be rapidly destroyed, the extracts enter M-phase and arrest there (Murray and Kirschner, 1989b).

These studies reveal that, in this system, the synthesis of cyclin B is all that is required to drive the entry into mitosis, and the destruction of the protein is necessary for exit from mitosis. They do not, however, indicate whether the cyclins directly activate MPF. Recent evidence suggests that they might. In clams (Dreatta et al., 1989) and sea urchins (Meijer et al., 1989) the $p34^{cdc2}$ protein kinase seems to associate directly with the cyclins, and it has been proposed that both the 62kD protein that co-purifies with $p34^{cdc2}$ in human cells (Dreatta and Beach 1988) and the 42-45kD protein that is part of Xenopus MPF (Lohka et al., 1988) may be cyclins (for review see Blow, 1989). As will be discussed later, there is also evidence from experiments in S. pombe that $p34^{cdc2}$ may interact directly with cyclin-like molecules.

The biochemical studies described above suggest that the

initiation of the G2/M transition involves a cascade of phosphorylation reactions in which one of the earliest events is the activation of MPF. This activation is controlled both by phosphorylation, and by interactions with other proteins. Once activated, MPF presumably alters the activities of other proteins to bring about the complex physiological changes associated with entry into M-phase.

1.3 The Cell Cycle in Yeast

While the systems described above have proved invaluable for cell biological and biochemical analyses of the cell cycle, the powerful genetic techniques available in yeasts have provided direct opportunities to identify, clone, and characterise the genes involved in controlling the cell cycle. These studies have focused primarily on the budding yeast *S. cerevisiae* (Hartwell, 1974; Pringle and Hartwell 1981) and the fission yeast *S. pombe* (Nurse, 1975, 1981). While many of the early studies on the cell cycle were performed in *S. cerevisiae*, the organisation of the cell cycle in this organism is different from that found in most other eukaryotes (see below), and many of the recent advances that have led to a unified model of how the G2/M transition is initiated have been based on proteins initially identified genetically in *S. pombe*.

The genetic approach involves isolating conditional mutants that can divide normally under permissive conditions (usually

lower temperatures) but arrest at a specific point in the cell cycle under restrictive conditions (usually higher temperatures). Such mutations are termed cell division cycle (cdc) mutants, and over 50 complementation groups have been identified in S. cerevisiae that have a cdc mutant phenotype. In S. pombe two important cell cycle control points have been identified from genetic studies. The first occurs late in G1 and is called "start". Once a cell traverses start it becomes committed to the mitotic cycle and can no longer undergo the alternative fate of conjugation and sporulation (Nurse and Bissett, 1981; Nurse, 1985). The second occurs late in G2 and determines when mitosis is initiated (Nurse, 1975). In S. cerevisiae the situation is slightly different and there may be only one major control point at start. This difference is probably due to the different organisation of the cell cycle in S. cerevisiae, where mitosis is initiated during S phase and so only one control point may be necessary (Fantes, 1989).

In S. pombe two genes have been implicated as being necessary to traverse start, cdc2 and cdc10 (Nurse, 1985; Hayles and Nurse, 1986). The cdc2 gene, along with at least three other loci, wee1 (Russell and Nurse, 1986a), cdc25 (Russell and Nurse, 1986b), and nim1 (Russell and Nurse, 1986c), also has a role in controlling the G2/M transition. These genes have been cloned, and a number of genetic and biochemical studies have suggested that the cdc2 gene product, a 34kD protein with protein kinase activity ($p34^{cdc2}$), is central in the

initiation of mitosis (Nurse, 1985; Lee and Nurse, 1988). The levels of this protein do not change detectably during the cell cycle. Its phosphorylation state, however, does vary in a cell cycle specific manner, and its kinase activity is dependent on its phosphorylation state (Simanis and Nurse, 1986; Moreno et al., 1989). Similarly, CDC28, the cdc2 homologue of S. cerevisiae (Beach et al., 1982), remains at constant levels throughout the cell cycle, but its protein kinase activity becomes maximal at about the time of the G1/S transition (Mendenhall et al., 1987). The wee1 and nim1 genes of S. pombe encode proteins with homology to protein kinases (Russell and Nurse, 1986a,c), and genetic studies have suggested that wee1 inhibits the mitosis-inducing activity of $p34^{cdc2}$, while nim1 inhibits wee1. The product of the cdc25 gene (which has no homology to known protein kinases), appears to activate $p34^{cdc2}$ in a dose dependent manner, suggesting that it plays a central role in initiating mitosis (Russell and Nurse, 1986b).

Two other gene products have been shown to interact closely with cdc2 in S. pombe. The suc1 gene encodes a protein of 13kD (Brizuela et al., 1987), and mutations in this gene interact with cdc2 mutations in an allele specific manner suggesting a direct physical interaction in vivo (Hayles et al., 1986). Similarly, mutations in the cdc13 gene have been shown to interact with certain cdc2 mutants in an allele specific manner, again suggesting a direct physical association between their products. It has been suggested

that the product of the cdc13 gene is a substrate for the $p34^{cdc2}$ protein kinase (Booher and Beach, 1987). There is also some evidence that the cdc13 gene product may interact with microtubules (Booher and Beach, 1988), possibly providing a link between $p34^{cdc2}$ and the cytoskeleton, which becomes dramatically re-arranged on entry into mitosis.

Thus, studies on S. pombe have identified a small number of genes that are crucial to the regulation of the G2/M transition. Central to this process is the $p34^{cdc2}$ protein kinase, whose activity is regulated both by phosphorylation and by association with other proteins. $p34^{cdc2}$ probably initiates entry into mitosis via a complex phosphorylation cascade, that is poorly understood. Thus, studies on yeast have led to a model of how the G2/M transition is controlled that is very similar in principle to models derived from the biochemical studies described in the previous section. Moreover, the main molecules involved seem to have been highly conserved as I shall now discuss.

1.4 The Same Family of Proteins Control Entry into M-phase in all Eukaryotes

The first direct evidence that the control of the G2/M transition might be similar in all eukaryotes came from the finding that human cells contained a cdc2 homologue. Thus, when a human cDNA library was expressed in yeast cells carrying a cdc2 mutation, it was found that a human sequence

was capable of rescuing the cdc2 mutant phenotype (Lee and Nurse, 1987), demonstrating that the human DNA encoded a protein capable of functioning in the place of cdc2 in S. pombe. When this human gene was sequenced it was found to be 63% identical at the amino acid level to $p34^{cdc2}$ from S. pombe. Subsequently, proteins homologous to $p34^{cdc2}$ and p13 (the product of the suc1 gene in S. pombe) were identified in human cells in culture, and the protein kinase activity of human $p34^{cdc2}$ was found to be maximal during the G2/M transition, suggesting that this protein might have a role in mitotic initiation in humans (Dreatta et al., 1987).

More recently, it has been shown that some of the proteins identified in yeast are homologous to the proteins identified in Xenopus. Antibodies raised against S. pombe $p34^{cdc2}$ were able to recognise the 32kD component of purified MPF (Gautier et al., 1988), suggesting that these proteins are related. At the same time, it was shown that p13 of S. pombe (known to bind directly to $p34^{cdc2}$ in both S. pombe and human cells) could bind to, and inhibit, MPF in a Xenopus cell-free system, suggesting that this was the Xenopus cdc2 homologue (Dunphy et al., 1988). Both of these groups reported that affinity columns prepared with either anti-$p34^{cdc2}$ antibodies (Gautier et al., 1988) or p13 (Dunphy et al., 1988) could be used to isolate both the 32kD homologue of cdc2 and a 42-45kD protein that was complexed to the 32kD protein. This 42-45kD protein is probably the same as the 45kD protein identified earlier in purified MPF (Lohka et al., 1988). Thus, the

activity identified as being crucial in initiating the G2/M transition in two completely different systems, using very different strategies, was in fact the same molecule - $p34^{cdc2}$.

Furthermore, the cloning and sequencing of the cdc13 gene from S. pombe (Booher and Beach, 1988; Hagan et al., 1988) revealed that this protein shows significant homology to B-type cyclins (Hagan et al., 1988; Solomon et al., 1988; Goeb and Byers, 1988). Thus, there is now evidence from yeasts, clams (Dreatta et al., 1989), and sea urchins (Meijer et al., 1989) that cyclins can directly interact with the $p34^{cdc2}$ protein kinase. This may also be the case in human cells (Dreatta and Beach, 1988) and in Xenopus (see Blow, 1989). Finally, recent studies on cellularised Drosophila embryos suggest that the product of the string gene acts as an initiator of mitosis (Edgar and O'Farrell, 1989). Sequencing of the string gene has revealed significant homology to the cdc25 gene of S. pombe, which acts as a dose dependent inducer of mitosis (Russell and Nurse, 1986c).

In summary, recent studies have demonstrated that many of the molecules responsible for controlling the G2/M transition have been highly conserved between the eukaryotes. The key molecule in initiating mitosis is $p34^{cdc2}$, whose activity is regulated by a number of other proteins, either by phosphorylation or via a direct association. Although the in vivo substrates of the $p34^{cdc2}$ kinase are largely unknown,

this kinase is thought to initiate a cascade of reactions that are responsible for the diverse physiological changes associated with entry into M-phase.

1.5 The Early Embryos of many Organisms have a Simplified Cell Cycle

As described above, the eggs and oocytes of marine invertebrates and Xenopus have been particularly useful for biochemical studies on the initiation of mitosis. The early embryos of these species have also proved to be useful systems for studying other aspects of cell cycle control, largely because their cell cycle is relatively simple. In somatic cells, cell cycle control is complicated by the need to respond to external signals. In single celled eukaryotes such as yeasts, the cells must sense their surroundings and only initiate a new cell cycle if growth conditions are appropriate. In higher eukaryotes, somatic cells have to respond to the complex growth requirements of a multicellular organism, where some types of cell divide for the lifetime of the organism, while others terminally differentiate and never divide again. In contrast, the cell divisions of early embryos from many species are comparatively simple; the embryo does not grow, and all of the requirements for the first few rounds of division have been laid down in the egg by the mother. Indeed, the cell cycle in many early embryos of insects, echinoderms, amphibians, and molluscs consists only of successive M and S phases with no G1 or G2 periods.

Thus, the cell cycle in these systems is often regarded as a "stripped down" cell cycle that has dispensed with some of the controls found in somatic cells.

For example, when a somatic cell is enucleated or treated with drugs to inhibit DNA synthesis, the cell can sense that the nucleus is not ready to divide and the cycle arrests (Rao and Johnson, 1970; Mitchison, 1971). In Xenopus embryos, however, neither enucleation (Hara et al., 1981) nor inhibition of DNA synthesis (Kimelman et al., 1986) completely blocks the cell cycle. Under these conditions, periodic surface contractions continue to occur, pointing to the existence of a cytoplasmic cell cycle oscillator that is largely independent of nuclear events (Kirschner et al., 1985). MPF is thought to be closely coupled to, or an integral part of, this oscillator and recently it has been shown that cycles of MPF activity continue to occur in enucleated Xenopus eggs (Daubavalle et al., 1988).

1.6 The Centrosome and the Cell Cycle

One aspect of cell cycle control that has been studied both in somatic cells and in early embryos is the control of the centrosome cycle. Before describing these studies, however, I will briefly introduce the centrosome. This organelle was first described by Van Beneden in 1876, who called it the polar corpuscle because of the position it occupied at the poles of the mitotic spindle. Bovari termed these organelles

centrosomes, and described them as comprising a tiny central body surrounded by a sphere of diffuse material. This central body is now known to consist of two centrioles (each comprising nine groups of three fused microtubules arranged in a hollow circle), and the diffuse material is now termed the peri-centriolar material (PCM) (Kuriyama and Borisy, 1981; Reider and Borisy, 1982).

The morphology of centrosomes varies widely from species to species, and even in a single cell type, the morphology of the centrosome can change dramatically during the cell-cycle (Bovari, 1901; Paweletz et al., 1984). For many years this variation obscured the central role of the centrosome in organising the mitotic spindle; because centrosomes were initially described as compact, well defined, structures, later investigators took the absence of such well defined structures in plant cells and some lower eukaryotes as proof that these cells did not contain centrosomes and that, therefore, these organelles were not essential components of the mitotic apparatus. It is now known, however, that the mitotic spindle consists mainly of microtubules (McIntosh, 1983), and all eukaryotic cells contain microtubule organising centres (MTOC's). In animal cells the MTOC usually takes the form of a compact centrosome, whereas in plants the MTOC lacks a centriole and is more diffuse, producing broader spindles than those found in animal cells. Despite this wide variation in morphology, it would be very surprising if all MTOC's were not related at the molecular

level (see Chapter 6).

In animal cells, centrosomes divide by the splitting apart of the centriolar pair generating two single centrioles. Each centriole nucleates the growth of a second centriole at right angles to the first. The two centriole pairs then move apart, producing two functional centrosomes per cell. The centrosomes migrate around the nucleus, and, on entry of the cell into mitosis, they nucleate many of the microtubules that form the bipolar spindle. Late in mitosis, the cell divides in two (cytokinesis), and each daughter cell inherits a single centrosome (for a more detailed description of centrosomes and the centrosome cycle see Alberts et al., 1989). It is obviously vital that the cell cycle and the centrosome cycle are co-ordinated so that the centrosome divides precisely once per cell cycle. Studies on somatic cells have revealed a close correlation between the onset of DNA synthesis and the replication of centrosomes (Robbins et al., 1968; Stubblefield, 1968; Brinkley and Stubblefield, 1970), and if DNA replication is inhibited by drugs, centrioles are able to split apart and nucleate the formation and elongation of a second centriole, but the centrosomes do not separate (Rattner and Phillips, 1973; Kuriyama and Borisy, 1981). Because the cell cycle is blocked in these cells, however, it impossible to say whether the lack of centrosome replication reflects an inability of the centrosome to divide in the presence of an un-replicated nucleus, or, whether the cell cycle is simply blocked at a

stage prior to the time when centrosome separation would normally occur.

In enucleated sea urchin embryos, by contrast, centrosomes continue to replicate in a co-ordinated fashion in the absence of the nucleus, although the timing of these divisions is slower than normal (Sluder et al., 1986). This observation demonstrates that there is no absolute requirement for the nucleus to provide a signal to trigger centrosome replication. On the other hand, experiments using aphidicolin (Ikegami et al., 1978), to inhibit DNA synthesis in sea urchin embryos, have yielded conflicting results on the ability of centrosomes to divide in the presence of an unreplicated nucleus. Two groups reported that centrosome division was either blocked (Nishioka et al., 1984), or occurred only rarely (Brachet and De Petrocellis, 1981), suggesting that an un-replicated nucleus inhibits centrosome replication. Sluder and Lewis (1987), however, reported that centrosome division occurred in a co-ordinated fashion in aphidicolin-treated sea urchin embryos, although the timing of the divisions was delayed. In this last study, the authors also followed the behaviour of the unreplicated nucleus. They found that despite the periodic doubling of the centrosomes, the nucleus did not proceed through cycles of nuclear envelope breakdown/reformation and chromosome condensation/decondensation, suggesting that either the signals that drive the nuclear cycle are not produced under these conditions, or, an unreplicated nucleus is unable to

respond to such signals.

I began my thesis work by studying the centrosome and nuclear cycles in early Drosophila embryos in which DNA synthesis was inhibited with aphidicolin, a specific inhibitor of DNA polymerase-α (Ikegami et al., 1978). The Drosophila embryo is a syncytium that undergoes thirteen rapid, nearly synchronous, nuclear divisions during the first two and a half hours of development (Rabbinowitz, 1941; Sonnenblick, 1950; Zalokar and Erk, 1976; Foe and Alberts, 1983). The first eight rounds of mitosis occur in the interior of the embryo. At telophase of nuclear cycle 8, the majority of the nuclei co-ordinately start to migrate to the cortex. Early in cycle 9 the nuclei that are destined to form the germ cells reach the posterior pole of the embryo. These nuclei undergo two further rounds of division before they pinch off to form separate pole cells (Warn et al., 1985). Early in cycle 10, the somatic nuclei reach the cortex of the embryo where they undergo a further four rounds of mitosis before cellularisation occurs at interphase of cycle 14. At this point the nuclei lose their mitotic synchrony and adopt a more typical somatic cell cycle, with G1 and G2 periods.

I found that the cytoplasmic cycles of centrosome replication and cortical budding (Foe and Alberts, 1983; Karr and Alberts, 1986) continue in Drosophila embryos after the inhibition of DNA synthesis with aphidicolin. Furthermore, the nuclei in these embryos appear to proceed through

multiple rounds of chromatin condensation/decondensation and nuclear envelope breakdown/reformation. These findings, described in chapter 3, demonstrate that Drosophila embryos, like many other early embryos, have a cytoplasmic cell cycle oscillator that operates largely independently of nuclear events. In Drosophila, this oscillator can drive both cytoplasmic and nuclear aspects of the cell cycle even when DNA synthesis is inhibited.

During this work, it became apparent that in aphidicolin-treated embryos, the replicating centrosomes dissociated from the non-replicating nuclei. I used aphidicolin to study the independent roles of the nucleus and the centrosome in the processes of nuclear migration and cortical cytoskeleton re-organisation. I found that an injection of aphidicolin at nuclear cycle 7-8, when the nuclei are starting to migrate to the cortex, inhibits nuclear migration, but centrosomes can continue to migrate to the surface in a co-ordinated manner. Once at the cortex, the centrosomes initiate many aspects of the reorganisation of the cortical cytoskeleton, including pole cell formation, in the absence of nuclei. These results, described in chapter 4, suggest that the centrosome, rather than the nucleus, normally plays the primary role in these developmental processes.

Finally, I looked at the mechanisms responsible for localising cyclin B mRNA in the developing Drosophila embryo. The Drosophila homologues of cyclin A and B were recently

cloned (Whitfield et al., 1989). An analysis of the mRNA distribution in the early embryo revealed that the cyclin B mRNA was initially homogeneously distributed throughout the embryo. By nuclear cycle 9-10, however, the mRNA had become dramatically concentrated at the posterior pole of the embryo, and also, to a lesser extent, at the rest of the embryo cortex. The levels of the cyclin B mRNA stayed high in pole cells throughout embryonic development, but levels progressively dropped around the somatic nuclei during cycles 10-14. Levels rose again in the somatic nuclei after cellularisation was complete, and cell divisions resumed.

In chapter 5, I describe experiments designed to investigate the mechanisms responsible for generating both the posterior accumulation and cortical accumulation of the cyclin B mRNA. I found that cyclin B mRNA is concentrated around nuclei, and co-migrates to the embryo cortex with the migrating nuclei, producing the cortical accumulation. I also found that cyclin B mRNA is located at the posterior pole in the earliest embryos, and in unfertilised eggs, suggesting that the mRNA becomes localised to the posterior pole during oogenesis, not embryogenesis as reported previously. I found that this posterior localisation of the cyclin B mRNA probably depends on the binding of the mRNA to a component (or components) of polar granules.

CHAPTER 2

Materials and Methods

2.1 Drosophila Culture

All flies were kept at 24°C, on yeast, treacle, cornmeal food (45ml brewer's yeast, 63ml treacle, 110ml maize meal, 15g agar, per litre of water; 30ml 10% Nipagen and 7ml propionic acid added after boiling). Stocks were raised in plastic vials of 25mm x 80mm, or in 1/3 pint milk bottles. Standard fly handling techniques were used (Roberts, 1986).

2.2 Embryo Collection

Flies were kept either in population cages for collections from Oregon-R stocks used in embryo injections, or in 1/3 pint bottles that had the bottom removed for collections from mutant stocks. Embryos were collected on petri dishes containing grape juice-agar (80g sucrose, 8g yeast, 25g agar per litre of water; 15ml 10% Nipagen and 7ml propionic acid added after boiling), supplemented with a small amount of live yeast suspension.

2.3 Embryo Fixation

Embryos were fixed in two different ways depending on whether they were to be stained with antibodies or hybridised with probes to detect mRNAs. For antibody staining, embryos were prepared using a slightly modified method from Dequin et al. (1984), which itself is based on the technique of Mitchison and Sedat (1983). Embryos were dechorionated in 60% domestic bleach, and then rinsed in 0.7% NaCl/0.1% Triton X-100. The embryos were transferred to a 7ml glass vial containing 25µl 37% formaldehyde (Fisons Scientific, UK),

100μl water, 25μl 10x buffer B (100mM potassium phosphate buffer pH 6.8, 450mM KCl, 150mM NaCl, 20mM $MgCl_2$), and 2.25ml heptane, and shaken vigorously for 10-15 minutes. If embryos were to be stained for microtubules, 0.5μM taxol (1.25μl of a 1mM solution in DMSO) was added to the mixture for 30 seconds prior to the addition of formaldehyde. While it is known that the microtubule structures observed in embryos fixed with this concentration of taxol are slightly different to those observed in non-fixed material (Warn et al., 1988; Kellog et al., 1989), these differences are unlikely to have affected any of the conclusions reached in this study. After shaking, 2.5ml of methanol was added and the mixture was shaken for a further 30 seconds; embryos that have lost their vitelline membrane sink to the bottom of the vial, while the rest remain at the heptane/methanol interface. The liquid and the non-devitellinised embryos were aspirated away, and the devitellinised embryos rinsed twice in methanol for a total of 10 minutes. The embryos were then re-hydrated in buffer A (15mM Tris-HCl pH 7.4, 60mM KCl, 15mM NaCl, 1.5mM spermine, 0.5mM spermidine) plus 0.1% Triton X-100 (buffer A+T). If the embryos were to be stored prior to antibody staining they were dehydrated in an ethanol series (30%, 50%, 70% - 5 minutes each) and then stored at -20°C in 70% ethanol.

If embryos were to be sectioned or hybridised to DNA probes as whole mounts, they were fixed using a slightly different method. The embryos were de-chorionated as above, but then

fixed by shaking for 10 - 20 minutes in a 1:1 mixture of freshly prepared 4% paraformaldehyde in PBS (PP): heptane. The aqueous phase was then removed and an equal volume of methanol was added; the embryos were devitellinised as described above. After rinsing in methanol for 10 minutes, the embryos were then rehydrated in a 7:3, 5:5, 3:7, series of methanol plus 0.1M EGTA: PP (5 minutes each). They were then post-fixed in PP for 20 minutes. If the embryos were not going to be used within one day, they were dehydrated and stored as described above.

2.4 Embryo Injection

Embryos that were to be injected were dechorionated by hand on a piece of double sided sticky tape (Scotch). The adhesive from standard Sellotape was dissolved in heptane and painted on to a coverslip. The heptane was allowed to evaporate leaving a transparent adhesive strip onto which embryos were placed for injection. The embryos were left to desiccate for a variable amount of time (usually 2-10 minutes), before they were covered with voltalef oil (10S, Atochem). The embryos were allowed to develop until they reached the desired age, and then injected in the middle of the embryo. The drugs used in this study were injected at the following concentrations: aphidicolin, 100μg/ml; colchicine, 1 x 10^{-3}M; cytochalasin D, 250μg/ml; α-amanatin, 400μg/ml. All of these were injected in injection buffer (5mM KCL, 0.1M sodium phosphate buffer pH6.8).

Injected embryos were fixed as follows: the coverslip holding the embryos was placed in a petri-dish; heptane was added and gently blown over the embryos with a pasteur pipette until the embryos had been dislodged from the glue, and the embryos were then fixed using either of the methods described above. Because this fixation process usually failed to remove the vitelline membrane, I found it more efficient to shake the embryos more gently in the fixation mixture for between one and two hours. The embryos were then sucked off from the interface and put in a watch-glass where they were washed twice in methanol. The embryos were re-hydrated in buffer A+T, and de-vitellinised by hand by rolling the embryos on a piece of double sided sticky tape (Scotch). The embryos were then either stained directly with antibodies or post-fixed in PP and dehydrated through an ethanol series for storage (as described above).

2.5 Observation of living embryos

Embryos were injected as described above. The coverslip holding the embryos was then inverted on two coverslips stuck to a slide to allow oxygen exchange. The embryos were observed using either Differential Interference Contrast (DIC) optics or phase contrast optics on a Nikon Microphot FX.

2.6 Antibody staining

Staining of embryos was carried out in small watchglasses. After incubation of embryos in buffer A+T plus 10% FCS for

one hour, embryos were hybridised with the first antibodies for 12-16 hours at 4°C. The embryos were washed for one hour with three changes of buffer A+T, and then hybridised to second antibodies for either 2-4 hours at room temperature, or 12-16 hours at 4°C. They were washed in buffer A+T for one hour (three changes) and then buffer A for 30 minutes (two changes). Finally the embryos were incubated for twenty minutes in 1μg/ml Hoechst 33258 (Riedal De Haen AG, Hannover, FDR) in water. The embryos were mounted in 85% glycerol containing 2.5% n-propyl gallate.

To antibody stain embryo sections, the paraffin sections were treated in Xylene (BDH) for 10 minutes and then re-hydrated in an ethanol series (100%, 80%, 60%, 30%, 2 minutes each). Slides were then antibody stained in the same way as described above, except that antibody and Hoechst solutions were placed on the sections and covered by a coverslip and the slides kept in a humid box. Washing of the slides was carried out in slide boxes.

2.7 Antibodies

The following antibodies have been used in this study: centrosomes were stained with Rb188 (1:500), a rabbit polyclonal sera that recognises a centrosome associated antigen (Whitfield et al., 1988); microtubules were stained with YL1/2 (1:5) (Kilmartin et al., 1982), a rat monoclonal antibody that recognises α-tubulin in Drosophila (Warn and Warn, 1985); lamins were stained with T47 (1:5), a mouse

monoclonal antibody kindly donated by Dr. H. Saumweber; actin was stained using a mouse monoclonal antibody (1:100) (Amersham International plc); the plasma membrane was stained with a rabbit polyclonal sera (1:100) that recognises phosphotyrosine, kindly donated by Dr. J. Wang (Wang, 1985). This antibody stains plasma membrane components in Drosophila embryos (Sullivan, 1987). All second antibodies were obtained from Jackson Immunoresearch Inc. (USA) and were used at dilutions of between 1:100 and 1:500, as appropriate.

2.8 Assay of ^{3}H-thymidine incorporation

Embryos were injected with injection buffer containing 1μCi/μl ^{3}H-thymidine with or without aphidicolin. They were allowed to develop for varying amounts of time, and then removed from the sticky tape as described above. The embryos were then homogenised in 200μl of 10mM Tris.HCl (pH8.0), 1mM EDTA (TE). Ten microlitres of the homogenate was placed on a GF/ filter (Whatman) and the rest was precipitated with two volumes of 10% TCA. The acid precipitate was washed onto a GF/A filter, and the filter was washed 3 times with 96% ethanol. Three millilitres of Aquasol (New England Nuclear research products) was added to the filters, which were then counted in a scintillation counter.

2.9 Preparation of ^{35}S-RNA probes

All probes were made from plasmid constructs containing cDNAs inserted between T3- and T7-RNA polymerase promoters (see Constructs below). The plasmids were linearised with a

suitable restriction enzyme. This reaction was then treated with 200μg/ml proteinase K (Sigma) at 37°C for 30 minutes, then phenol extracted, chloroform extracted, and the DNA ethanol precipitated using standard techniques (Maniatis et al., 1982). The DNA was re-suspended at a concentration of 1mg/ml in TE. RNA probes were prepared exactly as described in the Stratagene RNA Transcription kit (# 200 341), using ^{35}S-UTP (>800mCi/mMol, Amersham), and either T7- or T3-RNA polymerase, depending on the sense of the probe required. The reaction was left at 37°C for 30-40 minutes and terminated by the addition of 1/10 volume of 10x MS (10mM Tris pH7.5, 10mM $MgCl_2$, 50mM NaCl). The reaction was treated with 1μl of RNAse free DNAse (120mg/ml, BRL) for 7 minutes at 37°C, then a 1μl aliquot was TCA precipitated and counted in Aquasol on a scintillation counter to determine the percentage incorporation. 30μl of tRNA (10mg/ml, phenol extracted, ethanol precipitated, and resuspended in DEPC treated water) was added to the reaction, and the probe was phenol extracted, chloroform extracted and ethanol precipitated by adding an equal volume of 4M ammonium acetate and 2.5 volumes of ethanol. I could detect no difference on in situ sections using size-reduced and non-size-reduced probes, so I routinely omitted a size reduction step; the probe was therefore directly resuspended in 50% formamide (de-ionised, Rose), at between 0.5 - 2.5 x 10^6 cpm/μl and stored at -20°C until used.

2.10 Restriction digestion

All restriction digestions were carried out in accordance with the manufacturers instructions. Usually, 0.25-2μg of DNA was added to 1μl of enzyme buffer, 1μl of enzyme, and the reaction mixture made up to 10μl with ddH_2O. This was left at 37^{o}C for 1-2 hours.

2.11 Constructs

The following constructs have been used in this study: pKS1.7, a 1.8 kb cDNA containing the 3' end of the cyclin B mRNA (W. Whitfield, pers. comm.) in bluescript pKS (Stratagene), T3 RNA polymerase synthesises an antisense probe; pKS1.2, a 1.4 kb cDNA encoding almost the entire coding region of the cyclin A gene (W. Whitfield, pers. comm.) in bluescript pKS, T7 RNA polymerase synthesises an antisense probe; pSn9R1,Sph, a 0.6 kb cDNA encoding the first exon of the singed gene (J. Paterson, pers. comm.) inserted in to the pBS plasmid (Stratagene), T3 RNA polymerase synthesises an antisense probe; NBA1, a 3.4 kb cDNA encoding the 3' end of the lodestar mRNA (C. Girdham, pers. comm.), T7 synthesises an antisense probe; 10a2, a 2.1 kb cDNA containing the entire coding region of the polo gene (S. Llamezares, pers. comm.), T7 synthesises an antisense probe. These last two cDNAs were in the pNB40 vector (Brown and Kafatos, 1988).

2.12 Embryo Sectioning

Fixed embryos were dehydrated in an ethanol series (30%, 50%, 70%, 96%, 100% - 5 minutes each) and then incubated in Xylene

for 10 minutes twice. The embryos were then moved to an air oven at 60°C and allowed to warm up for 10 minutes. The Xylene was then gradually replaced with melted paraffin wax (BDH, melting point of 55°C), until the embryos were in 100% paraffin. They were transferred to a sectioning mould (Peel-a-way from Polysciences Ltd. USA), and allowed to cool slowly at room temperature for 30 -40 minutes; the mould was then kept at 4°C for at least 2 hours before 6-8μm sections were cut using a microtome. Individual sections were then floated on distilled water on a poly-lysine (Sigma) coated slide over night at 40°C. Slides were stored at room temperature until used.

2.13 Preparation, hybridisation, and washing of sections

The methods detailed below are essentially those described by Ingham et al, 1985. Slides were prepared for hybridisation in glass slide jars that had been baked O/N at 180°C. Slides were treated with xylene 2 x 10 minutes, then rehydrated in an ethanol series (100%, 96%, 80%, 60%, 30%, two minutes each). They were treated with 0.2M HCl for 20 minutes, washed in ddH_2O for 5 minutes, incubated in 2X SSC (1X SSC is 0.15M NaCl, 0.015M tri-Sodium Citrate) at 70°C for 30 minutes, and then washed in ddH_2O for 5 minutes. The sections were then incubated at room temperature with 0.125mg/ml Protease (Sigma) in 50mM Tris pH7.5, 5mM EDTA for 10 minutes, washed in PBS plus 0.2% glycine for 2 minutes, PBS for 1 minute, and then re-fixed in freshly made PP for 20 minutes. They were then rinsed in PBS for 5 minutes and de-

acetylated in 0.1M triethanolamine plus 0.5% acetic anhydride, with vigorous stirring, washed in PBS for 2 minutes, dehydrated through an ethanol series (30%, 50%, 70%, 96%, 100% - 2 minutes each), and air dried.

The ^{35}S-RNA probes were boiled for 2 minutes and then diluted 1/5 with hybridisation solution (50% formamide, 12.5% dextran sulphate, 0.375M NaCl, 12.5mM Tris pH7.5, 12.5mM sodium phosphate buffer pH6.8, 6.25mM EDTA, 1.25% Denhardts [1% ficoll, 1% polyvinyl pyrrolidone, 1% BSA], 12.5mM DTT, filtered through a millipore filter). 20μl of each probe was placed on a coverslip, and the slide with the tissue on it was used to pick up the coverslip, ensuring that the tissue was completely covered by probe. The coverslips were then sealed with rubber cement and incubated at 50^{o}C for 14-16 hours in a humid box.

The slides were dipped in wash solution (0.3M NaCl, 10mM Tris pH7.5, 10mM $NaPO_4$, 5mM EDTA, 10mM mercaptoethanol, 50% formamide - pre-heated to 50^{o}C) until the coverslips fell off, and then washed in this solution at 50^{o}C for at least 4 hours with hourly changes of buffer. The slides were incubated for 5 minutes in 1 x NTE (0.5M NaCl, 10mM Tris 7.5, 1mM EDTA), and then in 1 X NTE plus 20μg/ml RNAse (Sigma) at 37^{o}C for 30 minutes. They were then washed for 1 hour in 1 X NTE with 4 changes of buffer, dehydrated in an ethanol series as described above, and air dried. The slides were then dipped in a 50% solution of Ilford L-4 emulsion (melted at

45°C) in the complete dark, placed in a light tight box with silica gel, and left at 4°C in a radiation free fridge for an appropriate amount of time (2-7 days). The slides were then developed at 18°C in D-19 developer (Kodak) for 3 minutes, hardener (30% chromic potassium sulphite, 60% anhydrous sodium sulphate) for one minute, and fix (1/4 dilution of Kodak paper fix) for 5 minutes. The slides were rinsed for 10 minutes in ddH_2O, and then stained in a 10μg/ml solution of Hoechst 33258 for 3 minutes followed by a 20 minute wash in ddH_2O. The slides were air dried and mounted either in DPX mountant (Fluka), or GMM (1.75g of Canadian balsam/ml of methyl salicylate).

2.14 Preparation of Digoxygenin probes for Whole-Mount in situ's

The 3' 1.8 kb of the cyclin B cDNA was cut out of pKS1.7 (see constructs) using EcoR1. A 1/10 volume of loading buffer (2% ficoll, 0.05% bromophenol blue, 0.05% xylene cyanol) was added to the reaction, and this was run on a 1% Low Gelling Temperature agarose gel (dissolved in 40mM Tris-Acetate, 2.5mM EDTA (TAE), plus 1μg/ml ethidium bromide). Bands of DNA were visualised under short wave UV, and the appropriate band was cut out and the DNA purified using a geneclean kit (Bio 101). The DNA was resuspended in TE at 1mg/ml. Digoxygenin probes were made using a BRL kit (# 1093 657). The reaction is essentially an oligolabelling reaction (Feinberg and Vogelstein, 1983, and addendum, 1984) and was carried out exactly as described in the BRL kit.

2.15 Whole-Mount Hybridisation of Embryos to Digoxygenin Probes

The method described below is essentially that described by Tautz and Pfeiffe (1989). Embryos were fixed as described above, and the following steps were performed in an eppendorf tube using 1ml volumes (care was taken to ensure a minimum of RNAse contamination). The embryos were washed 3 x in PBS plus 0.1% Tween 20 (PBT) and incubated in PBT plus 50μg/ml Proteinase K (Sigma) for 3-4 minutes. The digestion was stopped by rinsing the embryos in 2mg/ml glycine in PBT for 2 minutes. The embryos were then washed in PBT 2 x 5 minutes, refixed in fresh PP for 20 minutes, followed by washing 5 x 5 minutes in PBT. They were washed in a 1:1 mixture of hybridisation solution (50% formamide, 5 x SSC, 100μg/ml sonicated salmon sperm DNA, 50μg/ml heparin, 0.1% Tween 20):PBT for 10 minutes, washed in hybridisation solution for 10 minutes, and then prehybridised in hybridisation solution for 1 hour at 45^{o}C. Most of this solution was then removed and 10μl of heat de-natured digoxygenin probe was added, mixed, and left to hybridise for 12-16 hours at 45^{o}C.

The embryos were washed for 20 minutes in hybridisation solution then 20 minutes each in a 4:1, 3:2, 2:3, 1:4 mixture of hybridisation solution:PBT. They were washed 2 x 20 minutes in PBT and then incubated for 1 hour in a 1:2000 dilution of horse anti-digoxygenin antibody (from the BRL

digoxygenin kit) that had been preadsorbed against fixed embryos for one hour. They were washed 4 x 20 minutes each in PBT, and then washed 3 x 5 minutes in 100mM NaCl, 50 mM $MgCl_2$, 100mM Tris pH9.5, 1mM Levamisol, 0.1% Tween 20. 4.5μl of NBT and 3.5μl of X-phosphate solution (both from the BRL digoxygenin kit) were added to the last wash. Colour develops over the next 10-30 minutes, and the reaction is stopped by a final rinse in PBT. The embryos were then stained with Hoechst (1μg/ml) for 20 minutes, and mounted in 85% glycerol, 2.5% propylgallate.

CHAPTER 3

Nuclear and Cytoplasmic Mitotic Cycles Continue in Drosophila Embryos in which DNA Synthesis is Inhibited with Aphidicolin

3.1 INTRODUCTION

As discussed in chapter 1, the early embryos of many organisms have a simplified cell cycle that dispenses with many of the controls imposed on somatic cells by the requirement to divide only under certain conditions. This is dramatically illustrated in Xenopus eggs, where enucleation does not inhibit periodic cycles of cortical contraction (Hara et al., 1980) or MPF activation (Daubavalle et al., 1988). These, and other, studies have pointed to the existence of a cytoplasmic cell cycle oscillator that is largely independent of the nucleus, and MPF is thought be a part of, or closely coupled to, this oscillator (Kirschner et al., 1985). Studies using aphidicolin, a specific inhibitor of DNA polymerase-α (Ikegami et al., 1978), have demonstrated that some cytoplasmic aspects of the early embryonic cell cycle also continue in the presence of an unreplicated nucleus. For example, cleavage divisions occur in aphidicolin-treated starfish embryos (Nagano et al., 1981), and periodic surface contractions occur in aphidicolin-treated Xenopus eggs (Kimelman et al., 1987). It has also been shown that centrosomes continue to divide in aphidicolin-treated sea urchin embryos (Sluder and Lewis, 1987), although previous reports suggested that this did not occur (Brachet and De Petrocellis, 1981; Nishioka et al., 1984).

Although it is clear that some cytoplasmic aspects of the

cell cycle continue in the absence of DNA synthesis, the behaviour of unreplicated nuclei in aphidicolin-treated embryos has only been examined in sea urchins (Sluder and Lewis 1987). In this case the nuclear envelope does not always break down, and when it does, the chromatin can be seen as an amorphous mass of fibres; the chromosomes do not condense, and the nuclear membrane does not reform, even though centrosome division continues. It seems, therefor, that although some parts of the cytoplasmic cycle continue in aphidicolin treated sea urchin embryos, the cycles of chromatin condensation and nuclear envelope breakdown are dramatically affected by the inhibition of DNA synthesis, suggesting that unreplicated DNA may interfere with these aspects of the nuclear cycle.

In this chapter I have examined the effects of aphidicolin on both cytoplasmic and nuclear aspects of the cell cycle in early Drosophila embryos. I have found that the cytoplasmic cycles of centrosome division and cortical budding continue in Drosophila embryos in which DNA synthesis is inhibited with aphidicolin, although the cycle time is slowed. More importantly, in contrast to the situation reported in sea urchins, it seems that nuclear cycles of chromatin condensation/decondensation and of lamin disassembly/assembly continue in such embryos, demonstrating, to my knowledge for the first time, that an unreplicated nucleus is capable of proceeding through multiple cycles of chromosome condensation and lamin disassembly.

3.2 Aphidicolin Inhibits DNA Synthesis and Nuclear Replication

In order to examine the effects of aphidicolin on nuclear division in Drosophila embryos, it was necessary to inject the drug at defined stages of development. As the first 8 nuclear divisions take place within the interior of the syncytium it is difficult to stage embryos during this period. At nuclear cycle 9, pole buds are formed as nuclei arrive at the posterior pole of the embryo, and early in cycle 10, the rest of the nuclei arrive at the cortex causing a distinctive cortical budding (Foe and Alberts, 1983; Karr and Alberts, 1986). I therefore injected aphidicolin during two developmental periods: (1) after a 30 minute egg collection, in which case all of the embryos should be at about nuclear cycle 3 or earlier, and (2) when cortical budding first became apparent at nuclear cycle 10. After injection, the embryos were left to develop for up to four hours. They were then fixed, stained with the DNA-binding fluorochrome Hoechst 33258, and examined by fluorescence microscopy. The number of nuclei seen per embryo was consistent with the expectation that nuclear division was inhibited at the time of aphidicolin injection (not shown). Embryos injected with injection buffer alone developed normally over the time course of the experiments and showed no unusual cytoskeletal or nuclear structures when stained by

indirect immunofluorescence with antibodies against tubulin, lamin, or centrosomes (not shown).

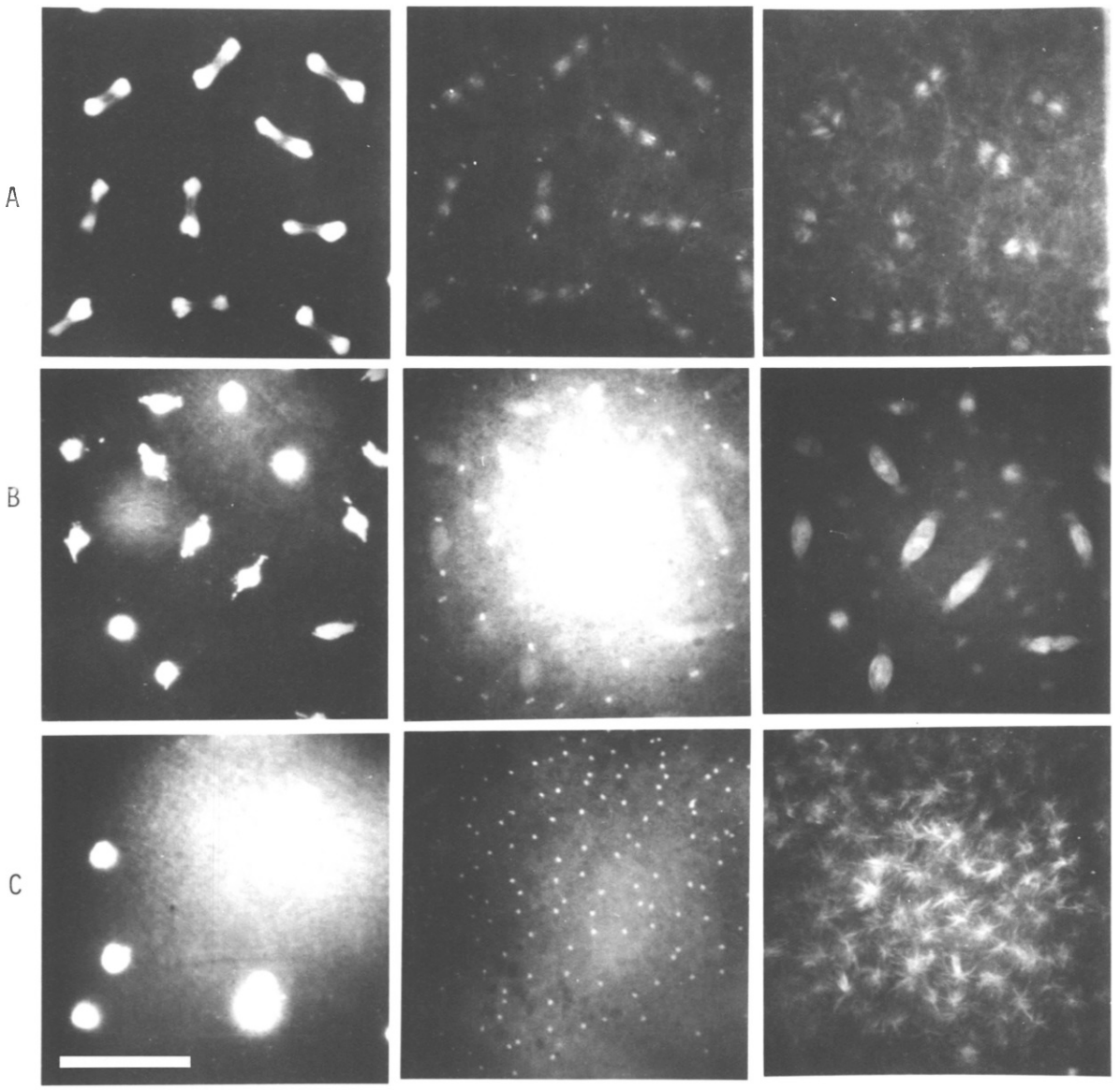
Aphidicolin has been shown specifically to inhibit DNA-polymerase-α-dependent DNA synthesis in many systems (e.g. Ikegami et al., 1979; Ikegami et al., 1978; Nagano et al., 1981). I tested its effectiveness in Drosophila embryos by co-injecting ^{3}H-thymidine and aphidicolin. In each of three experiments, aphidicolin inhibited ^{3}H-thymidine incorporation by 88 - 92% relative to control embryos injected with ^{3}H-thymidine alone. This is comparable to the inhibition achieved in other systems. The residual 10% incorporation could be due either to DNA repair or to DNA-polymerase β- and γ- dependent DNA synthesis.

3.3 Centrosomes Continue to Divide in Aphidicolin-Injected Embryos

In all subsequent experiments, aphidicolin was injected at about nuclear cycle 9-10. After injection, embryos were allowed to develop for varying lengths of time before they were fixed and stained by indirect immunofluorescence. Figure 3.1a shows a field of nuclei in an embryo that was allowed to develop for about 10 minutes following injection of the drug. The embryo is at the late telophase stage of mitosis as judged by the decondensing chromatin and the presence of the midbody, a structure characteristic of the

Figure 3.1

The Distribution of DNA, Centrosomes, and Microtubules in Aphidicolin-Injected Embryos at Various Times After Injection. Embryos were injected with aphidicolin at about the time of pole bud formation (nuclear cycle 9 - see text). They were allowed to develop for various lengths of time before fixation and staining with Rb188 (anti-centrosome) and Yl1/2 (anti-tubulin) followed by rhodamine-coupled goat anti-rabbit and fluoroscein-coupled mouse anti-rat antibodies. The embryos were finally incubated with Hoechst 33258 (see Materials and Methods). The figure shows fields from typical embryos fixed 10 minutes (A), 45 minutes (B), and 90 minutes (C), after injection of the drug. Scale bar = 20 μm

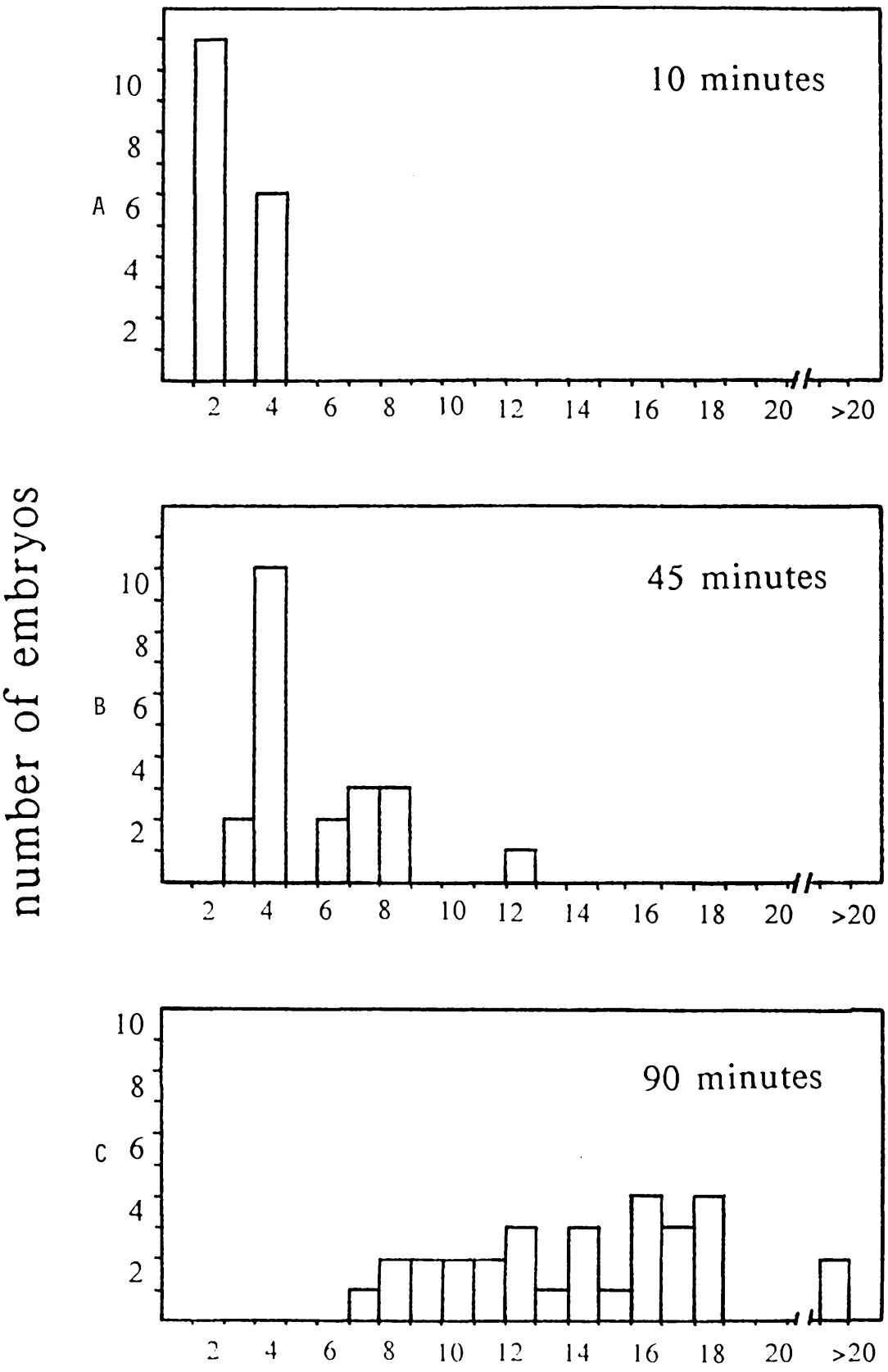

A
B
C
DNA
Rb188
Tubulin

telophase spindle. The centrosomes appear to have just divided, as often occurs at late telophase in Drosophila embryos. Normally, the chromosomes would be well separated by the telophase stage of mitosis, but in this case there is still a chromatin bridge linking the two daughter nuclei. This presumably reflects the inability of the spindle to separate DNA that has not been fully replicated. The effect of aphidicolin on nuclear division presumably depends on the proportion of the genome that has replicated at the time the drug was injected: if the genome was almost fully replicated, mitosis might proceed quite normally to produce two daughter nuclei each with two centrosomes, if the genome had just begun to replicate, mitosis might fail to separate the daughter nuclei, resulting in a single nucleus with four centrosomes. I observed that all of the embryos fixed 10 minutes after injection had a ratio of centrosomes to nuclei of two or four (Figure 3.2).

Fields from two embryos fixed 45 minutes and 90 minutes after injection with aphidicolin are shown in Figure 3.1b and 3.1c, respectively. In these particular fields the ratio of centrosomes to nuclei is approximately 7:1 (Figure 3.1b) and 24:1 (Figure 3.1c), demonstrating that centrosomes continued to divide following the injection of aphidicolin. The extra centrosomes did not remain associated with the nuclei. The ratios of centrosomes to nuclei in all of the embryos examined in these experiments are shown in Figure 3.2. While the ratios tended to increase with time, they did not

Figure 3.2

The Ratio of Centrosomes to Nuclei Following the Injection of Aphidicolin. Embryos were injected with aphidicolin and allowed to develop for varying lengths of time before fixation and staining. The ratios were obtained by counting the number of nuclei and centrosomes in photographs of embryos that had been allowed to develop for 10 minutes (A), 45 minutes (B), and 90 minutes (C), after injection of aphidicolin.


10 minutes
45 minutes
90 minutes
A
B
C
10
8
6
4
2
2 4 6 8 10 12 14 16 18 20 >20
number of embryos
ratio of centrosomes to nuclei

increase in a strict geometric progression as might be expected if all of the centrosomes divided in a co-ordinated fashion. I could not be certain, however, that I was counting all of the centrosomes in these experiments; although the majority were at the surface of the embryo, additional centrosomes were observed at different planes of focus throughout the embryo. It is likely that centrosome replication did occur in a co-ordinated fashion in aphidicolin-treated embryos, since I observed many examples of embryos in which all the centrosomes appeared to be in the process of dividing synchronously. This can be seen in Figure 3.1b and Figure 3.6b where almost all the centrosomes are closely paired.

As can be seen in Figure 3.1, the centrosomes in aphidicolin treated embryos were functional with respect to microtubule nucleation . The microtubular structures observed in treated embryos were similar to those seen in untreated embryos. Figure 3.1b, for example, shows condensed chromatin organising bi-polar spindles, each with one or two centrosomes at either pole. In addition, there are asters of microtubules nucleated by extra centrosomes that are not associated with chromatin. In Figure 3.1c, the majority of the centrosomes are at the surface of the embryo and are nucleating asters. The nuclei are below the surface and do not interact with the asters on the surface, although some of these nuclei have centrosomes associated with them (not shown).

3.4 Cycles of Cortical Budding Continue in Aphidicolin-Treated Embryos

Previous studies on Drosophila embryos using differential interference contrast (DIC) microscopy have shown that between nuclear cycle 10 to 14 the interphase portion of the cell cycle is associated with a cortical budding at the embryo surface. An interphase nucleus is associated with each bud (Foe and Alberts 1983). As the nuclei enter mitosis they move away from the surface of the embryo and the cortical budding recedes. Figure 3.3 shows the budding cycles in an untreated embryo studied by DIC microscopy. Alternate frames show successive minimal and maximal stages of budding. As illustrated, the number of buds increased progressively with the number of nuclei (frames at 4, 11, 21, 31, and 54 minutes) and the buds did not completely disappear at mitosis (frames at 8, 14, 24, and 37 minutes).

Embryos injected with aphidicolin at about nuclear cycle 10 also showed cycles of cortical budding, but the buds were much less pronounced than those in un-injected controls. Figure 3.4 shows one such embryo: budding was maximal at 12, 22, 56, and 77 minutes post-injection, and the number of buds in any given area of the embryo appeared to approximately double with each cycle. Cycles of cortical budding were observed in all 13 embryos followed in this way; the timing and length of each cycle, however, was more variable than in

Figure 3.3

Cortical Budding Cycles in an Untreated Embryo. The developing embryo was observed with DIC optics as described in Materials and Methods. Zero time represents the point at which cortical budding was first observed. This is one minute into interphase of cycle 10 (Foe and Alberts, 1983). Budding is maximal at 4, 11 ,21, and 31 minutes, (as shown), and at 41 minutes (not shown). The nuclei in the last frame at 54 minutes are in the process of cellularisation. The timing of successive cycles in 8 embryos injected with buffer is shown in Table 3.1 (page 78). Scale bar = 40μm

0
4
8
11
14
21
24
31
37
54

untreated controls (Table 3.1, page 78).

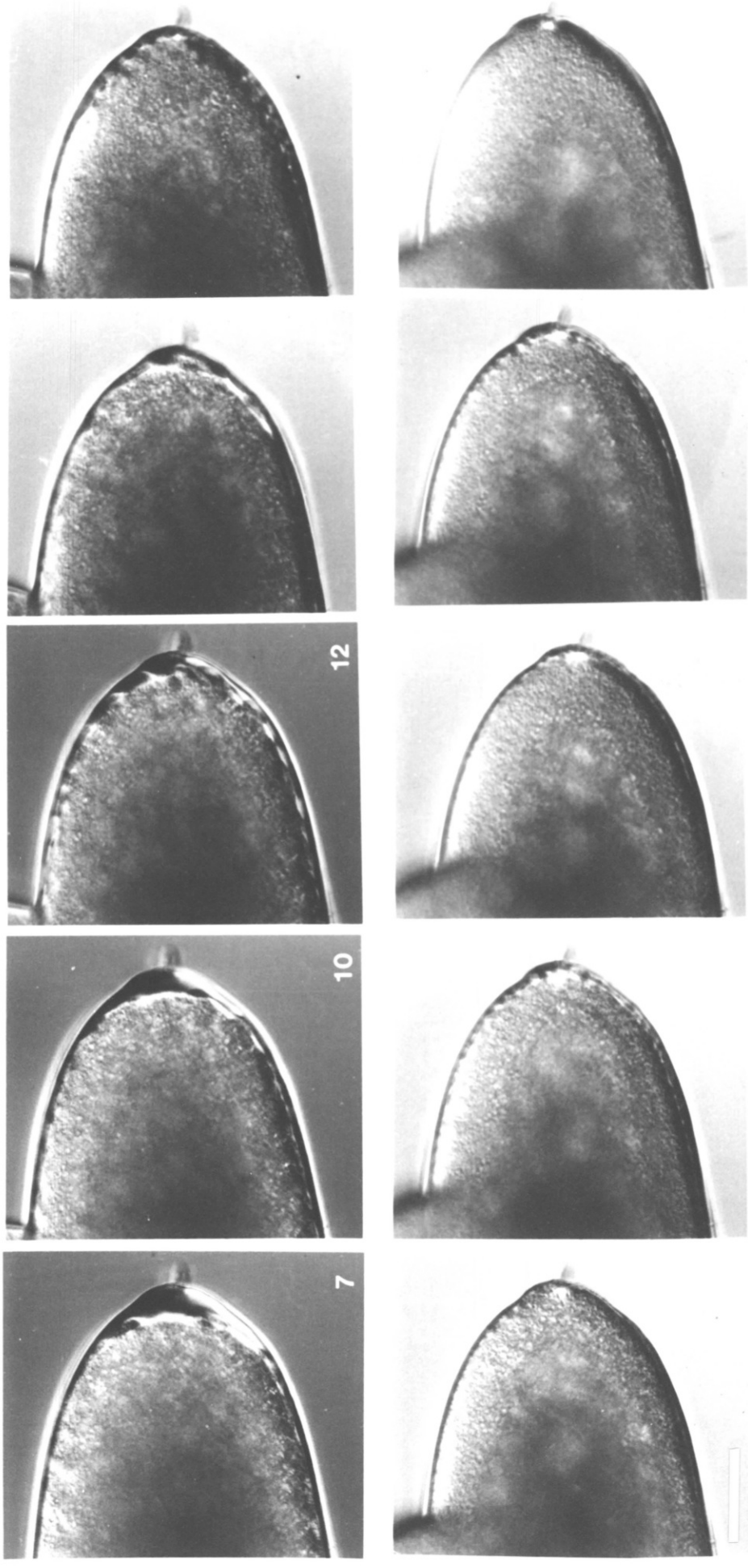
All of the embryos followed with DIC optics were fixed directly after observation, immunostained to reveal centrosomes, and counterstained with Hoechst 33258. This confirmed that nuclear division had not occurred whereas centrosome division had continued. Figure 3.5 shows the results of staining the embryo illustrated in Figure 3.4. The ratio of centrosomes to nuclei is approximately 14:1, suggesting that the centrosomes have gone through 3 to 4 rounds of division in the absence of nuclear division. I had observed by DIC microscopy that this embryo went through four rounds of cortical budding after aphidicolin injection (Figure 3.4). This approximate correlation between the number of budding cycles and the number of centrosome divisions in the absence of nuclear replication was observed in all embryos that were followed in this way.

3.5 Nuclear Cycles of Chromatin Condensation and Lamin Disassembly Continue in Aphidicolin-Treated Embryos

Although cortical budding cycles continued in aphidicolin-treated embryos, nuclei were rarely visible within the cortical buds. Because of this, it was not possible to see by DIC microscopy whether the nuclei were cycling in unison with the cytoplasmic budding cycles, as would be the case in an untreated embryo. I was, however, able to obtain indirect

Figure 3.4

Cortical Budding Cycles in an Aphidicolin-Injected Embryo. The embryo was injected with aphidicolin and observed with DIC optics (see Materials and Methods). Zero time is the point at which this embryo was injected with aphidicolin. As in Figure 3, this is one minute into interphase of cycle 10. Because of the time needed to prepare the embryo for observation, the first picture was taken after 7 minutes when the the cortical budding associated with interphase of nuclear cycle 10 was starting to fade. Budding was maximal at 12, 22, 56, and 77 minutes. The timing of successive cycles in embryos that were observed in this way is shown in Table 3.1 (page 78). Scale bar = 40 μm


12
10
7

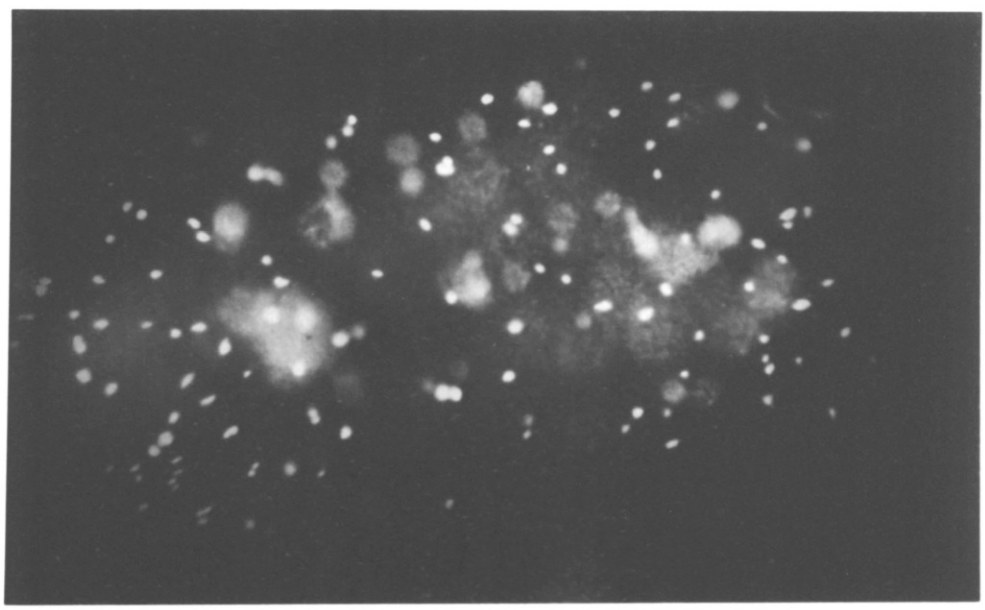
evidence that this was indeed the case.

Different embryos injected with aphidicolin and then fixed and stained with Hoechst after one or two hours had nuclei in different states of condensation, suggesting that the chromatin might be proceeding through cycles of condensation/decondensation even though DNA synthesis was inhibited. Figure 3.6 for example, shows fields of nuclei in two embryos fixed 90 minutes after injection and stained by indirect immunofluorescence using anti-centrosome and anti-lamin antibodies. In both cases there are extra centrosomes around the nuclei. In Figure 3.6a, however, the nuclei have the appearance of normal interphase nuclei with decondensed chromatin, whereas in Figure 3.6b the chromatin is condensed and some chromatin structure is visible. The abnormal tangled appearance of the chromatin in Figure 3.6b might reflect the inability of the spindle to separate unreplicated chromatin. The staining with the anti-lamin antibody indicated that the degree of chromatin condensation correlated with the state of the nuclear lamina. The lamins, which are major components of the nuclear envelope, are phosphorylated during nuclear envelope breakdown as the embryo enters M-phase (Gerace, 1986). This change in nuclear envelope structure can be followed with anti-lamin antibodies, which stain the nuclear envelope of interphase nuclei, but give a weaker, more diffuse staining in the rest of the mitotic cycle (Fuchs et al., 1983). Both staining patterns were seen in aphidicolin-treated embryos: the decondensed interphase-like nuclei were

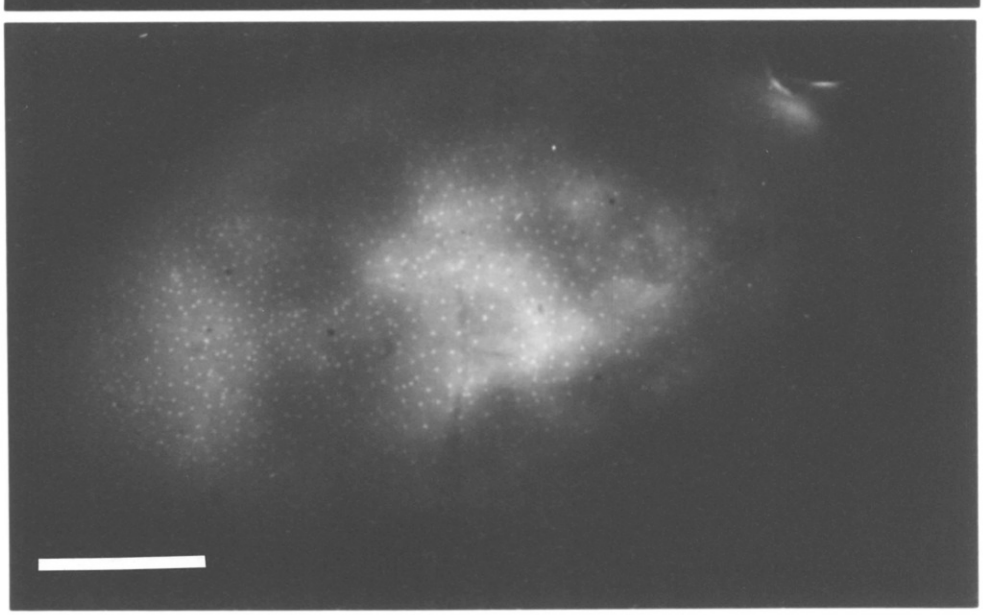
Figure 3.5

Fixed Preparation of the Embryo Illustrated in Figure 3.4 Following the observation of the cortical budding cycles, the embryo shown in Figure 3.4 was fixed and stained sequentially with Rb188, rhodamine-conjugated goat anti-rabbit, and Hoechst 33258. Scale bar = 50 μm

DNA



Rb188



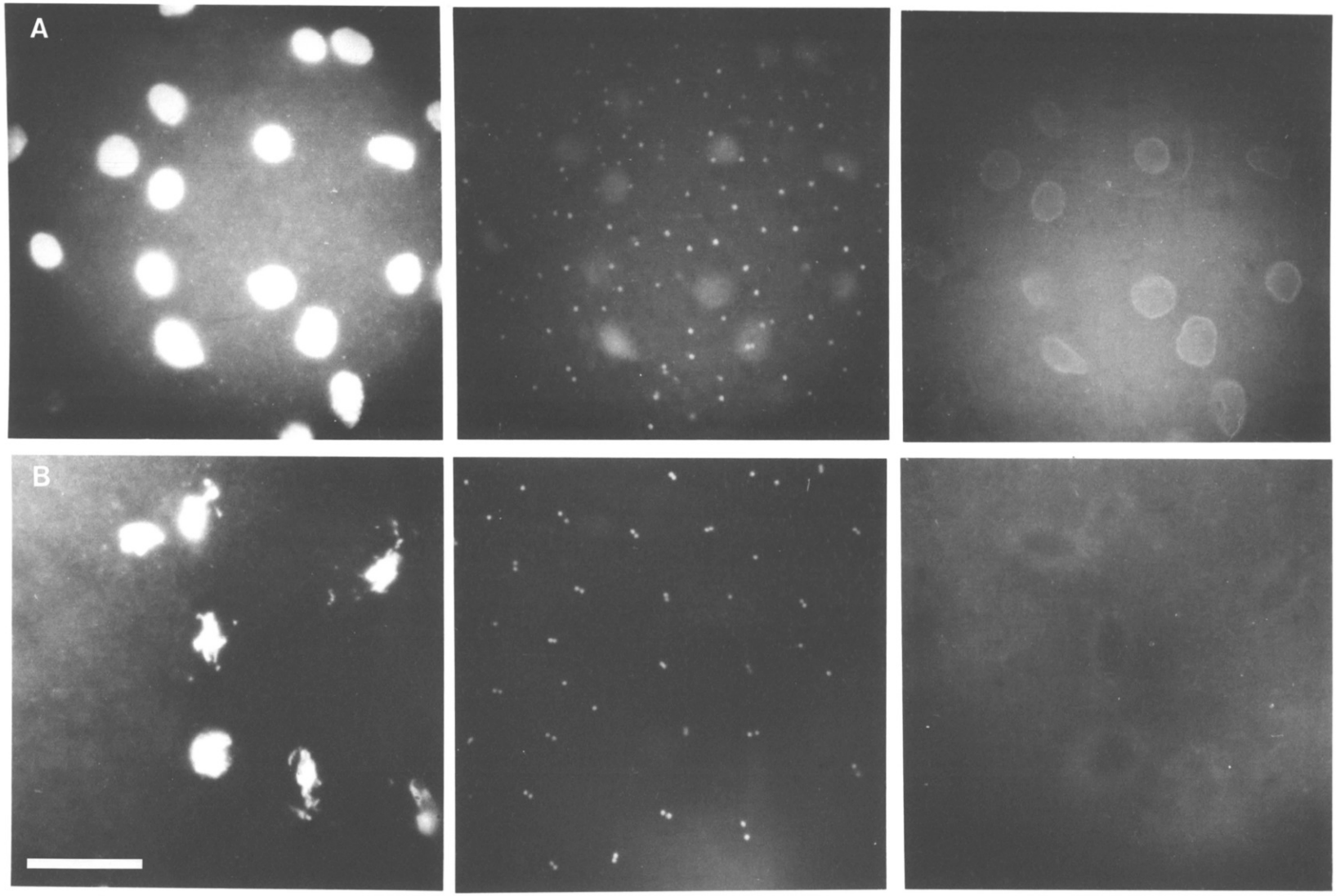
surrounded by bright annular lamin staining, whereas the staining around condensed chromatin was weaker and more diffuse (Figure 3.6).

A number of aphidicolin treated embryos were observed in which clearly defined areas contained nuclei in one or other of these two states, as might be expected if mitotic waves were still passing through the embryos. Normally the nuclei in a Drosophila embryo enter mitosis in waves that originate from both poles (Foe and Alberts 1983). These waves are also apparent in the cortical budding cycles of the embryo. In the aphidicolin-treated embryos the budding cycles often occurred in waves, although they often took longer to traverse the embryo (3 - 10 minutes) compared with the waves in control embryos (1/2-2 minutes). To test whether I could use the cortical budding cycles to predict the state of the chromatin, I allowed an aphidicolin-treated embryo to proceed through two cycles of budding and then fixed it just as the cortical buds were starting to fade from both poles. As predicted, the nuclei at both poles had lost their surrounding lamins while the nuclei in the middle had not (Figure 3.7a). Figures 3.7b,c show two fields from this embryo that illustrate this point more clearly.

In all of the embryos that were fixed when buds had faded (7 in total) most of the chromatin was condensed and had a diffuse anti-lamin antibody staining. In all of the embryos fixed when buds were present (6 in total), most of the

Figure 3.6

The Distribution of DNA, Centrosomes, and Lamins in Aphidicolin-Injected Embryos. Embryos were injected with aphidicolin at about nuclear cycle 10 and allowed to develop for 90 minutes before they were fixed and stained with Rb188 and T47 (anti-lamin) followed by fluoroscein-conjugated goat anti-rabbit and rhodamine-conjugated goat anti-mouse. The embryos were finally incubated with Hoechst 33258. Both embryos have many more centrosomes than nuclei. (A) A field from an embryo showing decondensed chromatin surrounded by bright lamin staining. (B) A field from a different embryo showing condensed chromatin and diffuse lamin staining. Scale bar = 10 μm


A
B
DNA
Rb188
Lamin

chromatin was decondensed and was surrounded by a nuclear membrane, as judged by the anti-lamin staining. The finding that I could use the cortical budding cycle to predict the state of the chromatin strongly suggests that cycles of chromatin condensation and lamin disassembly continued in synchrony with the cortical budding cycles in aphidicolin-treated embryos.

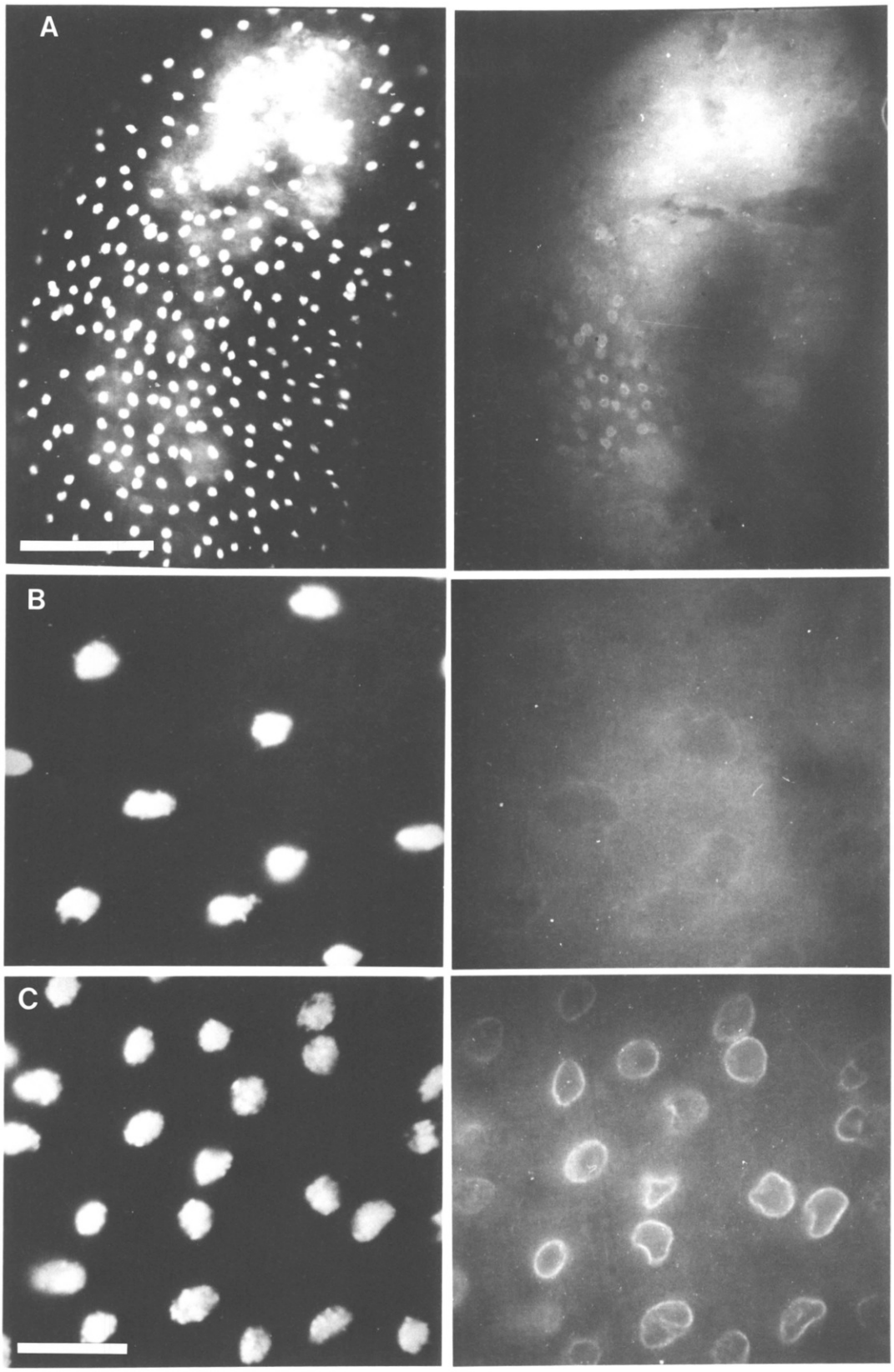
3.6 Mitotic Cycles Are Slowed in Aphidicolin-Injected Embryos.

The cycle times of the embryos followed in real time are shown in Table 3.1. Although the time of the first division cycle following injection was relatively unaffected, all of the subsequent cycle times in the aphidicolin-injected embryos were more variable and noticeably longer than in un-injected embryos. In most of the treated embryos the M phase of the cell cycle, the period where the buds were not apparent, was the most dramatically affected. The length of the S-phase equivalent, where buds were present, was variable but was often comparable to, or shorter than, the S-phase of untreated embryos. These real-time observations show that the cell cycle was slowed in aphidicolin treated embryos, and suggest that the major delay occurred in M-phase.

These findings are supported by my observations on fixed embryos. Ninety minutes after injection of aphidicolin into

Figure 3.7

An Aphidicolin-Injected Embryo Fixed as the Cortical Budding Receded from both Poles. The embryo was injected 4 minutes into interphase of cycle 10, and was observed with DIC microscopy through the cortical budding cycles equivalent to nuclear cycles 10 and 11. It was fixed as the buds associated with interphase of cycle 12 started to recede from the poles of the embryo. (A) A view of the whole embryo in which only the nuclei in the middle region are surrounded by a bright annular lamin staining. Scale bar = 50μm (B) A high power view of the anterior end of the embryo showing condensing chromatin and diffuse lamin staining. (C) A high power view of the middle of the embryo showing decondensed chromatin surrounded by a bright lamin staining. Scale bar = 10μm .


A
B
C
DNA
Lamin

a batch of embryos, the ratio of centrosomes to nuclei was variable, some embryos had ratios as low as 7:1, while others had ratios of greater than 20:1 (Figure 3.2). This implies that in some embryos the centrosomes divided only once or twice during the 90 minutes, while in others they have divided at least three or four times. In untreated embryos, on the other hand, the four rounds of mitosis that precede cellularisation usually take place in under one hour. My observations on fixed embryos also suggest that aphidicolin-treatment resulted in a delay to the cycle primarily in M-phase. Of the aphidicolin-treated embryos, approximately 55-65% had the majority of their chromatin in a condensed state (not shown). This is in contrast to non-injected embryos in which only 30-40% have condensed chromatin.

Table 3.1

Budding cycle times of aphidicolin injected embryos

Nuclear cycle	Length of time (minutes) when buds apparent				Length of time (minutes) when buds not apparent			
	aphidicolin *		control +		aphidicolin *		control +	
	mean	range	mean	range	mean	range	mean	range
10	6.2	(6-7)	5.7	(5-7)	3.4	(3-4)	3.8	(3-4)
11	7.2	(6-15)	6.8	(5-7)	7.2	(3-21)	3.3	(3-5)
12	8.0	(4-10)	8.2	(6-9)	16.1	(8-30)	4.1	(3-5)
13	9.0	(5-15)	14.0	(13-15)	12.6	(6-15)	5.8	(5-7)

* Ten embryos were injected with aphidicolin and timed accurately through cycle 10, 13 were observed for cycle 11 and 12, and 9 for cycle 13.

\+ Eight embryos were injected with buffer and observed through cycles 10 to 14.

The changes in budding were measured by eye, and are therefore subject to an error of approximately ± 1 minute.

3.7 DISCUSSION

The results presented in this chapter demonstrate that there is a cell cycle oscillator in the early Drosophila embryo that can drive both cytoplasmic and nuclear aspects of the cell cycle when DNA synthesis has been inhibited.

The Cytoplasmic Cell Cycle

It has previously been reported that embryos laid by Drosophila females homozygous for the mutation gnu develop a small number of giant nuclei (Freeman et al., 1986; Freeman and Glover 1987). The centrosomes of these embryos continue to divide many times, however, demonstrating that centrosome replication and nuclear division can be uncoupled. By injecting aphidicolin into wild type Drosophila embryos, I have now demonstrated that centrosomes can proceed through multiple rounds of division in the absence of DNA replication. Similar experiments examining centrosome replication in aphidicolin-treated sea urchin embryos have yielded conflicting results. Two groups reported that centrosome division either did not occur or was very rare (Nishioka et al., 1984; Brachet and De Petrocellis, 1981), while another found that centrosomes did divide, but the timing of the divisions was significantly slowed (Sluder and Lewis, 1987). My findings are consistent with the demonstrations that centrosome division continues when DNA replication is inhibited in both sea urchin (Sluder and

Lewis, 1987) and starfish embryos (Nagano et al., 1981).

Cortical budding cycles also continue in aphidicolin-injected Drosophila embryos. As in untreated embryos, the cortical budding occurs in waves, usually spreading from both poles. The number of buds at the cortex of the treated embryos roughly doubles with each new budding cycle, even though the number of nuclei do not increase. This suggests that some cytoplasmic component, not associated with the nucleus, is doubling every cell cycle, and this component is capable of organising the cortical buds. An obvious candidate for this component is the centrosome, and in the next chapter I go on to show that these buds are indeed organised by the centrosome.

The finding that the cytoplasmic cycles of centrosome replication and cortical budding can continue in aphidicolin-treated Drosophila embryos demonstrates that this embryo behaves like many other early embryonic systems, and cytoplasmic aspects of the cell cycle can be uncoupled from DNA synthesis. These findings suggest that in somatic cells, where inhibiting DNA synthesis blocks centrosome replication, this block is not due to an inability of centrosomes to divide in the presence of an unreplicated nucleus; rather, there must be a specific control mechanism that prevents centrosomes dividing while DNA synthesis remains incomplete.

The Nuclear Cycle

In aphidicolin-injected embryos fixed when cortical buds are apparent at the surface, the majority of nuclei are in an interphase-like state with decondensed chromatin surrounded by a nuclear envelope, as judged by the bright annular staining with an anti-lamin antibody. In treated embryos fixed when the cortical budding has receded, the majority of the chromatin is condensed and not enveloped by lamins. Treated embryos fixed during the progression of the budding-waves have nuclear structures consistent with the spreading of a mitotic state through the embryo. Thus, cycles of cortical budding seem to occur in synchrony with cycles of chromatin condensation and lamin disassembly, suggesting that the nuclear cycle continues in aphidicolin-treated embryos.

It has recently been demonstrated that cycles of MPF activity can occur in activated *Xenopus* oocytes devoid of any nuclear components (Dabauvalle et al., 1988), suggesting that the signals that drive the nuclear cycle (which are presumably dependent on MPF) are produced in the cytoplasm and oscillate in the absence of a nucleus. The results presented in this chapter suggest that these signals continue to cycle in the presence of an unreplicated nucleus in aphidicolin-treated *Drosophila* embryos, and unreplicated nuclei are able to respond to these signals. This is in contrast to the situation reported in aphidicolin-treated sea urchin embryos. Here, nuclear envelope breakdown is variable from embryo to

embryo, and in some cases it never occurs, even though the centrosomes proceed through multiple rounds of replication (Sluder and Lewis 1987). In embryos where the nuclear envelopes do break down, the chromatin can be seen as an amorphous mass of fibres; it does not condense properly, and nuclear envelopes do not reform. Thus, in aphidicolin-treated sea urchin embryos, nuclei in which DNA synthesis has been inhibited may be unable to respond to oscillating cytoplasmic signals. Alternatively, a subset of cytoplasmic signals that drive the nuclear cycle might not be produced in these embryos while other aspects of the cycle continue. It is not clear why Drosophila and sea urchin embryos should differ in this respect, but it is possible that some aspect of embryo organisation imposes an additional set of constraints upon the mitotic cycle in sea urchin embryos.

The Delay in the Cell Cycle

In common with aphidicolin-treated sea urchin, Xenopus, and starfish embryos there is a considerable delay in the cell cycle of aphidicolin-treated Drosophila embryos. The reason for the delay is not known. Although aphidicolin is a specific inhibitor of DNA polymerase-α (Ikegami et al., 1978), it could have non-specific effects that slow the cell cycle. Another possibility is that the cell-cycle oscillator might be coupled to DNA replication, but this coupling can be overridden after a certain delay. Thus, the inhibition of DNA replication would only cause a delay in the cell cycle

rather than a complete block. In its simplest form, this model would predict that the cell cycle would be delayed in S phase while the oscillator waited for some DNA-synthesis-dependent signal. My results, however, suggest that the delay in the cell cycle occurs mainly in M phase. Perhaps the most likely explanation is that the disorganised state of the aphidicolin-treated embryo in some way delays the cell cycle. There is some evidence that in sea urchin embryos the spatial organisation of tubulin has a role in the timing of mitotic events. Under conditions where tubulin polymerisation is inhibited (Sluder, 1979), or the mitotic spindle is rearranged (Sluder and Begg, 1983), the embryo spends much longer in M phase while the S phase of the cell cycle is unaffected. In the aphidicolin-injected Drosophila embryo the presence of extra centrosomes could affect tubulin kinetics. This, together with the presence of unreplicated nuclei that cannot be separated by mitotic spindles, could cause a delay in M phase.

CHAPTER 4

Centrosomes, and not Nuclei, Initiate Pole Cell Formation in Drosophila Embryos

4.1 INTRODUCTION

In the preceding chapter, I showed that an injection of aphidicolin into the early Drosophila embryo effectively inhibits DNA synthesis and, as a consequence, nuclear replication. Under these conditions, many aspects of the cell cycle continue; in particular, centrosomes continue to replicate and become dissociated from the non-replicating nuclei. This finding prompted me to use aphidicolin as a tool to investigate the relative contribution of the centrosome and the nucleus in the processes of nuclear migration and cortical cytoskeleton re-organisation.

In the Drosophila embryo, the first 8 rounds of mitosis occur within the interior of the embryo. Then, at telophase of nuclear cycle 8, the majority of nuclei co-ordinately start to migrate to the embryo cortex (Foe and Alberts, 1983). Early in cycle 9, the nuclei destined to form the germ cells reach the posterior pole of the embryo, where they form characteristic protrusions termed pole buds; after 2 further rounds of mitosis the pole buds pinch off to form separate pole cells (Warn et al., 1985). The somatic nuclei reach the surface of the embryo early in interphase of cycle 10, following which there is a dramatic re-arrangement of the cortical cytoskeleton around the nuclei (Karr and Alberts, 1986; Warn et al., 1984, 1987; Kellog et al., 1988). These nuclei undergo a further 4 rounds of mitosis before

cellularisation occurs at cycle 14.

It has been demonstrated previously that nuclear migration in Drosophila embryos is blocked by drugs that inhibit microtubule assembly (Zalokar and Erk, 1976), but it is not clear whether the microtubule-dependent transport apparatus acts on the nucleus, the centrosome, or both. It is also unclear what the relative importance of the centrosome and the nucleus is in re-organising the cortical cytoskeleton following their migration to the surface of the embryo. In this chapter, I show that an injection of aphidicolin before the nuclei reach the surface of the embryo effectively inhibits nuclear migration. Centrosomes, however, can continue to migrate to the cortex in a co-ordinated fashion. Once at the surface, the centrosomes initiate many aspects of the re-organisation of the cortical cytoskeleton, including pole cell formation, in the absence of nuclei. These results suggest that the centrosome, rather than the nucleus, normally plays the primary role in these developmental processes.

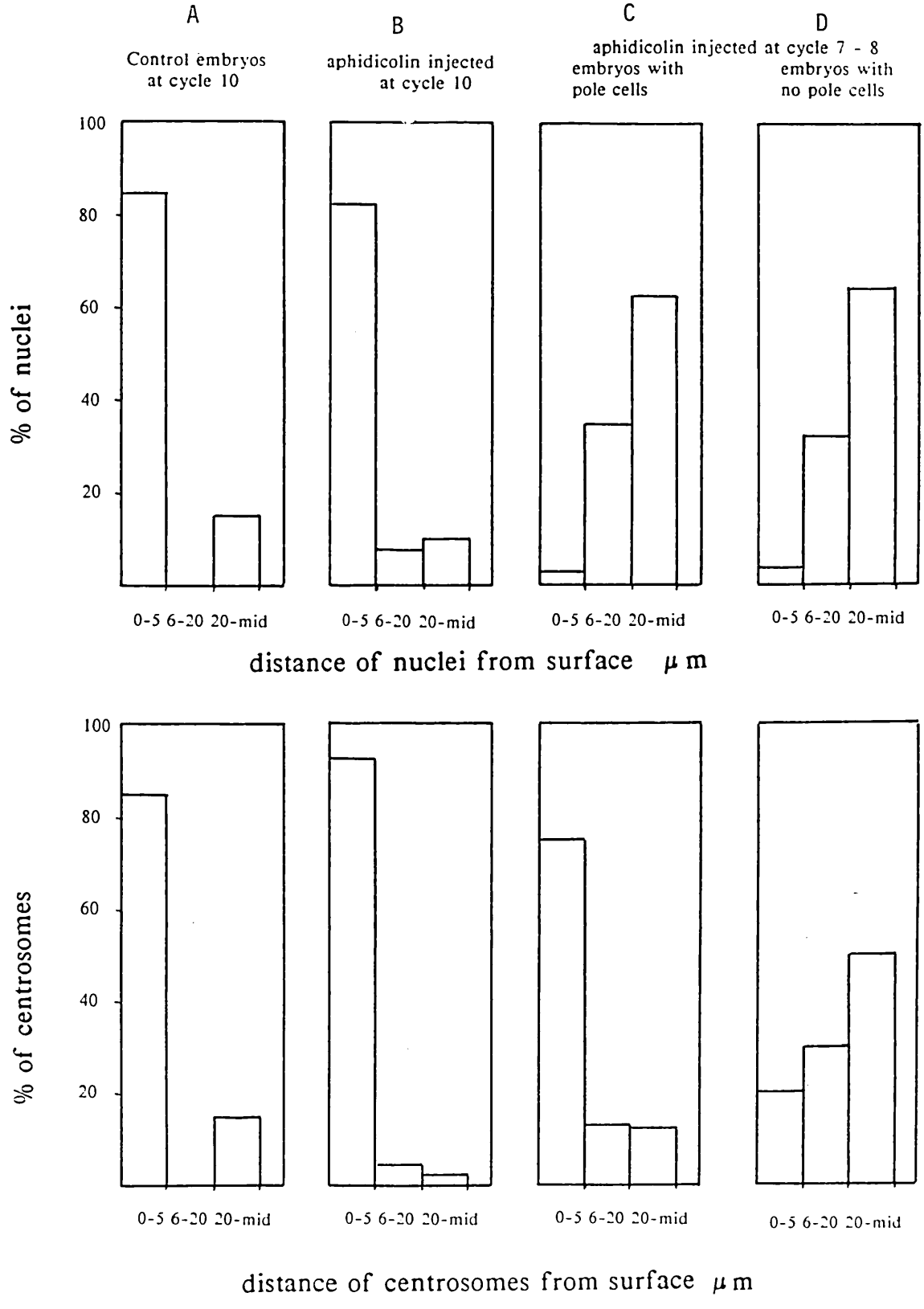
4.2 Aphidicolin Blocks Nuclear but not Centrosome Migration

To test whether nuclear migration was blocked by inhibiting DNA synthesis and nuclear replication, I injected aphidicolin into embryos just before pole buds were due to form. Embryos were collected for 20 minutes and allowed to develop for 60 minutes (24^{o}C) before aphidicolin was injected into the middle of the embryo. In this way the injection was given between nuclear cycles 7-8, approximately 0-20 minutes before the nuclei at the posterior pole of the embryo would have reached the surface and initiated pole bud formation. These embryos were observed under the light microscope for 40 minutes before they were fixed and stained to visualise the nuclei and centrosomes.

The distribution of nuclei and centrosomes in control and aphidicolin-injected embryos is shown in Figure 4.1. In control embryos injected with buffer alone at nuclear cycle 7-8 and allowed to develop until nuclear cycle 10-11 before fixation (Figure 4.1a), the nuclei and centrosomes remained associated, and approximately 85% of them reached the surface of the embryo (the nuclei that remain in the middle of the embryo eventually drop out of cycle with the rest of the nuclei and form the polyploid yolk nuclei). In embryos injected with aphidicolin at nuclear cycle 10 (when the nuclei were already at the surface) and allowed to develop

Figure 4.1

The Distribution of Nuclei and Centrosomes in Aphidicolin Injected Embryos. (A) Control embryos injected with buffer at nuclear cycle 7-8 and allowed to develop until cycle 10-11. (B) Embryos injected with aphidicolin at cycle 10 and allowed to develop for 40 minutes (C) Embryos injected with aphidicolin at cycle 7-8 that formed pole buds during the 40 minutes they were allowed to develop. (D) Embryos injected with aphidicolin at cycle 7-8 that did not form pole buds. The distance from the embryo surface was calculated by calibrating the focusing ratchet on the microscope so that the number of turns corresponded to a known distance. The number of centrosomes and nuclei in each focal section of the embryo were then counted as the microscope was focused from the surface to the interior of the embryo, in a small area halfway between the two poles. Note that aphidicolin blocks nuclear migration in all embryos that are injected 0-20 minutes before pole cells are due to form (see text), but centrosome migration apparently continues in embryos that initiate pole cell formation. A total of 10 embryos were counted in (A) and (B) and 15 in both (C) and (D).

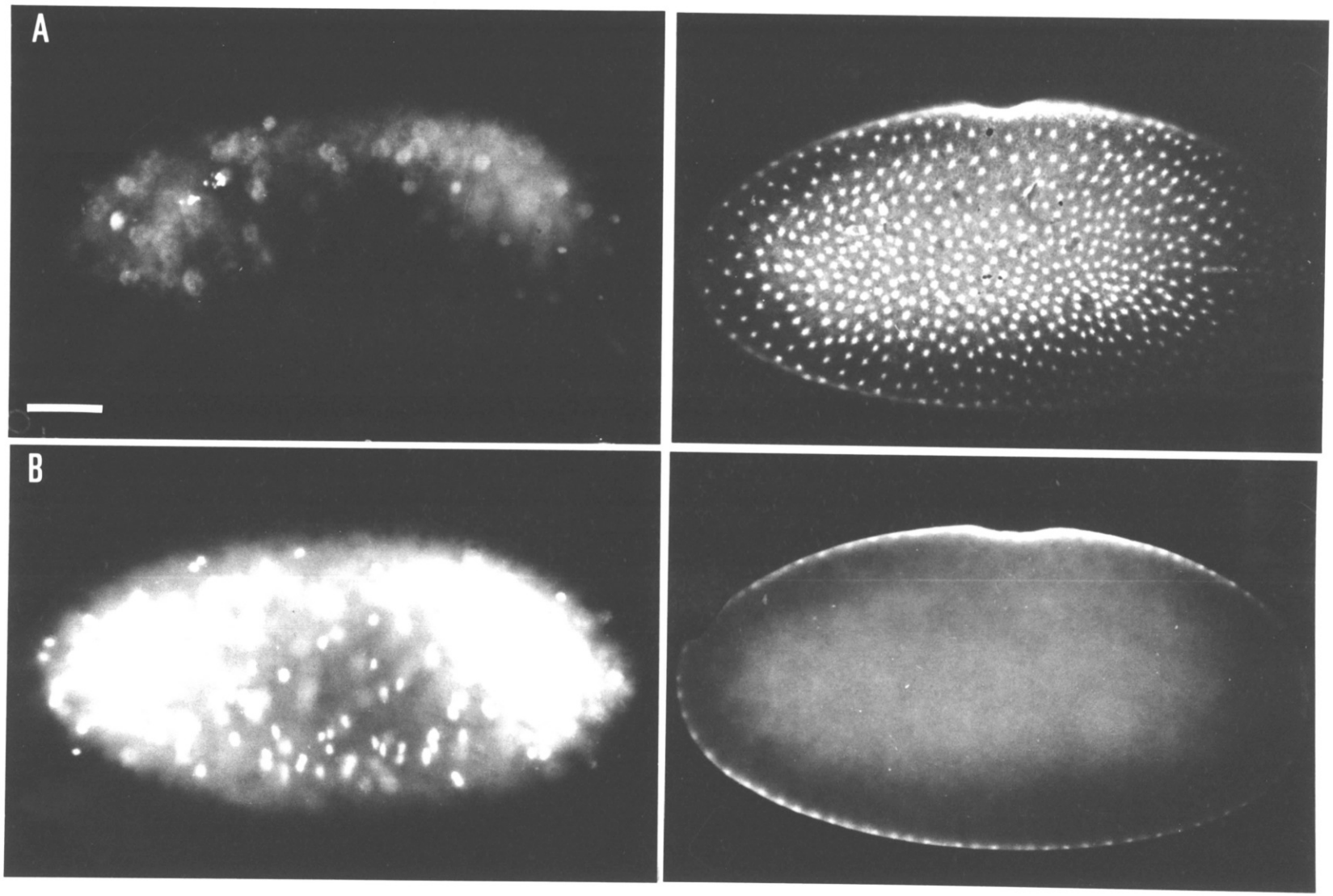

A
Control embryos at cycle 10
B
aphidicolin injected at cycle 10
C
aphidicolin injected at cycle 7 - 8
embryos with pole cells
D
embryos with no pole cells
% of nuclei
100
80
60
40
20
0-5 6-20 20-mid
distance of nuclei from surface μm
% of centrosomes
distance of centrosomes from surface μm

for 40 minutes before fixation (Figure 4.1b), the nuclei and centrosomes were dissociated, but their distribution was similar to that of the buffer injected controls. Thus, aphidicolin did not interfere with the processes that keep nuclei and centrosomes at the surface of the embryo over the time course of these experiments. The embryos injected with aphidicolin at nuclear cycles 7-8 fell into 2 classes, 64% (n=47) initiated pole cell formation during the 40 minute observation period (Figure 4.1c) and 36% did not (Figure 4.1d). When these embryos were fixed, however, I found that in both classes only 2-5% of nuclei had migrated to the surface of the embryo. Thus, injecting aphidicolin before pole bud formation blocked the migration of the somatic nuclei to the surface.

While the distribution of nuclei was similar in embryos that initiated pole cell formation and those that did not, the distribution of centrosomes was very different in these two classes of embryo. In embryos that formed pole cells, the distribution of centrosomes was similar to that found in the buffer injected controls. One such embryo is shown in Figure 4.2. Focusing on the surface of the embryo (Figure 4.2a) and in the middle of the embryo (Figure 4.2b), it was apparent that most of the centrosomes, but almost none of the nuclei, were at the surface. In contrast, in those embryos that did not form pole cells only about 20% of their centrosomes were at the surface, and no centrosomes were found within 2-3μm of the posterior pole.

Figure 4.2

The Distribution of Nuclei and Centrosomes in an Embryo that Formed Pole Cells. This embryo was injected with aphidicolin at nuclear cycle 7-8 and observed for 40 minutes under the light microscope, during which time it formed pole cells. The embryo was then fixed and stained with YL1/2 to reveal tubulin and the DNA binding fluorochrome Hoechst 33258 to reveal nuclei (see Materials and Methods). (A) Focusing at the surface of the embryo and (B) focusing in the interior of the embryo, one can see that centrosomes have migrated to the surface, but nuclei have not. Bar=50μm



DNA

Tubulin

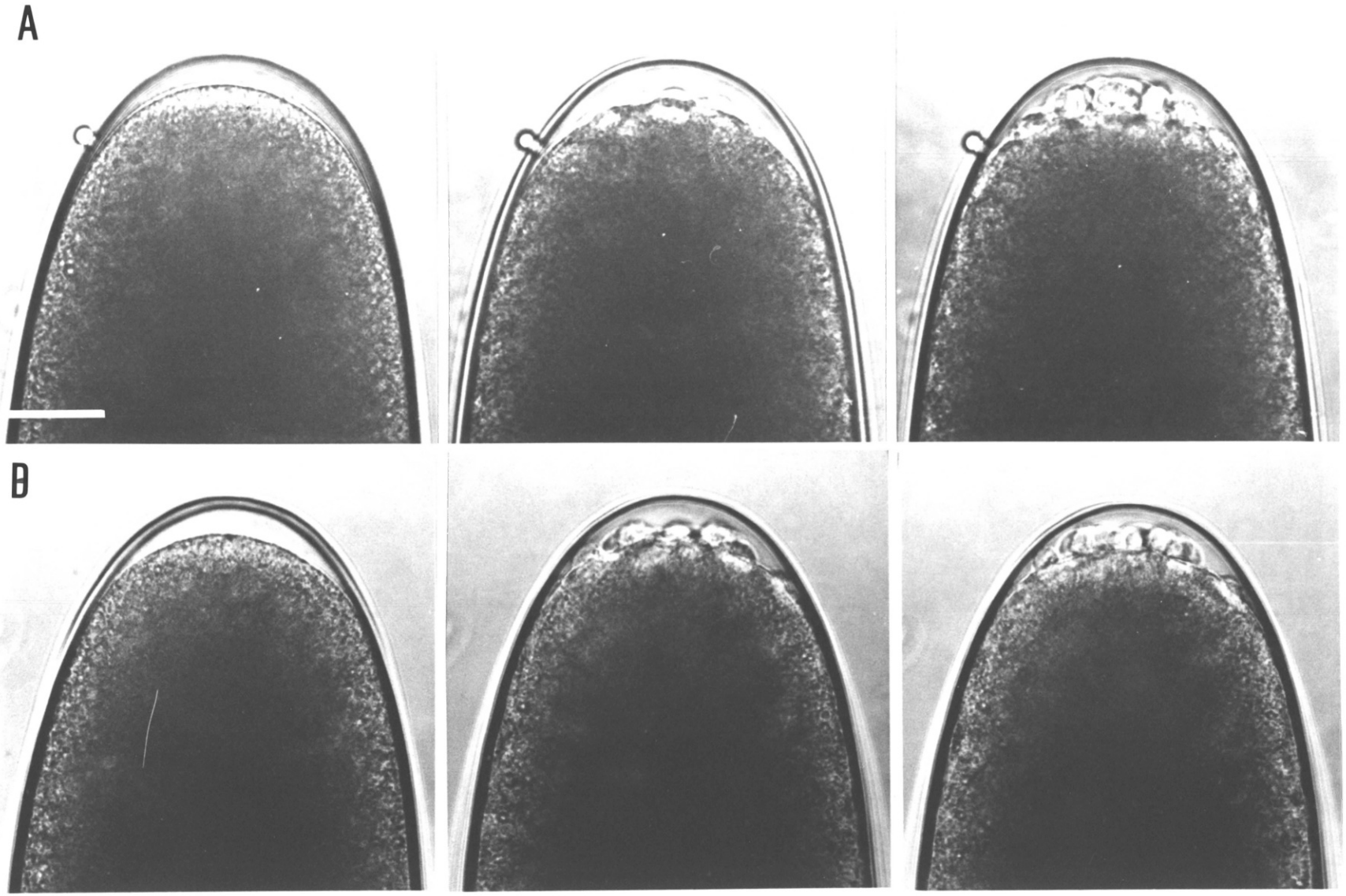
4.3 Pole Cells without Nuclei are Formed around Centrosomes in Aphidicolin-Injected Embryos

Since the formation of pole cells has generally been assumed to be a consequence of the interactions of nuclei with the posterior pole plasm, I examined the formation of pole cells in aphidicolin-injected embryos in more detail. In embryos that were collected for 20 minutes and aged for 60 minutes before injection (as described above), 64% (n=47) initiated pole cell formation within 30 minutes of injection; no embryos initiated pole cell formation after this time. Figure 4.3 illustrates the process of pole cell formation in a buffer-injected and aphidicolin-injected embryo. The process is almost indistinguishable between the 2 types of embryo, and in both cases the pole buds appear to divide one or two times before the buds pinch off to form pole cells. When aphidicolin-injected embryos that had formed pole cells were fixed and stained, I found that in all cases the pole cells contained only a single or doubled centrosome and usually no nuclei (Figure 4.4). In embryos where pole buds formed within 5 minutes of the aphidicolin injection, a few of the resulting pole cells sometimes contained nuclei, although the majority did not. If pole buds were formed more than 5 minutes after aphidicolin injection then the resulting pole cells only very rarely contained a nucleus.

4.4 Centrosome Migration and Pole Cell Formation are Inhibited by Colchicine

Figure 4.3

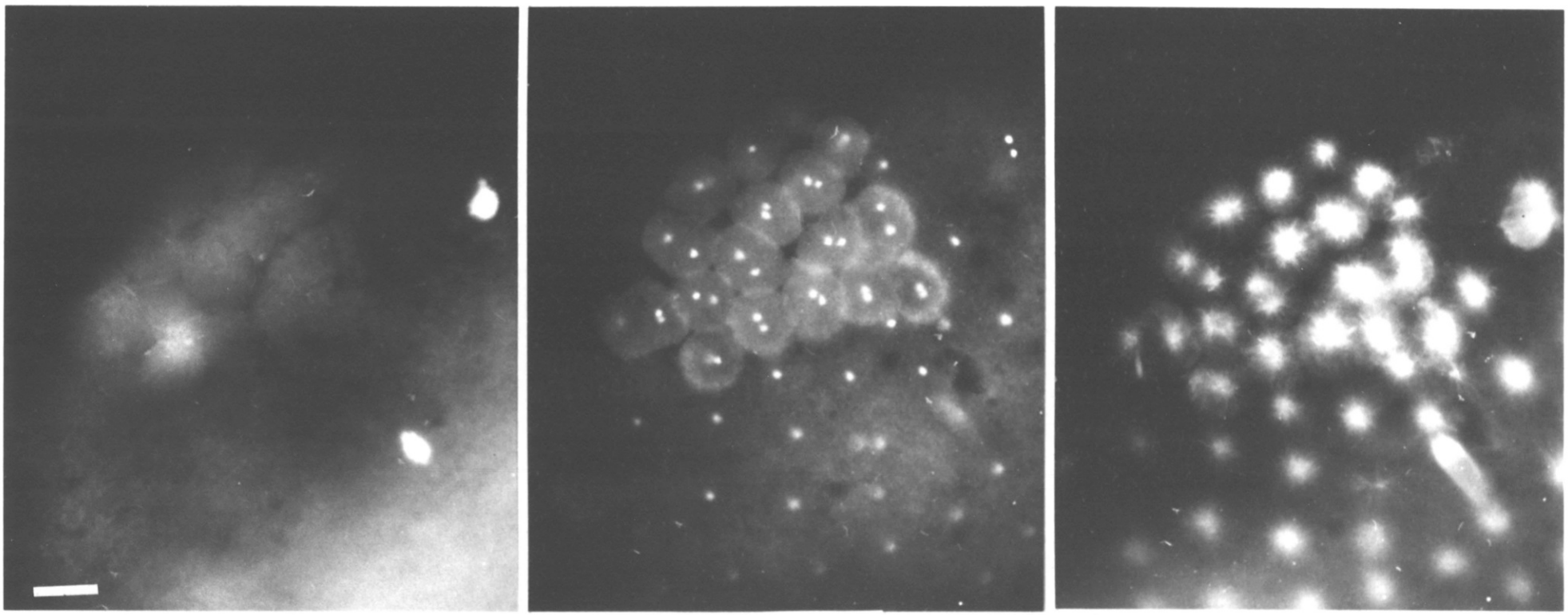
Pole Cell Formation in Buffer-Injected and Aphidicolin-Injected Embryos. The embryos were injected with buffer (A) or aphidicolin (B) at nuclear cycle 7-8 and observed under the light microscope for 40 minutes. Both embryos were photographed at the time of injection, 13 minutes after injection, and 40 minutes after injection (left to right). Bar=25μm


A
B

The accumulation and regular distribution of centrosomes at the surface of aphidicolin-treated embryos suggests that centrosomes can move to the cortex in a co-ordinated manner even when the nuclei do not. In an attempt to exclude the possibility that the accumulation of centrosomes at the surface of such embryos reflected a passive process of random centrosome movement, I co-injected aphidicolin together with colchicine, an inhibitor of microtubule polymerisation. Zalokar and Erk (1976) have reported that the movement of nuclei to the surface in Drosophila embryos is colchicine-sensitive. I found that when colchicine was injected at nuclear cycle 7-8, either on its own or in combination with aphidicolin, it completely inhibited both nuclear and centrosome migration and pole cell formation (not shown). Thus, the co-ordinated movement of centrosomes that I observe in aphidicolin injected embryos would appear to be an active process mediated by microtubules. Also, pole cell formation is not initiated when both nuclei and centrosomes are inhibited from migrating to the posterior pole. One must be cautious in interpreting results obtained with colchicine, however, since it can have wide ranging effects on the developing embryo (see discussion). An injection of cytocholasin-D, an inhibitor of actin filament polymerisation, did not block nuclear or centrosome migration, although the movement was somewhat patchy and irregular (not shown). An irregular pattern of migration has been observed previously in cytocholasin treated Drosophila embryos, and it may be a consequence of the uneven

Figure 4.4

Centrosomes Organise Pole Cells. The posterior pole of an embryo injected with aphidicolin at nuclear cycle 7-8, and observed for 40 minutes before fixation. During the observation period, the embryo initiated pole cell formation. After fixation, the embryo was stained to reveal the distribution of centrosomes, tubulin, and DNA. In this embryo, the anucleate cells appear to have just divided, and each cell contains two closely paired centrosomes. Bar=5μm.



DNA Centrosome Tubulin

distribution of nuclei in such embryos prior to nuclear migration (Zalokar and Erk, 1976; my own unpublished observations).

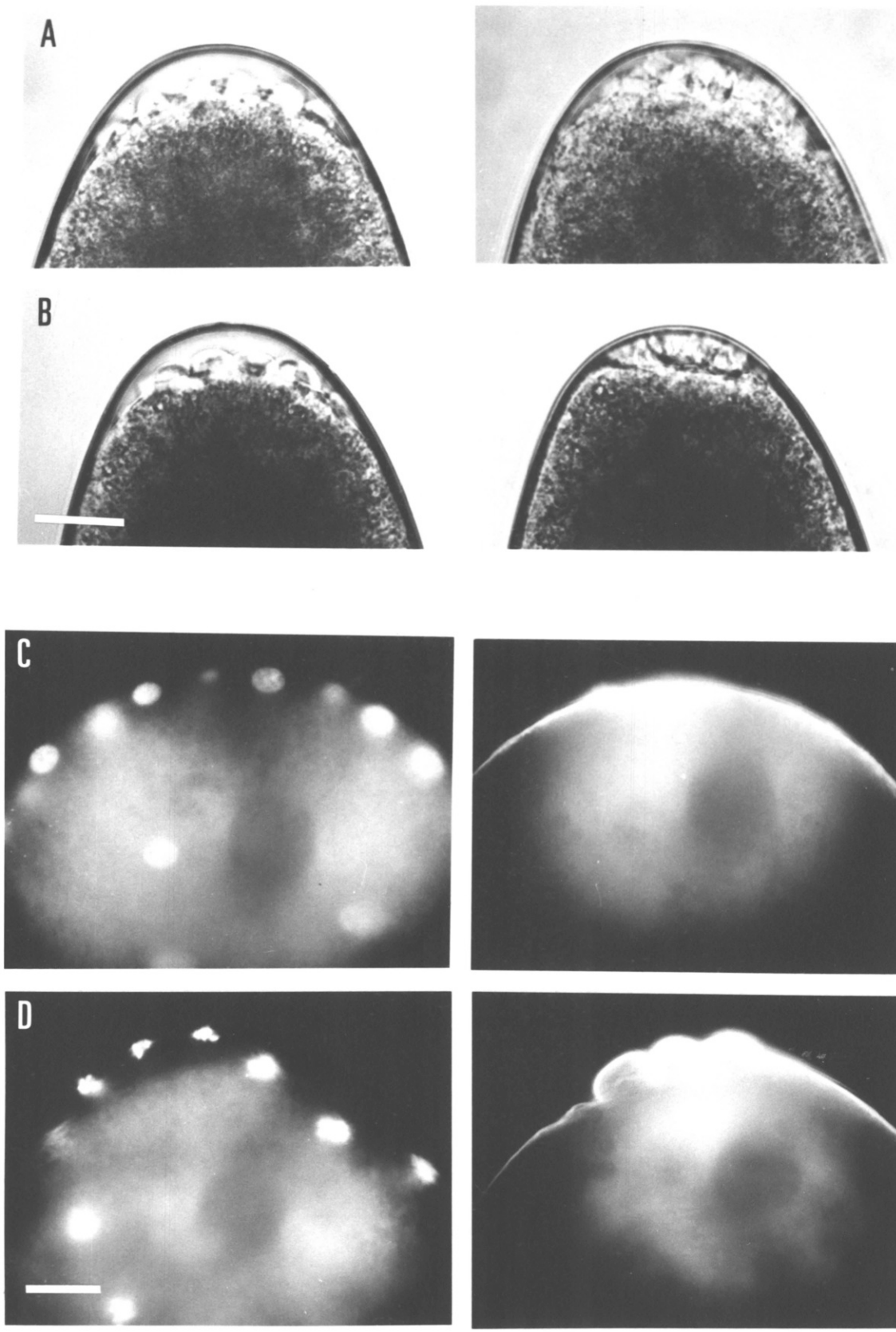
Although cytocholasin-D did not block nuclear or centrosome migration, it completely inhibited pole cell formation, even though nuclei and centrosomes were present at the posterior pole. Even if the drug was injected after pole buds had formed, the buds became disorganised and collapsed within 5-10 minutes (Figure 4.5a,c). Colchicine, on the other hand, had a less dramatic effect on pole buds if it was injected just after they had started to form. Although the pole buds did not proceed through any further rounds of division and no other nuclei reached the cortex of the embryo, demonstrating that the drug had reached the posterior pole, the pole buds were quite stable and hints of pole buds (not cells) were still visible 40 minutes after colchicine injection (Figure 4.5b,d). Thus, although it appears that centrosomes are required to initiate pole bud formation, the process is dependent on actin filaments, as might be expected from previous studies on the distribution of actin during pole cell formation (Warn et al., 1985).

4.5 Centrosomes Organise Cortical Actin and the Overlying Plasma Membrane

In the previous chapter, I showed that cycles of cortical budding that normally occur as the nuclei at the surface of

Figure 4.5

The Effects of Colchicine and Cytochqlasin-D on Pole Bud Formation. Two embryos are shown in which cytochalasin-D (A,C) or colchicine (B,D) were injected as pole buds were being initiated. (A) Cytochalasin-D injected embryo. Left hand panel shows the embryo just before the drug was injected, right hand panel shows the same embryo 10 minutes later; the pole buds have almost completely collapsed. This embryo was fixed at this point and stained to observe the nuclei (C - left hand panel) and actin (C - right hand panel); this confirms that there is no trace of any pole buds. (B) Colchicine injected embryo. Left hand panel shows the embryo just before colchicine injection, right hand panel shows the same embryo 40 minutes later; hints of pole buds are still apparent. This embryo was fixed at this point and stained to reveal nuclei (D - left hand panel) and actin (D - right hand panel); this confirmed that hints of pole buds were still present. Bar(A,B)=25μm, Bar(C,D)=15μm.

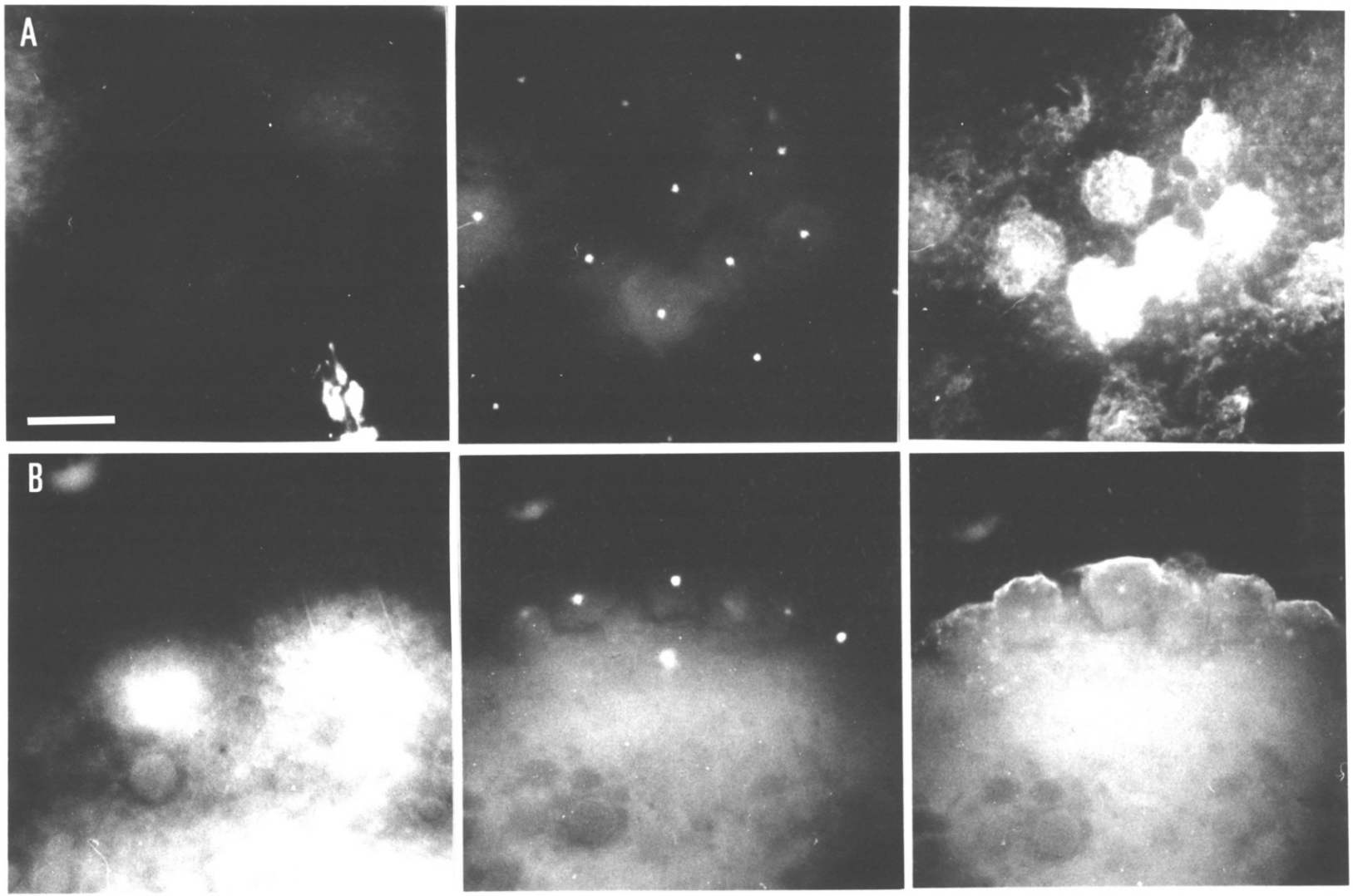

A
B
C
D

the Drosophila embryo enter and leave mitosis continue in embryos treated with aphidicolin at nuclear cycle 9-10. In such embryos the number of cortical buds appeared to double at successive nuclear cycles, even though the number of nuclei did not increase, and I suggested that the centrosome may be organising these budding cycles. In the present experiments, when aphidicolin was injected at nuclear cycle 7-8, cortical budding was usually very weak, even in those embryos that initiated pole cell formation. When these embryos were fixed and stained with an anti-actin antibody, however, I found that all of the centrosomes that reached the surface of the embryo were organising regions of actin (Figure 4.6). No regions of actin organisation were seen apart from those around centrosomes. In addition I repeated experiments in which aphidicolin was injected at nuclear cycle 9-10. The embryos were observed through a number of budding cycles and then fixed and stained with an anti-actin antibody. These experiments confirmed that the cortical budding cycles were associated with cycles of actin cap formation organised around each centrosome (not shown).

I also found that all of the centrosomes that reached the surface of embryos injected with aphidicolin at nuclear cycle 7-8 were organising regions of the plasma membrane detected with an anti-phosphotyrosine antibody (Wang, 1985). No regions of plasma membrane organisation were seen apart from those around centrosomes. Figure 4.7a shows a field of centrosomes, revealed by staining with an anti tubulin

Figure 4.6

Centrosomes can Organise Actin in the Absence of Nuclei. Both of the embryos shown were injected with aphidicolin and observed to form pole cells as described in the text. The embryos were then fixed and stained with Rb188 to reveal centrosomes and a mouse monoclonal antibody (Amersham International plc.) to reveal actin. (A) The surface of an embryo showing centrosomes organising actin caps. (B) A lateral view of centrosomes organising actin caps at the anterior pole. Bar=5μm

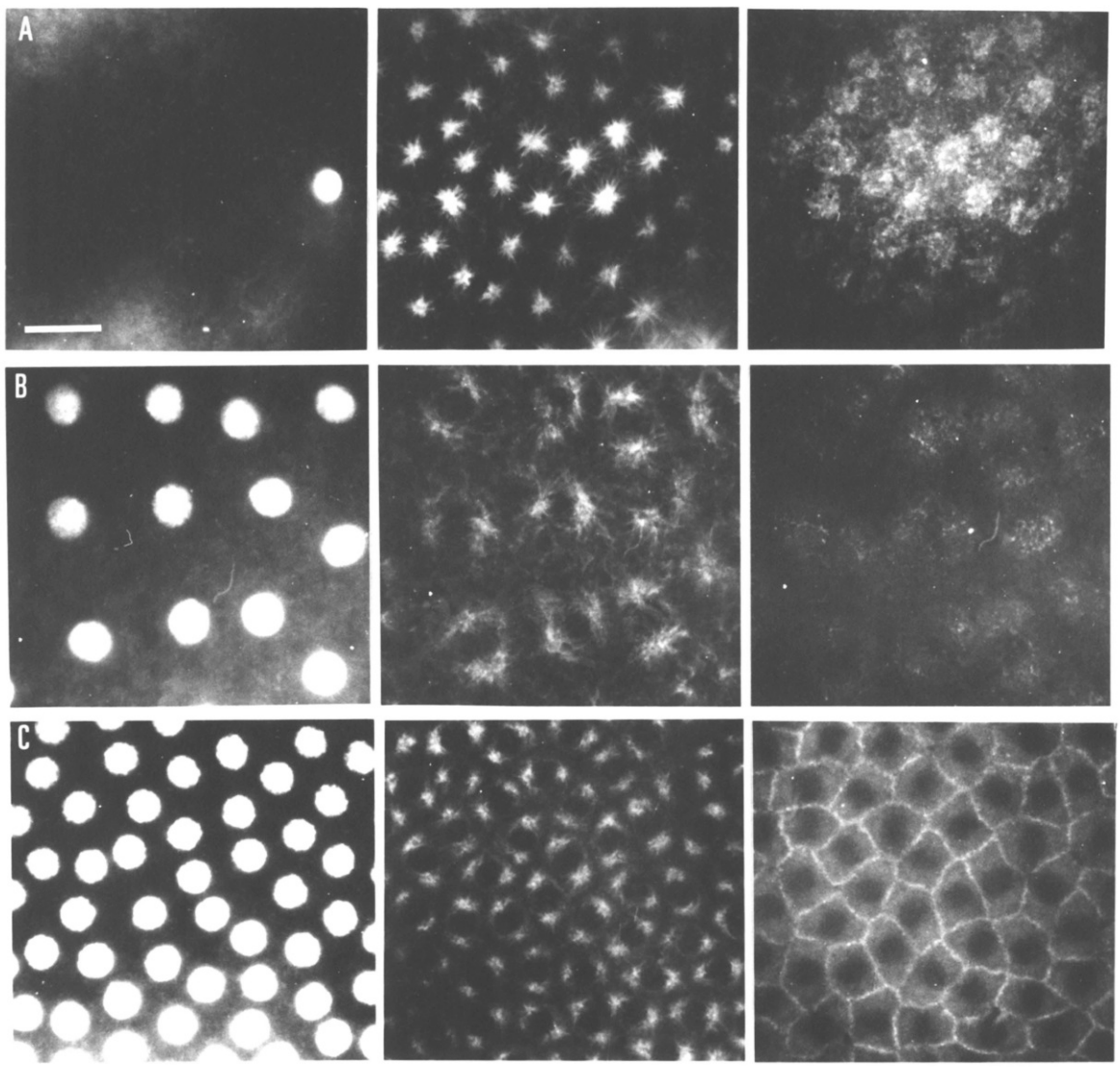

A
B
DNA
Centrosome
Actin

antibody, organising plasma membrane components. I see similar staining patterns above each of the nuclei as they reach the cortex of control embryos at nuclear cycle 10 (Figure 4.7b). The staining pattern with this antibody becomes more reticulate at successive nuclear cycles (Figure 4.7c) and the membranes can be seen to invaginate around the nuclei as cellularisation begins at nuclear cycle 14 (not shown, see Sullivan, 1987).

In aphidicolin-injected embryos, no cells other than those at the posterior pole formed around centrosomes at the cortex, even when the embryos were allowed to develop for much longer than is normally required for somatic cellularisation to occur.

Figure 4.7

Centrosomes can organise plasma membrane components in the absence of nuclei. Embryos were fixed and stained with YL1/2 to reveal tubulin and an anti-phosphotyrosine antibody (Wang, 1986) that recognises plasma membrane components in Drosophila (Sullivan, 1988). (A) A field from an aphidicolin-injected embryo that was observed to form pole cells. Every centrosome is organising a region of the plasma membrane in the absence of nuclei. (B) An un-injected embryo at nuclear cycle 10 when the nuclei have just reached the surface. Note how the plasma membrane appears to be concentrated around the astral regions. (C) An un-injected embryo at nuclear cycle 12. The plasma membrane staining has become more reticulate, forming a lattice around the nuclei. Bar=10μm


A
B
C
DNA
Tubulin
membrane

4.6 DISCUSSION

The results presented in this chapter provide strong indirect evidence that centrosomes play a crucial role in the co-ordinated movement of nuclei to the cortex of the developing Drosophila embryo. They also provide direct evidence that centrosomes can initiate the reorganisation of the cortical cytoskeleton and the overlying plasma membrane, including the formation of pole cells at the posterior pole of the embryo, in the absence of nuclei.

Nuclear Migration

It has been known for many years that inhibitors of microtubule polymerisation interfere with nuclear migration in Drosophila embryos suggesting that this process is dependent on microtubules (Zalokar and Erk, 1976). A network of radial microtubules that stretches between the inner yolk mass and the cortex is believed to be responsible for the saltatory motion of small particles that can be seen with a light microscope in embryos between cycles 10 and 14. Although these microtubules could provide tracks for the nuclei to move along, saltatory motion along these tracks has thus far only been observed after the nuclei have reached the surface of the embryo (Foe and Alberts, 1983).

I have found that an injection of aphidicolin into Drosophila

embryos at nuclear cycle 7-8 dramatically inhibits nuclear migration. In many of these embryos, however, centrosomes continue to migrate to the surface in a co-ordinated fashion, raising the possibility that nuclear migration is normally mediated via forces acting on the centrosome associated with the nucleus rather than the nucleus itself. In support of this hypothesis, previous studies on the gall midge Wachtliella persicaniae have revealed that during the migration of nuclei in this embryo, microtubules extend from the centrosome to the embryo surface, and from the centrosome to the nucleus (Wolf, 1978). I found that the migration of both centrosomes and nuclei in aphidicolin-treated embryos was inhibited by colchicine, an inhibitor of microtubule assembly, but not by cytocholasin D, an inhibitor of actin filament assembly. This is consistent with the view that centrosome migration is dependent on microtubules and not on actin filaments. Since colchicine dramatically slows the cell cycle in Drosophila (my unpublished observations) and sea urchin (Sluder, 1979) embryos, one must be cautious in interpreting results obtained with the drug.

Nuclear migration occurs almost exclusively at the telophase stage of mitosis in Drosophila embryos (Foe and Alberts, 1983). In the previous chapter, I have shown that inhibiting DNA synthesis with aphidicolin indirectly inhibits the progress of the nucleus to the telophase stage of mitosis, as the un-replicated DNA cannot be separated by the mitotic spindle. This presumably leads to the dissociation of the

centrosomes and nuclei during anaphase B. Thus, aphidicolin may block nuclear migration by causing the dissociation of the nuclei from the centrosomes; the nuclei are then left behind as the centrosomes migrate to the surface. In support of this explanation, I found that embryos that initiate pole bud formation within 0-5 minutes after aphidicolin injection often have nuclei present in some of their pole cells, whereas embryos that initiate pole bud formation after 5 minutes very rarely have nuclei in their pole cells. Apparently, if DNA synthesis in nuclear cycle 8 is nearly complete (this would be expected at approximately 3-4 minutes before pole bud initiation) then the nuclei destined to form the pole cells can complete that round of mitosis and thus migrate to the surface with their centrosome.

Reorganisation of the Cortical Cytoskeleton and Pole Cell Formation

In order to address the question of the relative importance of the nucleus and the centrosome in reorganising the cortical cytoskeleton of the *Drosophila* embryo, I took advantage of the fact that these organelles dissociate from each other in aphidicolin-treated embryos. I observed that the centrosomes that reach the posterior pole of such embryos are able to initiate pole cell formation in the absence of nuclei. When, however, both nuclear and centrosome migration were blocked with the drug colchicine, pole cell formation was completely inhibited. I also observed that in the 36% of

aphidicolin-injected embryos that did not initiate pole cell formation, centrosomes were not found within 2-3μm of the posterior pole. Thus, pole cell formation is apparently only initiated if centrosomes are present at the posterior pole. The formation of pole cells, although apparently organised by centrosomes, is dependent on actin filaments, since cytocholasin-D completely inhibited pole cell formation even if injected after pole buds had formed. It seems, therefore, that the centrosome must be able to interact with actin filaments during this process. Moreover, I found that all of the centrosomes that reached the cortex were able to organise actin and the overlying plasma membrane. Presumably, this organising ability of the centrosome is mediated via the microtubules it nucleates (McIntosh, 1983).

The behaviour of the cortical cytoskeleton, during both pole cell formation and the four rounds of mitosis that precede cellularisation, has been well characterised (e.g. Warn et al., 1985; Warn and Magrath, 1983; Karr and Alberts, 1986; Warn et al., 1987; Kellog et al., 1988). Many of these authors have noted that the centrosome appears to play a role in organising actin filaments at the cortex. For example, the actin cap organised above each nucleus is densest over the centrosome (Karr and Alberts, 1986), and microtubules have been implicated in the formation and maintenance of actin caps (Warn et al., 1987). The results presented in this chapter provide the first direct evidence of the crucial role of the centrosome in organising pole cell formation,

cortical actin, and the overlying plasma membrane in Drosophila embryos.

Several previous studies have suggested that centrosomes can autonomously organise the cytoskeleton in early embryonic systems. In 1925, for example, Wilson details a number of reports describing asters organising cleavage furrows in embryonic systems. Similarly, an aster was found inside each anucleate cell in aphidicolin-treated starfish embryos that were observed to cleave abnormally (Nagano et al., 1981). Most convincingly, parthenogenetically activated Xenopus embryos were found to undergo cleavage only if exogenous centrosomes were injected into the embryos (Maller et al., 1976; Bornens et al., 1987). These observations, together with the results presented here, strongly argue that the centrosome plays a crucial role in orchestrating many of the cytoskeleton-dependent events in early embryos.

Although centrosomes are able to direct the formation of pole cells in the absence of nuclei, cellularisation did not occur around the centrosomes in the rest of the embryo cortex, even when the embryos were allowed to develop far beyond the time when cellularisation would normally occur. A possible explanation for this finding is that aphidicolin-injected embryos might never attain some critical nuclear:cytoplasmic ratio required to trigger cellularisation. Previous studies have suggested that the initiation of cellularisation in Drosophila embryos requires the attainment of a critical

nuclear:cytoplasmic ratio (Edgar et al., 1986). In both Drosophila (Edgar and Schubiger, 1986; Edgar et al., 1986) and Xenopus embryos (Newport and Kirschner, 1982a; Newport and Kirschner, 1982b; Kimelman et al., 1987), an increase in the nuclear:cytoplasmic ratio also seems to trigger the lengthening of the cell cycle that occurs when maternal products are depleted and zygotic transcription is required for development to proceed.

How might the arrival of the centrosomes at the posterior pole initiate pole cell formation? In the Drosophila embryo, it has been shown that determinants localised in the posterior pole plasm direct the nuclei that reach this region to participate in pole cell formation (Okada et al., 1974; Illmensee and Mahowald, 1974; Illmensee and Mahowald, 1976). Although the molecular nature of these determinants is unknown, structures called polar granules can be seen in the posterior pole plasm (Counce, 1963; Mahowald, 1962; Mahowald, 1968). The polar granules are composed of RNA and protein (Mahowald, 1971) and they are absent in a number of mutants that fail to form pole cells. Several genes whose products are required for pole cell formation have been defined (Niki and Okada, 1981; Niki, 1984; Boswell and Mahowald, 1985; Lehmman and Nusslein-Volhard, 1986) and one of these genes has recently been cloned (Lasko and Ashburner, 1988; Hay et al., 1988). My finding that centrosomes can initiate pole cell formation, raises the question of how an interaction between polar determinants and the centrosome (or the

microtubules it nucleates) might direct the formation of pole cells. This aspect of anucleate pole cell formation will be discussed in more detail in chapter 6.

CHAPTER 5

Two Distinct Mechanisms are Involved in Localising cyclin B mRNA within the Early Drosophila Embryo.

5.1 INTRODUCTION

As discussed in chapter 1, the cyclins play a major role in initiating the entry into mitosis. The Drosophila homologues of the cyclin A and B genes have recently been cloned (Whitfield et al., 1989). Hybridisation of antisense RNA probes to embryo sections revealed a striking pattern of cyclin B mRNA distribution. Initially, the mRNA was homogeneously distributed throughout the embryo. At some time prior to pole bud formation, the mRNA accumulated dramatically at the posterior pole, although high levels remained evenly distributed throughout the rest of the embryo. As the nuclei arrived at the cortex, the mRNA at the posterior pole became incorporated into pole cells (where levels remained high throughout embryonic development). At the same time, the distribution pattern of mRNA in the rest of the embryo changed, producing a concentration of the mRNA at the embryo cortex. Levels of mRNA at the cortex gradually decreased, and the mRNA completely disappeared from the somatic nuclei during cellularisation. Levels of the mRNA rose again as gastrulation started, and somatic cell divisions resumed.

In this chapter, I describe experiments designed to investigate the mechanisms that generate the accumulation of cyclin B mRNA both at the cortex and at the posterior pole. By injecting α-amanatin, an inhibitor of transcription, I have established that the changing pattern of cyclin B mRNA

distribution does not depend on de novo transcription: it is due entirely to a re-distribution of maternally supplied mRNA. I found that cyclin B mRNA is closely associated with nuclei during development, and co-migrates to the cortex with the migrating nuclei. Drugs such as colchicine or aphidicolin that indirectly block nuclear migration also block mRNA migration, suggesting that the cortical re-localisation of the mRNA depends on this co-migration with nuclei.

Surprisingly, I found that the posterior accumulation of cyclin B mRNA was present in even the earliest embryos, and in unfertilised eggs, suggesting that this localisation develops during oogenesis, not embryogenesis as reported previously (Whitfield et al., 1989). Polar granules, which are found exclusively in the posterior pole plasm, are obvious candidates for the activity responsible for localising the cyclin B mRNA to the posterior pole. These organelles become concentrated around the centrosomes arriving at the posterior pole (Rabbinowitz, 1941; Counce, 1963; Mahowald, 1968; Mahowald et al., 1976), and I found that the cyclin B mRNA localised at the posterior pole is also concentrated around centrosomes, raising the possibility that the mRNA is bound directly to polar granules. This possibility is supported by the finding that the posterior localisation of the cyclin B mRNA is disrupted in mutants that have no polar granules and fail to form pole cells. Together, these findings strongly suggest that the cyclin B

mRNA is localised to the posterior pole by an association with a component (or components) of polar granules.

5.2 The Pattern of Cyclin B mRNA Distribution does not Depend on Zygotic Transcription

In the early Drosophila embryo, there is very little, if any, transcription during the first 9-10 nuclear cycles. After this, transcription levels gradually increase at successive nuclear cycles (Edgar et al., 1986; Edgar and Schubiger, 1986), and at nuclear cycle 14, most maternal mRNAs are depleted and the embryo requires zygotic transcription to drive cellularisation and further embryonic development. Although embryos apparently develop normally in the absence of zygotic transcription until cycle 14 (Edgar et al., 1986; Wieschause and Sweeton, 1988; Merrill et al., 1988), there is evidence that at least one gene may be zygotically active as early as nuclear cycle 6 (Karr et al., 1989).

To test whether de novo transcription played any part in generating the pattern of cyclin B mRNA distribution, I injected the transcription inhibitor α-amanatin (400μg/ml) into early embryos. It has previously been shown that this concentration of α-amanatin effectively inhibits RNA polymerase II dependent transcription (Edgar et al., 1986). I found that this concentration of α-amanatin had little effect on the nuclear cycles of the early embryo. At nuclear cycle 14, however, cellularisation was completely inhibited, and the embryo rapidly became disorganised and necrotic.

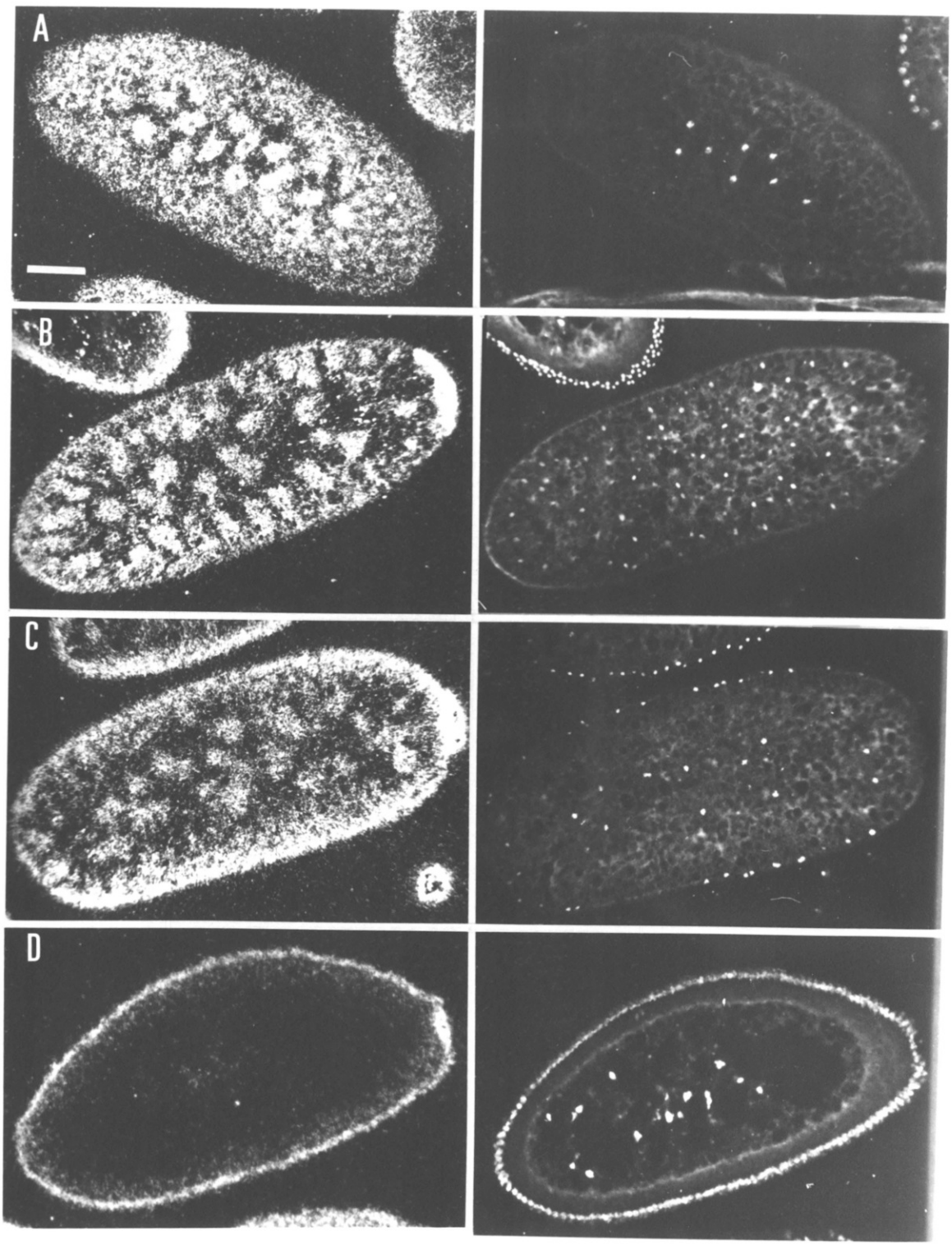
When α-amanatin-injected embryos were fixed, sectioned, and hybridised to cyclin B antisense RNA probes, the pattern of mRNA distribution was found to be the same as in untreated embryos (not shown). Thus, the spatial pattern of cyclin B mRNA distribution does not depend on zygotic transcription: it must be generated by a redistribution of maternally derived mRNA, possibly combined with the selective degradation of a subset of the maternal mRNA.

5.3 Cyclin B mRNA is Localised Around, and Co-Migrates with, Nuclei

To look more closely at the cortical accumulation of cyclin B mRNA, I repeated the hybridisation of embryo sections to antisense cyclin B RNA probes (Whitfield et al., 1989), but instead of staining these sections with Geimsa stain, I stained them with the DNA binding fluorochrome Hoechst 33258. I found that the cyclin B mRNA was accumulated around nuclei in the early embryo (Figure 5.1a), and, as the nuclei migrated to the cortex, they carried large amounts of the mRNA with them (Figure 5.1b). This resulted in the clearing of the mRNA from the middle of the embryo, producing the dramatic cortical localisation (Figure 5.1c,d) reported previously (Whitfield et al., 1989). The nuclei that did not migrate to the cortex (destined to become the yolk nuclei), initially retained the mRNA concentrated around them (Figure 5.1c). This accumulation becomes less obvious during cycles 11-12, when the nuclei have dropped out of the mitotic cycle

Figure 5.1

The Distribution of Cyclin B mRNA During Early Embryo Development. Sections of wild-type embryos were hybridised to an antisense RNA probe of cyclin B. Embryos were at nuclear cycle 5-6 (A), 8 (B), 10 (C), and early cycle 14 (D). The left hand panels are dark field images and the right hand panels Hoechst fluorescence. The mRNA is concentrated around nuclei (A,B), and becomes localised to the embryo cortex(C,D). In some parts of the sections there are regions of mRNA accumulation that are devoid of nuclei; this occurs because the mRNA is accumulated in a sphere around each nucleus, and so some sections of the embryo only contain the mRNA and not the actual nucleus around which the mRNA is accumulated (e.g. in (A), there are more regions of mRNA accumulation than there are nuclei). Scale bar=40μm.


A
B
C
D

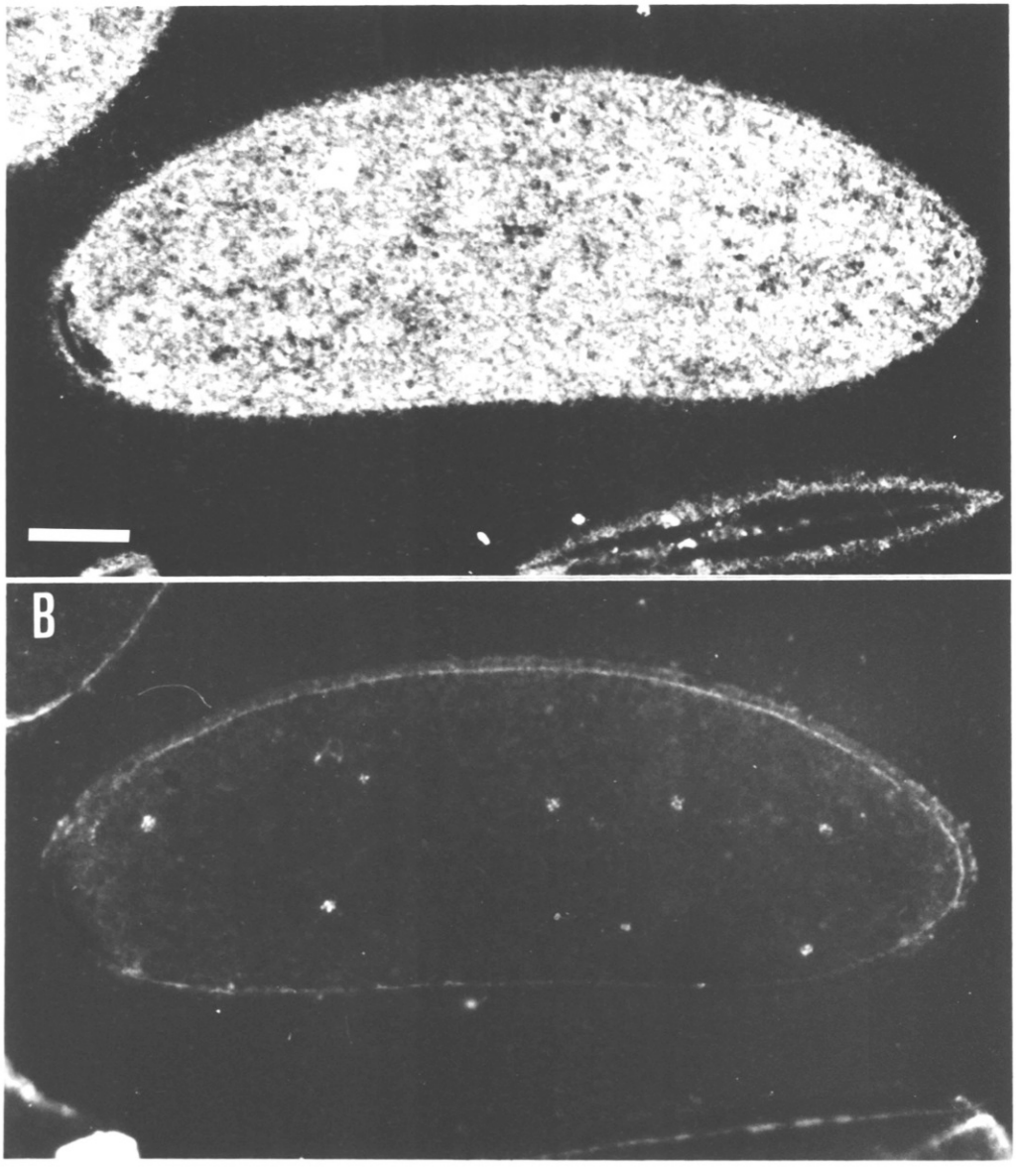
and are becoming polyploid (Foe and Alberts, 1983). By cycle 14, these nuclei had lost all traces of the mRNA (Figure 5.1d).

The cortical migration and the nuclear association of the cyclin B mRNA were inhibited by colchicine, an inhibitor of microtubule polymerisation (Figure 5.2), but not by cytochalasin D, an inhibitor of microfilament polymerisation (not shown). Since nuclear migration is also inhibited by colchicine (Zalokar and Erk, 1976; chapter 4 of this thesis), the block to mRNA migration could be due to the block in nuclear migration. Alternatively, the mRNA could be associating with other organelles that are moved to the embryo cortex by the microtubules, or with the microtubules themselves. As described in the previous chapter, injecting aphidicolin into cycle 7-8 embryos, often allows the centrosomes to migrate to the cortex while the nuclei are left behind. Aphidicolin treatment, therefore, provides a way of testing whether the cyclin B transcripts are carried to the cortex by associating (either directly or indirectly) with microtubules or nuclei.

I injected aphidicolin into embryos that were collected over a one hour period and aged so that they would all be at approximately cycle 6-12. The embryos were allowed to develop for 40-60 minutes before they were fixed, sectioned, and hybridised with a cyclin B antisense RNA probe. Because of technical difficulties in antibody staining sections that

Figure 5.2

The Distribution of Cyclin B mRNA in a Colchicine-Injected Embryo. This embryo was injected with colchicine at about nuclear cycle 6-8 and allowed to develop for one hour before it was fixed, sectioned, and hybridised with a cyclin B antisense probe. If the embryo had not been injected, it would have been at nuclear cycle 11-13 at the time of fixation, and both the nuclei and mRNA would have been localised to the embryo cortex. Here, both nuclear and mRNA migration to the cortex have been inhibited (the polar localisation, however, has not been affected). The mRNA is also no longer concentrated around the nuclei. Scale bar=30μm.


B

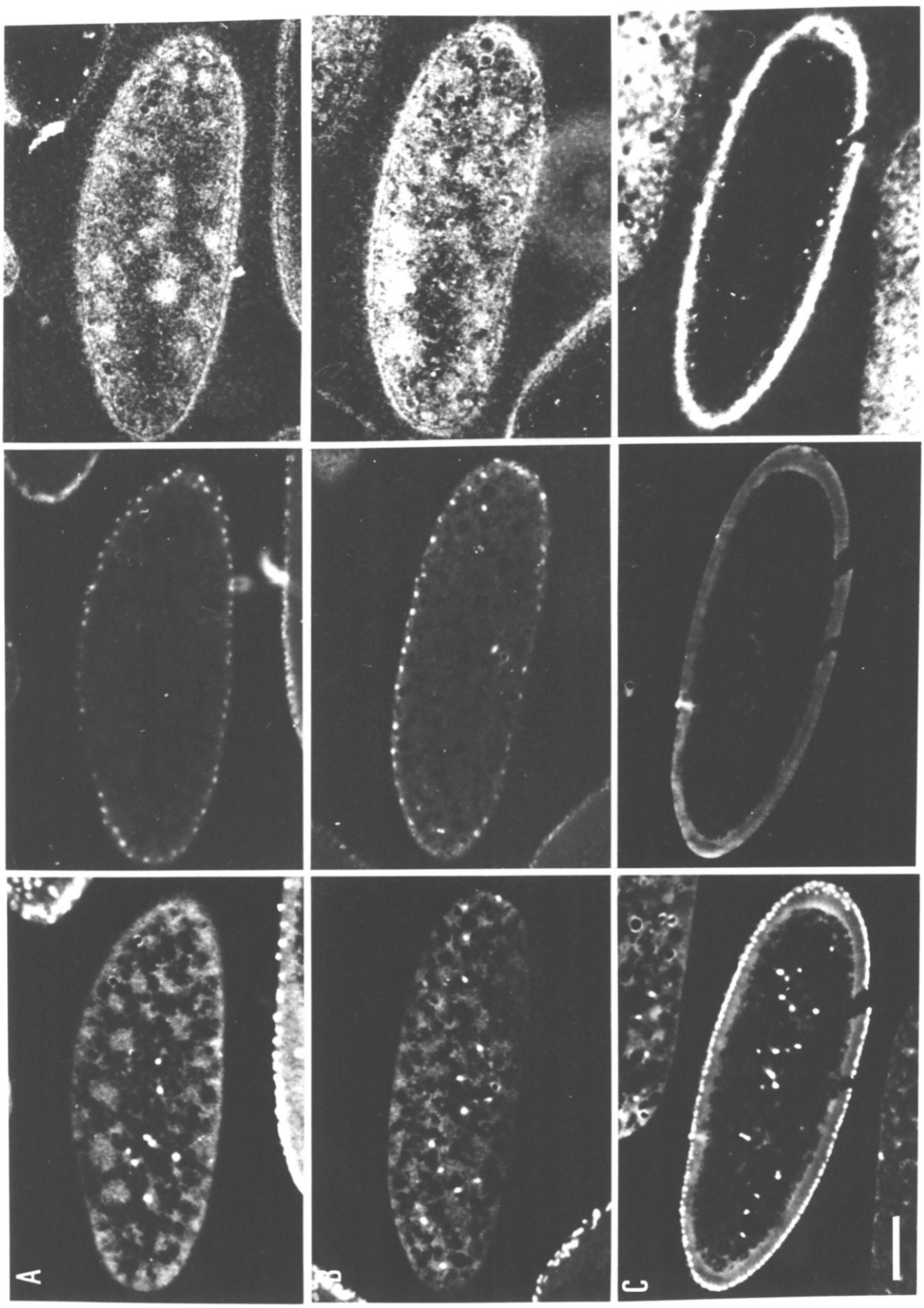
were hybridised to RNA probes, alternate sections were either hybridised to cyclin B antisense probes or stained with antibodies against tubulin, to reveal the distribution of centrosomes in the injected embryos. Figure 5.3 shows sections of three aphidicolin-injected embryos hybridised with cyclin B antisense RNA probes, together with the alternate section showing the distribution of nuclei and centrosomes. In two of the embryos (Figure 5.3a,b), the centrosomes have migrated to the cortex leaving the nuclei behind. In these embryos, the bulk of the mRNA has remained around the nuclei and has not migrated to the cortex with the centrosomes. In the other embryo (Figure 5.3c), the nuclei were already at the surface at the time of aphidicolin injection, and the mRNA is tightly localised to the cortex, demonstrating that the drug does not interfere with the processes that maintain the cortical localisation. By studying a large number of aphidicolin-injected embryos, I concluded that the bulk of cyclin B mRNA does not migrate to the cortex with the centrosomes alone.

5.4 Other Maternal mRNAs are Accumulated Around Nuclei, but not as Dramatically as Cyclin B mRNA

Each nucleus in the syncytial Drosophila embryo is surrounded by a yolk-excluding zone (Foe and Alberts, 1983), and it is possible, therefore, that all maternally contributed mRNAs accumulate in this region. To test this possibility, I performed a series of hybridisations to early embryo sections

Figure 5.3

The Distribution of Cyclin B mRNA in Aphidicolin-Injected Embryos. Embryos were injected with aphidicolin, allowed to develop for 40-60 minutes, fixed, and sectioned. As described in the text, alternate sections were either hybridised to cyclin B antisense RNA probes (right hand panels), or stained with antibodies to reveal the distribution of tubulin (middle panels), and Hoechst 33258 to reveal the distribution of nuclei (left hand panels). Two embryos are shown (A,B) in which the centrosomes have migrated to the cortex without the nuclei. Most of the mRNA, however, has not migrated to the cortex, and has remained associated with the nuclei. (C) shows an embryo in which the nuclei, centrosomes, and mRNA had already migrated to the cortex at the time of aphidicolin injection. Scale bar=50μm.


A
B
C

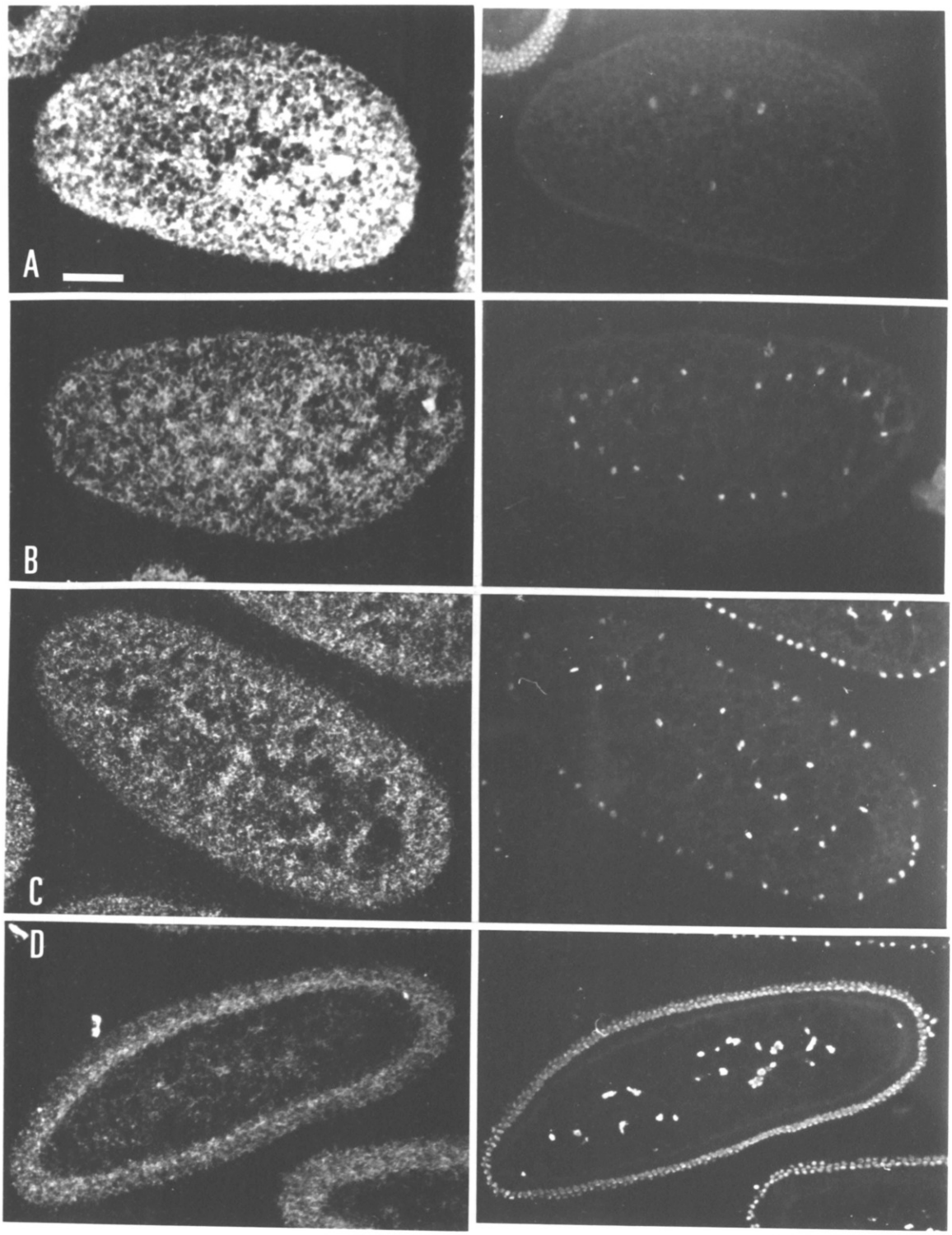
with a number of probes from genes that were known to be maternally derived: cyclin A, polo (S. Llamazares, pers. comm.), lodestar (C. Girdham, pers. comm.), and singed (J. Paterson, pers. comm.). The distribution of signal with all of these probes, was very similar; the mRNAs were evenly distributed in the early embryo, and then appeared to be gradually excluded from the inner yolk region, leading to an accumulation in the cytoplasmic region below the cortex. This accumulation at the cortex is not as dramatic as that seen for the cyclin B mRNA (compare Figure 5.4, showing the distribution of cyclin A mRNA, to Figure 5.1), and a similar distribution pattern has been reported previously for at least two other maternally supplied mRNAs (Mlodzik and Gehring, 1987; Gerttula et al., 1988). While all of the mRNAs I tested were accumulated around nuclei to some degree, none were localised as dramatically as the cyclin B mRNA (compare Figures 5.4 (cyclin A) and 5.1 (cyclin B)). This probably explains why the cortical accumulation of cyclin B mRNA is the most dramatic. It is not clear whether this localisation of cyclin B mRNA has any functional significance (see discussion).

5.5 Cyclin B mRNA is Localised at the Posterior Pole in Early Embryos and Unfertilised Eggs

In order to determine precisely when the posterior localisation of cyclin B mRNA occurred, I looked at the mRNA distribution in whole mount embryos. I found that even in

Figure 5.4

The Distribution of Cyclin A mRNA During Embryo Development. Sections of wild-type embryos were hybridised to antisense probes of cyclin A. Embryos were at nuclear cycle 5-6 (A), 8 (B), 10 (C), and early cycle 14 (D). The left hand panels are dark field images and the right hand panels are Hoechst fluorescence. The nuclear localisation and cortical accumulation of this mRNA are much less dramatic than that observed for cyclin B (figure 5.1). Scale bar=40μm.


A
B
C
D

very early embryos with only a few nuclei, the posterior localisation was already apparent (Figure 5.5a), and I did not observe any embryos that did not have cyclin B mRNA localised at the posterior pole. The posterior localisation was also apparent in unfertilised eggs (Figure 5.5b), suggesting that the mRNA is localised to the posterior pole during oogenesis, not embryogenesis as previously reported (Whitfield et al., 1989). The posterior localisation of the cyclin B mRNA is not always apparent in sections (because the sections do not always contain the region of the posterior pole to which the mRNA is localised - see Figure 5.1a, for example), and this is probably why the mRNA was originally thought to be homogeneously distributed in the early embryo (Whitfield et al., 1989).

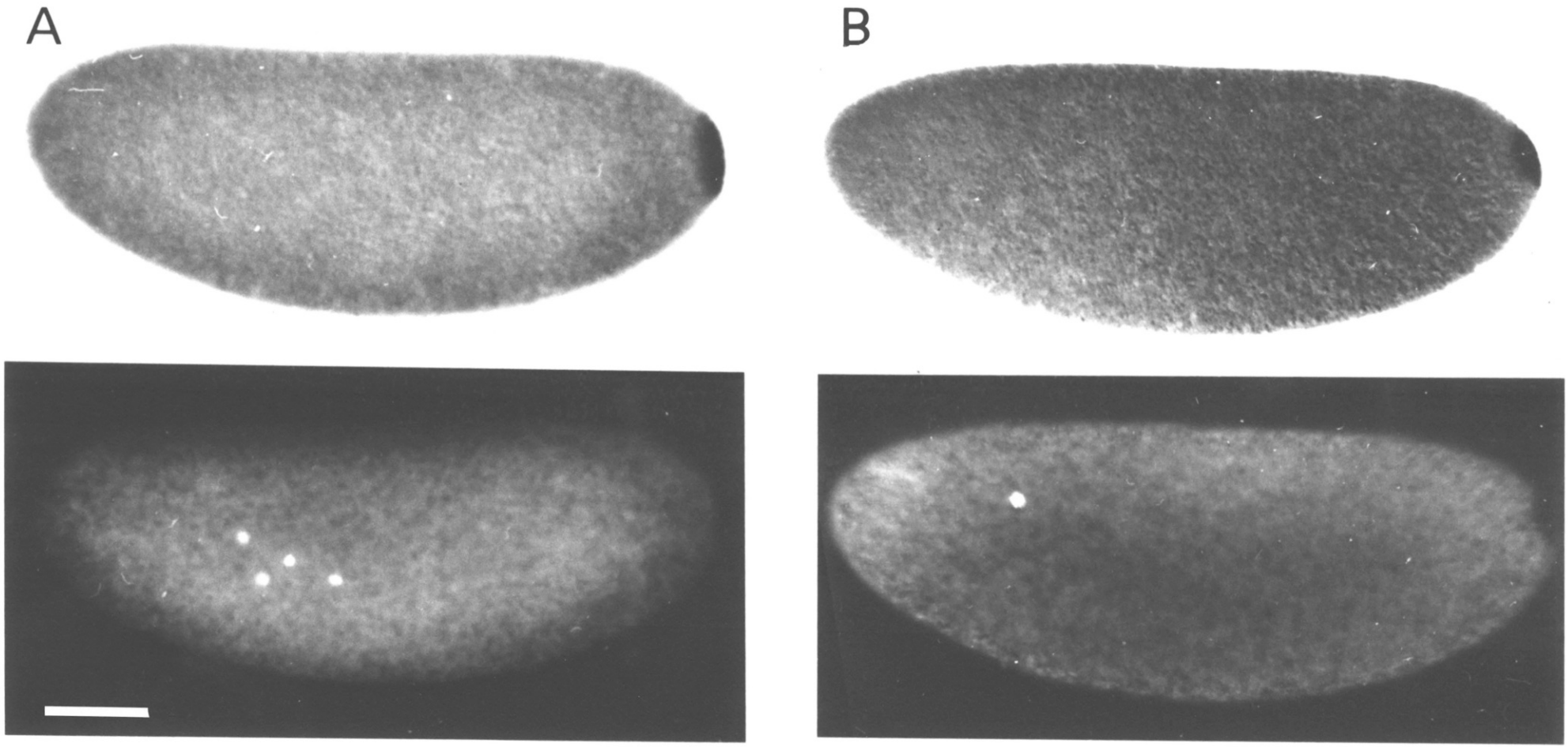
5.6 Cyclin B mRNA is Probably Localised to the Posterior Pole by an Association with Components of Polar Granules

Obvious candidates for the activity that localises cyclin B mRNA to the posterior pole are the polar granules that develop during oogenesis and are believed to play a role in germ cell determination. To test whether these organelles were responsible for localising cyclin B mRNA to the posterior pole, I used two approaches.

The first was to test if cyclin B mRNA became localised around centrosomes at the posterior pole. It has previously been shown that polar granules become concentrated around the

Figure 5.5

Cyclin B mRNA is Localised to the Posterior Pole in Early Embryos and Unfertilised Eggs. Eggs and embryos were fixed, hybridised with a cyclin B digoxygenin probe, and then stained to reveal the distribution of cyclin B mRNA and nuclei (see Materials and Methods). Top panels are bright field, lower panels are Hoechst fluorescence. (A) Wild-type embryo at nuclear cycle 3 with four nuclei present. (B) an unfertilised egg (in this example, the four polar bodies have fused into one). In both instances, cyclin B mRNA is accumulated at the posterior pole. Scale bar=50μm.

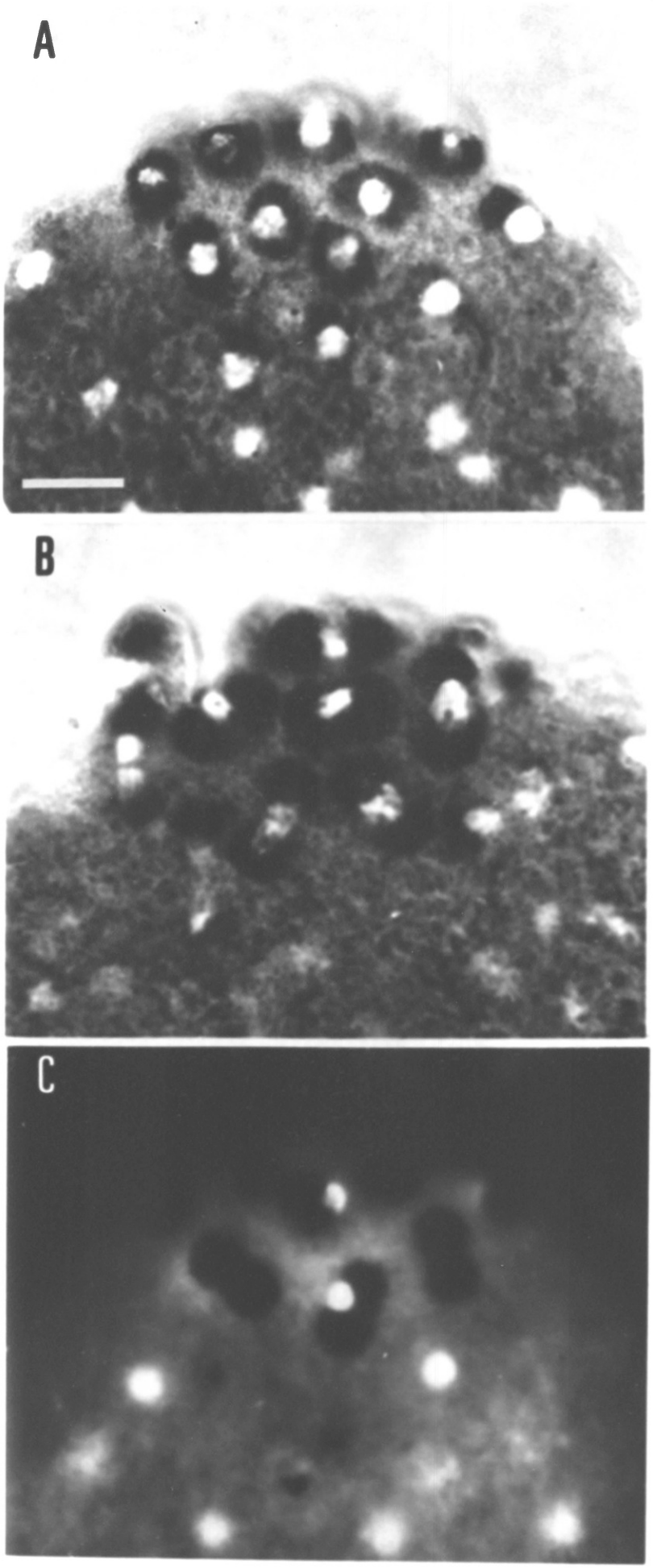

A
B

microtubules nucleated by centrosomes arriving at the posterior pole (Rabbinowitz, 1941; Counce, 1963; Mahowald, 1968; Mahowald et al., 1976). When wild-type embryos are stained to visualise components of polar granules, the polar granules become re-arranged in a bipolar fashion around the nuclei at the posterior pole, presumably because of their association with centrosomes (see Hay et al., 1988, Figure 1b). I looked at the distribution of cyclin B mRNA at the posterior pole in wild-type embryos and found that, like polar granules, the mRNA became concentrated around nuclei in a bipolar fashion (Figure 5.6a). This bi-polar arrangement becomes more obvious at metaphase/anaphase (Figure 5.6b).

To confirm that the mRNA was concentrated around centrosomes, I looked at the posterior distribution in embryos injected with aphidicolin at nuclear cycle 7-8. As described in chapter 4, the centrosomes in such embryos can migrate to the cortex and organise pole buds in the absence of a nucleus. These buds are only initiated when a centrosome reaches the posterior pole, and each bud always contains a centrosome. I found that the mRNA at the posterior pole of these embryos became localised in pole buds that were organised by centrosomes in the absence of nuclei (Figure 5.4c). Thus, the mRNA at the posterior pole is organised by centrosomes, as would be expected if the mRNA was bound to polar granules. The mRNA at the cortex of the rest of the embryo is not concentrated around centrosomes (not shown), demonstrating that the mRNA on its own is not organised by centrosomes.

Figure 5.6

Cyclin B mRNA is Concentrated Around Centrosomes at the Posterior Pole. Embryos were fixed, hybridised with a cyclin B digoxygenin probe, and stained to reveal the distribution of the cyclin B mRNA and nuclei. The posterior pole of two wild-type embryos in interphase (A), and metaphase/anaphase (B), of nuclear cycle 10. The figures show both the bright field image revealing the distribution of the mRNA, and the fluorescence image of the nuclei, superimposed. Panel (C) shows the distribution of the mRNA in an aphidicolin-injected embryo (see text). Here only the fluorescence of nuclei is shown, and the cyclin B mRNA can be seen by the bleaching of the embryos auto-fluorescence. In all of these embryos, the mRNA is concentrated around centrosomes. Scale bar=15μm.

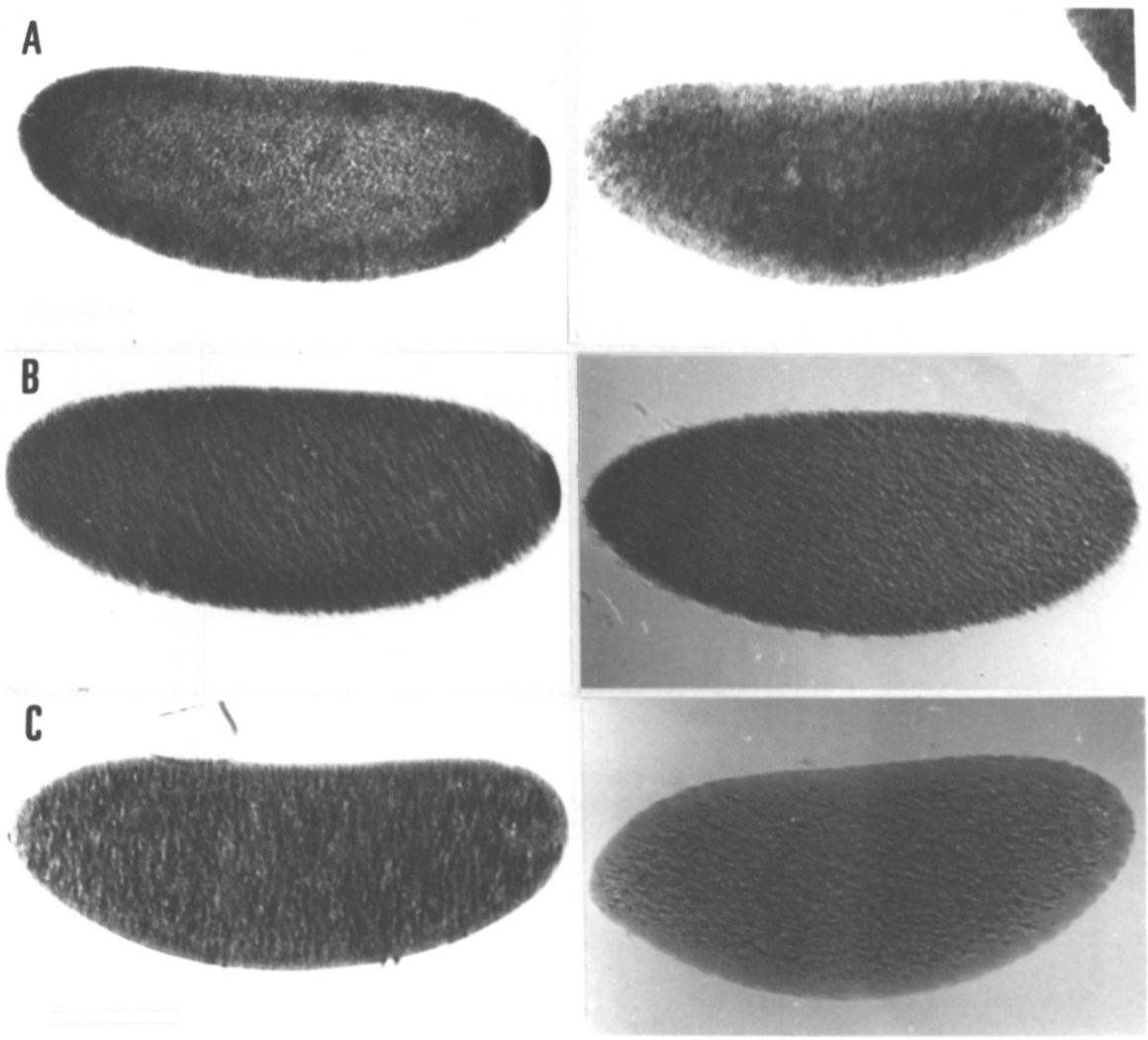

A
B
C

The second approach I used to investigate the possible interaction between polar granules and cyclin B mRNA was to look at the distribution of the mRNA in mutant embryos that fail to form pole cells. Five genes have been genetically identified as having a role in polar granule formation, vasa, oskar, valois, tudor, and, staufen (Boswell and Mahowald, 1986; Schupbach and Wieschaus , 1986; Lehman and Nusslein-Volhard, 1986). Embryos laid by females homozygous for any one of these mutations lay embryos that have no polar granules, fail to form pole cells, and usually have a variety of other defects associated with the absence of abdominal structures.

I tested the distribution of cyclin B mRNA in the stock osk^{301}, a weak oskar allele, where homozygous females produce embryos that are viable at 24°C, but fail to form pole cells due to the absence of polar granules. The viability of these embryos suggests that their only abnormality (at 24°C) is a lack of polar granules. Even though these embryos lack polar granules (Lehman and Nusslein-Volhard, 1986), I found that the posterior localisation of cyclin B mRNA was present in early embryos. By cycle 5-10 (variable from embryo to embryo), however, this localisation was lost (Figure 5.7b). On the other hand, in embryos laid by females homozygous for the mutation $vasa^{PD23}$ (a strong vasa allele), the posterior localisation was completely absent, even in the earliest embryos (Figure 5.7c). I am currently analysing the

Figure 5.7

The Posterior Localisation of Cyclin B mRNA is Disrupted in Embryos that Lack Polar Granules. Embryos were fixed, hybridised, and stained to visualise the cyclin B mRNA distribution. All of the embryos shown were stained with Hoechst 33258 to reveal the distribution of nuclei, although this is not shown. The left hand panels show embryos at nuclear cycle 2-5, the right hand panels show embryos at cycle 10. (A) Wild-type embryos, (B) embryos laid by females homozygous for the mutation *oskar*^{301}, (C) embryos laid by females homozygous for the mutation *vasa*^{PD23}. Scale bar=100μm.


A
B
C

distribution of cyclin B mRNA in embryos laid by females missing wild type copies of the other genes that effect polar granule formation. It is apparent, however, that mutations which disrupt polar granules also disrupt the posterior localisation of cyclin B mRNA.

DISCUSSION

The results presented in this chapter demonstrate that two independent mechanisms are responsible for generating the spatial distribution of cyclin B mRNA in the early *Drosophila* embryo: (1) the mRNA is concentrated around nuclei during early embryo development, leading to a dramatic cortical localisation as the mRNA migrates to the cortex with the nuclei; (2) the mRNA is localised to the posterior pole during oogenesis (not embryogenesis as previously reported); this localisation probably depends on the mRNA binding to a component (or components) of polar granules.

Nuclear Localisation of Cyclin B mRNA

I found that cyclin B mRNA was concentrated around nuclei at the earliest stages of embryo development. As the nuclei migrated to the cortex, they took large amounts of cyclin B mRNA to the cortex with them, producing a clearing of the mRNA from the middle of the embryo. This process presumably explains the dramatic cortical re-localisation of the mRNA (Whitfield et al., 1989), and drugs that prevent nuclear migration, such as colchicine and aphidicolin, also block the accumulation of the mRNA at the embryo cortex.

The nuclei in the *Drosophila* syncytium are surrounded by a region of cytoplasm that is relatively free of yolk particles (Foe and Alberts, 1983). It was possible, therefore, that

all maternally supplied mRNAs would preferentially accumulate in this yolk-free region. To test this, I performed hybridisations to embryo sections with antisense probes from a number of genes whose mRNAs are known to be maternally supplied. I found that all of these mRNAs were initially homogeneously distributed throughout the embryo, and were then gradually excluded from the thickening inner yolk region during cycles 10-14. This distribution pattern may be common to most maternally derived mRNAs, and a similar pattern has been reported for at least two other maternal mRNAs (Mlodzik and Gehring, 1987; Gerttula et al., 1988). Although the mRNAs I tested were accumulated around nuclei, this localisation was not as obvious as that seen for cyclin B mRNA, and their cortical redistribution during nuclear migration was correspondingly less dramatic. The mechanisms responsible for localising cyclin B mRNA around nuclei are unknown. It is possible that the mRNA contains a localisation signal that binds it to some component localised in the yolk-free region around each nucleus. Alternatively, it may be that the cyclin B mRNA is localised around nuclei by the same process (also unknown) that concentrates many other maternal mRNAs around nuclei, but for some reason this localisation appears more obvious in the case of cyclin B.

Although it is not clear that the nuclear localisation of cyclin B mRNA has any functional significance, it is interesting to speculate about the possible functions this localisation might have. It has previously been shown that

the level of cyclin A protein detected with antibodies in whole mount Drosophila embryos does not vary appreciably during the first 14 rounds of division (Lehner and O'Farrell, 1989), and this is also true for the cyclin B protein (G. Maldonado-Cordina, pers. comm.). This is in contrast to some other organisms, where cyclin protein levels can be seen to rise and fall in synchrony with the cell cycle in whole embryo extracts (Evans et al., 1983). This cycling pattern, however, is found in the Drosophila embryo after cycle 14 when the nuclei have cellularised. In each cell the cyclin proteins accumulate during the cell cycle, and are then rapidly degraded at metaphase/anaphase (Lehner and O'Farrell, 1989, G. Maldonado-Cordina pers. comm.). The apparent lack of cyclin protein cycles in early Drosophila embryos is puzzling, especially in the light of experiments showing that the destruction of exogenously added cyclin B protein is necessary to allow an exit from mitosis in an amphibian in vitro system (Murray and Kirschner, 1989b). It is possible that in the early (pre-cycle 14) Drosophila embryo, the concentration of cyclin proteins vary only in a localised region around each nucleus rather than over the whole embryo, and there is some evidence that this is the case for cyclin A (Lehner and O'Farrell, 1989). If this were true of cyclin B, then the nuclear association of cyclin B mRNA could act to concentrate the mRNA where the protein is required. Such a dramatic nuclear association, however, is obviously not required for cyclin A mRNA function.

It is intriguing that the nuclei that remain behind after the others have migrated to the cortex initially retain cyclin B mRNA concentrated around them. By nuclear cycle 12, however, most of this mRNA has disappeared, and at this stage these yolk nuclei have dropped out of the mitotic cycle and are undergoing endomitosis and polyploidisation (Foe and Alberts, 1983). It may be that this lack of cyclin B mRNA plays some role in this transition; if cyclin B mRNA has been exhausted, perhaps these nuclei can no longer enter mitosis, unlike the nuclei at the embryo cortex, which are still surrounded by cyclin B mRNA.

The Posterior Localisation of Cyclin B mRNA.

I have shown that cyclin B mRNA is accumulated at the posterior pole in the earliest embryos and unfertilised eggs. This is in contrast to the previous report which suggested that this mRNA became localised to the posterior pole sometime during the early nuclear divisions of the embryo, prior to pole bud formation (Whitfield et al., 1989). Two separate lines of evidence suggest that this localisation depends on the binding of the mRNA to a component of polar granules: (1) cyclin B mRNA, like polar granules, becomes concentrated around centrosomes arriving at the posterior pole; (2) mutations that disrupt polar granule formation disrupt the posterior localisation of cyclin B mRNA.

In the early embryos of many organisms, there exists a region

of specialised cytoplasm that will be incorporated into the forming germ line cells (Beams and Kessel, 1974; Eddy, 1975). In Drosophila, it has been demonstrated that this cytoplasm is both necessary (Okada et al., 1974; Warn, 1975) and sufficient (Illmensee and Mahowald, 1974) to initiate germ line development. This cytoplasm is ultrastructurally distinctive, and contains a large number of electron-dense organelles called polar granules (Counce, 1963; Mahowald, 1962, 1968), which are thought to be germ line determinants. This hypothesis is supported by the fact that polar granules are absent in a number of mutations that abolish the ability of embryos to form pole cells (Boswell and Mahowald, 1985; Lehman and Nusslein-Volhard, 1986; Schupbach and Wieschause, 1986). It is known that polar granules contain both protein and RNA (Mahowald, 1971), but the nature of the RNA present in these organelles is unknown. The studies described in this chapter suggest that one of these RNAs codes for the Drosophila cyclin B protein.

Genetic studies have implicated five genes in the formation of polar granules, vasa, staufen, valois, oskar, and tudor (Boswell and Mahowald, 1986; Schupbach and Wiechause, 1986; Lehman and Nusslein-Volhard, 1986). Embryos laid by females homozygous for mutations in any of these genes lay embryos that lack polar granules and fail to form pole cells. The vasa gene has recently been cloned (Hay et al., 1989; Lasko and Ashburner, 1989), and the vasa protein was found to be present in polar granules (Hay et al., 1988). This protein

has homology to RNA binding proteins, suggesting it may play a role in localising at least some of the RNA that is known to be present in polar granules.

I found that the posterior localisation of cyclin B mRNA is abolished in embryos laid by females homozygous for <u>vasa</u>^{PD23}. In this mutant there is no <u>vasa</u> protein localised at the posterior pole of the early embryo (Hay et al., 1988). In embryos laid by females homozygous for the mutation <u>osk</u>^{301}, however, the posterior localisation of cyclin B mRNA is initially present, but this is then lost during the early stages of embryo development. These observations suggest that cyclin B mRNA is binding to a component of polar granules that is absent in embryos laid by females homozygous for <u>vasa</u>^{PD23}, but is initially present and then lost from the posterior pole in embryos laid by females homozygous for <u>osk</u>^{301}. Recent studies have revealed that the <u>vasa</u> protein is present at the posterior pole of embryos laid by females homozygous for a number of mutants involved in polar granule formation, but, like the localisation of cyclin B mRNA in <u>osk</u>^{301} mutant embryos, this localisation is lost as development proceeds (P. Lasko pers. comm.). It will obviously be interesting to look at the distribution of <u>vasa</u> protein in embryos laid by females homozygous for <u>osk</u>^{301}, and I am currently investigating the relationship between cyclin B mRNA and <u>vasa</u> protein localisation in some of these mutant stocks. Preliminary results are consistent with the hypothesis that cyclin B mRNA may be binding directly to the

vasa protein.

It is intriguing to speculate why the Drosophila embryo has evolved in such a way that cyclin B mRNA is sequestered into the germ line by associating with polar granules. An obvious specialised function of the germ line is to eventually undergo meiosis, and it has recently been demonstrated that in clam oocytes, a pool of intracellular cyclin B protein is released on fertilisation, and this drives meiosis I (Westendorf et al., 1989; Luca et al., 1987). In Drosophila, there seems to be no accumulation of cyclin B protein in the pole cells of the early embryo (G. Maldonado-Cordina pers.comm.), suggesting that the mRNA is not being translated into protein at this time, but is being stored for later use. Indeed, the pole cells do not undergo any division until much later in development when the gonads are forming (Sonnenblick, 1941; 1950). Why the embryo has to store maternally supplied cyclin B mRNA, rather than transcribe it at some later stage when the protein is required is unclear. Perhaps the pole cells are transcriptionally inactive for a time during embryo development, and the stored cyclin B mRNA acts as a source of cyclin B protein that drives the later division(s) of these cells. The availability of antibodies raised against Drosophila cyclin B (W. Whitfield pers. comm.) should allow these questions to be addressed.

CHAPTER 6

Discussion

6.1 Summary of Results

The experiments described in this thesis examine aspects of the cell cycle and the organisation of the cytoskeleton in the early Drosophila embryo. I show that there is a cell cycle oscillator in the early embryo that can drive both nuclear and cytoplasmic components of the cell cycle, even when DNA synthesis and nuclear replication have been inhibited with aphidicolin. I noticed that an injection of aphidicolin into early embryos leads to the disocciation of replicating centrosomes from non-replicating nuclei. This observation allowed me to investigate the relative importance of these organelles in nuclear migration and in the re-organisation of the cortical cytoskeleton. I found that centrosomes are able to migrate to the embryo cortex without their accompanying nuclei, suggesting that nuclear migration is normally mediated by forces acting on the centrosome rather than the nucleus. Once at the surface, the centrosomes are able to organise much of the cortical cytoskeleton, including the process of pole cell formation, in the absence of a nucleus.

The recent cloning of the Drosophila cyclin B gene, revealed that its mRNA became dramatically accumulated at the posterior pole and at the embryo cortex during embryo development (Whitfield et al., 1989). Because of my interest in the role of the cytoskeleton in nuclear migration and in organising positional information at the posterior pole, I

went on to investigate how the spatial distribution of cyclin B mRNA is controlled. I found that cyclin B mRNA is closely associated with nuclei during the early stages of embryo development. As a result of this association, the mRNA is dramatically re-localised with the nuclei as they migrate to the embryo cortex. I found that the posterior accumulation of cyclin B mRNA probably develops during oogenesis, not embryogenesis as reported previously. Its distribution at the posterior pole in both wild type and mutant embryos, suggests that the mRNA is probably binding to components of polar granules.

The initial aim of my project was to study aspects of cell cycle control in the early Drosophila embryo. As the project progressed, however, my interest focused on the centrosome, which seems to play a crucial part in organising many events in the early embryo. In this last chapter, therefore, I shall concentrate on centrosomes, discussing the replication of these organelles, and their role in cellular organisation. Finally, I shall speculate on the approaches that are likely to reveal how the centrosome functions at the molecular level.

6.2 The Reproduction of Centrosomes

The mode of centrosome replication has been a subject of controversy for many years (Wilson, 1925; Karsenti and Maro, 1986). In particular, it is unclear whether centrosomes can

form de novo, or whether replication requires a pre-existing centrosome to act as a template. The latter view implies a continuity from generation to generation, like that of the genetic information, and means that a faithful copy of each centrosome must be passed on to each daughter cell. As described in chapter 1, centrosomes generally replicate by the splitting apart of the centriole pair. Each centriole then nucleates the assembly of a second centriole at right angles to the first. When elongation of the second centriole is complete, two daughter centrioles have been formed, and these then move apart generating two centrosomes. This mechanism of centrosome replication would appear to be template driven, as it requires a pre-existing centrosome. It is still unclear, however, whether centrosomes contain genetic information in the form of nucleic acids. The results presented in this thesis and elsewhere (Sluder and Lewis, 1987), demonstrate that DNA synthesis driven by DNA polymerase-α is probably not required for centrosome replication. Centrioles may, however, contain functional RNA (Heidemann, et al., 1977), and, in principle, this could act as a source of genetic information. In Chlamydomonas, a set of genes that encode proteins involved in forming basal bodies (organelles that contain centrioles and act as MTOC's for the microtubules in cilia) are carried on a discrete genetic element that segregates independently of the major chromosomes (Ramanis and Luck, 1986). The nature and location of this element, however, remains to be identified.

While in general centrosome replication is thought to be template driven, there are a number of examples where centrosomes can apparently form de novo. This phenomenon is mainly found in the early embryos of marine invertebrates, where various treatments can lead to the formation of centrosomes where there were none before (Wilson, 1925; Mazia, 1961; Karsenti and Maro, 1986). These observations would appear to rule out the possibility that centrosomes absolutely require a template for replication. These examples, however, occur under unusual circumstances, in which eggs that are loaded with the precursors that will drive embryo development (including precursors for centrosome formation) are subjected to some non-physiological treatment. The situation in these examples is further complicated, by the fact that these eggs do contain a centrosome, but it is in some way inactivated so that the paternal centrosome (supplied by the incoming sperm) is usually used by the developing embryo (Sluder et al., 1989). Thus, although these observations show that centrosomes can form de novo in special circumstances, the physiological relevance of this type of centrosome formation is not clear.

6.3 The Centrosome as an Organiser of Microtubules

While a detailed description of microtubule dynamics is beyond the scope of this discussion, it is worth considering how the centrosome is thought to organise microtubules. Microtubules are polymers formed from tubulin molecules, each

of which is a heterodimer of α- and β-tubulin. Each dimer tightly binds a GTP molecule that is hydrolysed soon after the dimer is added to the polymer. The tubulin molecules assemble into linear protofilaments, 13 of which are arranged side by side to form a hollow, cylindrical microtubule. Unlike actin filaments, which, when formed in vitro, can reach a steady state where all of the filaments maintain a constant length, microtubules formed in vitro are in a state of "dynamic instability" (Mitchison and Kirschner, 1984a), where some microtubules are slowly growing and others are rapidly shrinking. Microtubules in cells also appear to behave in this way (Horio and Hotani, 1986; Sammak and Borisy, 1988). The mechanisms underlying dynamic instability are complex, and controversial, and I will only describe them superficially here (for a more complete discussion see Kirschner and Mitchison, 1986).

Microtubules, like actin filaments, are polar structures, with a slow growing minus end, where depolymerisation is favoured, and a fast growing plus end, where polymerisation is favoured. Under physiological conditions, depolymerisation from a free minus end is rapid. For this reason, microtubules are only stable in a cell if the minus end is stabilised by binding to a microtubule organising centre. This is the function of the centrosome. Because the minus end is almost invariably stabilised in the cell, it is only necessary to consider the kinetics at the plus end. Here, GTP-tubulin subunits add to the polymer, and because

the hydrolysis of GTP occurs with some delay after subunit addition (Carlier and Pantaloni, 1981), it is thought that a "GTP cap" exists at the plus end of a growing microtubule. Since GTP-tubulin dissociates from the polymer less readily than GDP-tubulin, the GTP-cap protects the plus end from depolymerising. If the cap is lost, however - because tubulin subunit addition has not been fast enough to maintain it, for example - then the microtubule starts to depolymerise rapidly. This may proceed until the microtubule is completely depolymerised, or until enough GTP-tubulin subunits bind to the microtubule to create another protective GTP cap, at which point the microtubule can start to grow again.

Although GTP hydrolysis is not required for the addition of subunits to the polymer, it acts as a source of energy, which enables the microtubule to escape from the more rigorous kinetic constraints that would be placed on a simple equilibrium polymer (where subunit assembly and disassembly are governed only by the concentration of subunits and the affinity of the subunits for the polymer - see Kirschner and Mitchison, 1986). It should be stressed, however, that the model described above is only a hypothesis designed to explain the observed behaviour of microtubules in vitro and in vivo, and at present there is no proof that a GTP cap exists at the plus end of microtubules.

The dynamic instability of microtubules is essential to their

function. This is best illustrated by considering the formation of the mitotic spindle. In an interphase cell, most microtubules will be nucleated by the centrosome. This array of microtubules plays an important role in directing intracellular transport (Vale, 1987), and in positioning other organelles such as the Golgi apparatus and the endoplasmic reticulum (Allan and Kreis, 1986; Dabora and Sheetz, 1988). In mitosis, the cytoplasm undergoes a change that makes the interphase array of microtubules much less stable, so that they rapidly depolymerise. The centrosome now nucleates a large number of short microtubules, which are in a very dynamic state, with plus ends growing out from the centrosome and then rapidly depolymerising. The kinetochores of mitotic chromosomes can capture, and stabilise, the plus ends of microtubules (Nicklas, 1987). It is thought that this process of random microtubule growth from the centrosome followed by capture and stabilisation by the kinetochore that initiates the formation of the bipolar mitotic spindle (Kirschner and Mitchison, 1986).

While the dynamic behaviour of microtubules is well established, the mechanisms that allow centrosomes and kinetochores to stabilise microtubules are largely unknown. It is clear that they do not simply bind to the ends of microtubules and prevent subunit turnover, since subunit addition and loss occurs at both ends of microtubules during mitosis (Mitchison, 1989a). The molecular basis of the complex events that occur during mitosis, such as chromosome

congression, anaphase A, and anaphase B, are also poorly understood and remain highly controversial (Mitchison, 1989b).

6.4 Centrosomes as Organisers of the Cytoskeleton

Whereas centrosomes directly organise microtubules, one of the main conclusions of the work presented in this thesis is that centrosomes can indirectly organise actin filaments. This is most impressively demonstrated by the ability of the centrosome to initiate pole cell formation, a process that is dependent on actin filaments (Warn et al., 1985; chapter 4 of this thesis). Although pole cell formation is presumably initiated by interactions between microtubules nucleated by the centrosome and the actin rich cortex, injecting a microtubule depolymerising drug soon after anucleate pole buds have started to form does not lead to the collapse of the pole buds. This suggests that while the centrosome initiates a change in the actin filament distribution, once organised, the actin filaments are no longer dependent on the integrity of microtubules.

That centrosomes can organise actin filaments has previously been suggested from a number of studies in embryos. Early studies on starfish (McClendon, 1908) and sea urchin (Wilson, 1925) embryos showed that centrosomes could direct cleavages in enucleated embryos, and this has been confirmed in more recent experiments (Maller et al., 1977; Nagano et al., 1981;

Bornens et al., 1987). It is now known that cleavage is brought about by the interactions of actin and myosin filaments, which form a contractile ring between the two spindle poles. This ring gradually contracts, pinching the two daughter cells apart (cytokinesis). The mechanism responsible for generating the force for this contraction is is thought to be similar to that involved in muscle contraction (see Alberts et al., 1989). If centrosomes can organise cleavage, they must be able to organise actin and myosin filaments. It has recently been shown that the contractile ring forms half way between two centrosomes, even when there is not a spindle between them (Rappaport, 1986). This presumably explains how multiple rounds of cytokinesis can occur in an anucleate embryo, where centrosome division continues in the absence of a nucleus. The results presented in this thesis add to the growing body of evidence that centrosomes are able to somehow interact with actin filaments. The mechanisms underlying this interaction remain unclear; presumably, microtubules nucleated by the centrosome are able to bind to actin filaments via an unidentified protein assembly that binds both to actin filaments and to microtubules.

One of the most interesting aspects of anucleate cell formation in the Drosophila embryo is that it occurs only in a specialised region of the embryo. Although all of the centrosomes that reach the cortex organise actin buds around themselves, only the centrosomes at the posterior pole are

incorporated into actual cells. This mimics the normal behaviour of the nuclei in the Drosophila embryo, where nuclei at the posterior pole cellularise before the somatic nuclei do. In aphidicolin-treated embryos, however, the centrosomes at the remainder of the embryo cortex do not become incorporated into cells at a later time. This may be because cellularisation of the somatic nuclei requires the attainment of a critical nuclear/cytoplasmic ratio (Edgar et al., 1986) which is never attained in aphidicolin-treated embryos (see discussion of chapter 4). Thus, in normal embryos, information localised at the posterior pole dictates that the nuclei arriving here will be incorporated into cells before nuclear cycle 14. My findings demonstrate that centrosomes alone can interact with this information, and initiate cell formation only at the posterior pole.

In many embryos, there exists a specialised region of cytoplasm that becomes incorporated into the developing germ line (Beams and Kessel, 1974; Eddy, 1975). In Drosophila embryos, polar granules are found exclusively in this region, and these organelles are thought to be germ cell determinants (see discussion of chapter 5). Polar granules become concentrated around the centrosomes arriving at the posterior pole (Counce, 1963; Mahowald, 1968; Rabbinowitz, 1941; Mahowald, 1976). Perhaps it is because of this interaction that the centrosomes initiate cell formation rather than just actin cap formation. The binding of polar granules to the microtubules nucleated by the centrosomes at the posterior

pole might change the properties of these microtubules, allowing the centrosomes to pinch off from the embryo surface. As discussed by Warn et al., (1985), the mechanism underlying this type of cleavage must be different from that underlying the cleavage that occurs during cell division, as in the former case, cleavage does not occur between two centrosomes. Maybe the binding of polar granules to the centrosome-microtubule assembly enables this distinctive mode of cleavage to occur, ensuring that only centrosomes at the posterior pole pinch off into pole cells. Whatever the mechanism, positional information located at the posterior pole of the Drosophila embryo must act to change the behaviour of the cytoskeleton in this region. It is interesting to note that cellularisation around the somatic nuclei occurs by the slow invagination of a cell membrane between the nuclei starting from the embryo cortex. This is another unusual form of cleavage and presumably utilises yet another special mechanism.

6.5 Towards a Molecular Understanding of the Centrosome?

Although the centrosome was described over a hundred years ago, it still remains an enigma: both its structure and mode of action are poorly understood. Electron microscopic studies have revealed that its structure is extremely complex (e.g. Bornens et al., 1987). The recent purification of centrosomes to near homogeneity, has allowed their protein composition to be analysed; not surprisingly, they were found

to comprise many different proteins (Bornens et al., 1987).

It is clear that in order to understand how the centrosome works, one will need to analyse the macromolecules from which it is formed. The recent advances in molecular biology have enabled investigators to characterise some centrosome associated proteins by cloning the cDNAs encoding them (e.g. Whitfield et al., 1987; Huang et al., 1988). So far, there is very little information about the function of any of these proteins, although one of them has homology to both Ca^{++} binding proteins, and to the protein encoded by the CDC31 gene of S. cerevisiae that is known to function in spindle pole formation (Huang et al., 1988). Although this protein was originally identified in the basal bodies of Chlamydomonas, a related protein is also found in mammalian centrosomes (Baron and Salisbury, 1988).

While it is important to characterise the individual macromolecules that make up the centrosome, it is unlikely that studying these molecules in isolation will reveal how the centrosome works. The centrosome is a complex protein machine, and to fully understand it, one will have to reconstitute its functions in vitro, so that the functional role of each component can be analysed. This type of approach has already proved successful for analysing other complex protein machines, such as the one that mediates DNA replication (Formosa and Alberts, 1984).

The development of *in vitro* systems for analysing centrosome function has already begun in a number of laboratories (Mitchison and Kirschner, 1984; Karsenti, 1988). A particularly promising system is based on the *Xenopus* cell-free extracts which have been used for studying cell cycle control. When purified centrosomes are added to extracts made from interphase-arrested *Xenopus* oocytes, the centrosomes nucleate long, relatively stable arrays of microtubules. If, on the other hand, centrosomes are added to extracts made from mature *Xenopus* eggs, which are arrested in M-phase, the centrosomes nucleate much shorter and more dynamic microtubules (Karsenti, 1988). Thus, the exogenously added centrosomes can respond to cytoplasmic signals that change during the cell cycle. This offers a powerful system in which to study the molecular basis of the cell cycle regulation of the centrosome-microtubule assemblies. Relevant to this is the recent observation that a subset of the $p34^{cdc2}$ protein in mammalian cells is localised to the centrosome (Riabowol *et al.*, 1989). This suggests that many of the changes in centrosome properties brought about on entry into M-phase may depend on phosphorylation events either directly or indirectly mediated by the activation of the $p34^{cdc2}$ protein kinase.

In the long term, a combination of molecular cell biology and molecular genetics will allow many of the components of the centrosome to be isolated. By using the type of system described above, it should be possible to assay the function

of combinations of the isolated components and so reconstruct a functional centrosome *in vitro* from its individual components. Once this kind of molecular understanding is achieved, we should at last be able to resolve some of the problems that have been puzzling cell biologists for over a hundred years.

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