GNU: A NUCLEAR REPLICATION MUTANT OF DROSOPHILA

Matthew John Aylmer Freeman

Department of Biochemistry

Imperial College of Science and Technology

London SW7 2AZ

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ABSTRACT

This thesis describes aspects of the phenotype of the *Drosophila* maternal effect lethal mutation, *gnu*. Embryos from females homozygous for the *gnu* mutation (GNU embryos) are defective in nuclear division, but not DNA replication, which leads to the formation of a small number of giant nuclei in the syncytial blastoderm. I have shown that many of the structural components of mitosis are present and functional in GNU embryos. In particular, centrosomes are found to replicate independently of nuclei, and are able to nucleate microtubule asters. In contrast to wild type eggs, GNU eggs develop even if unfertilized. Another distinguishing characteristic of GNU embryos is that the DNA of their polar bodies undergoes replication, whereas in wild type eggs this never happens. The *gnu* mutation thus appears to cause a 'replicative environment' in GNU embryos, allowing inappropriate DNA synthesis to occur. I have proposed that the primary lesion caused by *gnu* lies in the system that represses DNA synthesis in normal unfertilized eggs, and I have provided further evidence for this model. The *gnu* gene has been mapped genetically and cytologically, and this will allow its isolation. Since repression of DNA synthesis is a feature common to unfertilized eggs of a wide range of species, the molecular characterisation of the *gnu* gene will be of general interest.

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LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
CAT	Chloramphenicol Acetyltransferase
cdc	Cell Division Cycle
Ci	Curie
CSF	Cytostatic Factor
DMSO	Dimethyl Sulphoxide
DNAse	Deoxyribonuclease
dNTP	Deoxyribonucleoside Triphosphate
dpm	Radioactive Decays per Minute
EDTA	Diaminoethanetetra-acetic Acid
IgG	Immunoglobulin Class G
kb	Kilobase: 1000 Nucleotides or Nucleotide Pairs of Nucleic Acid
LSP	Larval Serum Protein
M	Molar
mh	Maternal Haploid
mM	Millimolar
MPF	Maturation Promoting Factor
mu	Map Units
pers. comm.	Personal Communication
RNAse	Ribonuclease
SDS	Sodium Dodecyl Sulphate
SSC	Saline Sodium Citrate
TE	Tris-EDTA
Tris	Tris(hydroxymethyl)-aminoethane
ts	Temperature Sensitive

CHAPTER 1

Introduction

Mitotic cell division is the process by which somatic cells divide during most periods of growth, and is of fundamental importance in all eukaryotic organisms. One cell replicates its genetic material and then divides, giving rise to two daughter cells with an identical genetic content. Since the nineteenth century there have been detailed descriptive accounts of mitosis (see for example Van Beneden, 1883), but remarkably little is known about its mechanism or regulation. The eukaryotic cell cycle is divided into two main stages of activity, S-phase, in which DNA replication occurs, and M-phase, when the nucleus divides. These phases are separated by 'gaps', G1 between M- and S-phase, and G2 between S- and M-phase. Although no dramatic biochemical or physical changes which affect the cell cycle occur in G1 or G2, there is evidence that important regulatory events happen during these phases (Nurse and Bisset, 1981). (For reviews of mitosis, see Pickett-Heaps et al., 1982; McIntosh, 1984). In addition to the necessary replication and division of the DNA content of a cell during the cell cycle, there is also a need to partition the cytoplasmic components of the mother cell. Although many of these are probably passively and randomly allotted, there are others that show a division cycle analagous to the nuclear cycle (Frasch et al., 1986). Perhaps the best characterised of these 'cytoplasmic cycles' is the replication and division of the centrosome. The centrosome is the organelle which nucleates microtubules at the pole of the mitotic spindle,

and so it must replicate strictly in unison with the nuclear cycle in order to ensure that each new cell inherits the ability to produce a spindle pole. The centrosome replicates during G1 or S-phase of the cell cycle and the two new centrosomes then each form one of the spindle poles of the dividing cell, thereby allowing the necessary transmission of a centrosome to each of the daughter cells (Wilson, 1928). It can be seen that the eukaryotic mitotic cycle is a complex set of interacting nuclear and cytoplasmic events which needs to be regulated in a manner that allows the faithful replication and division of somatic cells.

One approach that has been successful in the analysis of mitosis is the genetic dissection of the various processes involved in the yeast cell cycle (for reviews see Pringle and Hartwell, 1981; Nurse, 1981). The method used has been to isolate temperature sensitive (ts) mutant strains of yeast in which stage-specific mitotic defects are observed at the restrictive temperature. Over 50 such cell division cycle (cdc) complementation groups have been isolated, and there is evidence that these only represent a proportion of all the necessary functions for the cell cycle (Pringle and Hartwell, 1981). The genetic analysis of these cell cycle mutants has led to a fairly detailed characterisation of the yeast cell cycle although, as will be described, it is not easy determine the how similar this is to the cell cycle of multicellular eukaryotes.

Many cdc mutations which block an event early in the

cell cycle also block other events that take place later. For example, Hartwell et al. (1974) showed that S. cerevisiae mutants blocked in nuclear division also became blocked in further cytokinesis and DNA replication, and that mutants blocked in DNA replication became blocked in nuclear division and cytokinesis. In contrast, mutants blocked in cytokinesis continued to undergo DNA replication and nuclear division. These, and similar observations, led to a model of the yeast cell cycle in which there are two linked sequences of events, the nuclear cycle and cytokinesis, and in which the events within a sequence show a linear dependence upon each other. Consistent with the idea that the events of mitosis occur in such a linear pathway, in which each event is dependent on the action of the previous one, has been the temporal mapping of the different cdc functions. The time at which a particular function has completed its part in the cell cycle can be estimated by shifting ts mutants to the restrictive temperature and measuring how many cells go on to complete division. Only those cells that have completed the temperature sensitive function will be capable of completing division, and the proportion of cells so capable can be used to calculate, with some ambiguity, the time at which the function completed its role; this time has been called the 'execution point' (Hartwell, 1974). The execution points of many cdc genes have been determined, and they are found to occur in clusters along the time course of the cell cycle, rather

than being evenly spaced. This observation has been interpreted as meaning that there are only a small number of rate limiting steps throughout the cycle (Nurse, 1981). However, the strictly linear model of mitosis is not upheld by characterising the interdependence of mutations that affect various different parts of the cell cycle, and there has evolved a more complex model in which the processes of cytokinesis, nuclear replication and division, nuclear migration, and spindle pole body (the yeast centrosome analogue) replication, all lie on connected but parallel pathways (Pringle and Hartwell, 1981). Another observation that is consistent with a more complex scheme than a strictly linear pathway is that the CDC2 gene of S. pombe appears to be an important regulator of the cell cycle, but is found to act at two different times, in G1, and late in G2 (Nurse and Bisset, 1981).

Molecular studies have led to the assignment of several of the cdc genes to specific enzymes, and this approach is further elucidating the mechanisms which underlie parts of the cell cycle. Johnston and Nasmyth (1978) showed that cdc9 mutants were defective in DNA ligase activity, and the recent cloning of the the CDC9 gene has confirmed that it is the structural gene for this enzyme (Barker and Johnston, 1983; Peterson et al., 1985). Similarly, the CDC21 gene is thought to code for thymidylate synthetase, and the cdc19-1 mutation appears to be defective in pyruvate kinase (Pringle and Hartwell, 1981). The CDC25 gene of S. pombe has been cloned and over-expressed in

yeast cells (Russell and Nurse, 1986), and has been found to be a dose dependent activator of mitosis; the higher the level of CDC25 expression, the earlier the cell enters mitosis. This observation is reminiscent of the major finding of a completely different approach that has been adopted by others to the study of the cell cycle, namely the discovery of a factor, maturation promoting factor (MPF), which appears to induce metaphase in the Xenopus oocyte.

This biochemical approach to cell cycle analysis was initiated when Rao and Johnston (1970) showed that when mammalian cells are fused, cells in mitosis can induce the onset of metaphase in non-mitotic cells, and that an S-phase cell can induce a cell in G1 to undergo DNA synthesis. This led to the idea that there are diffusible factors which can trigger the various stages of the cell cycle. MPF was first discovered when it was shown that the cytoplasm from unfertilized amphibian eggs was able to induce the maturation of oocytes, including the entry into metaphase of meiosis II (Masui and Markert, 1971; Smith and Ecker, 1971). Subsequently, similar activities have been found in mitotic cells from all species that have been examined (for example, Nelkin et al., 1980; Kishimoto et al., 1982), and it has been proposed that an MPF activity plays a role in the induction of mitotic metaphase in all cells, as well as having a role in maturation and meiosis. Thus MPF appears to be a dose dependent activator of mitosis in a way similar to the

product of the CDC25 gene. This result is encouraging as it suggests that fundamental cell cycle mechanisms may be similar in unicellular eukarotes such as yeast, and complex metazoans such as Xenopus, even though mitotic regulation in the latter is expected to be considerably more complicated than in the former due to the necessity of maintaining fine spatial and temporal control over the proliferation of the different cell types within a single organism. Indeed, the model which has been proposed for the regulation of mitosis from its study in amphibians, differs in a fundamental way from that proposed for yeast. Hara et al. (1980) have shown that there exists a 'master oscillator' function in Xenopus eggs that continues to cycle even when the egg is enucleated and consequently all the normal apparatus of mitosis is removed. This has led to the suggestion that the cell cycle is regulated by a basic clock function that controls the timing of the various rate limiting steps, rather than by a continuous pathway of mutually dependent events. There are clearly some fundamental differences between the two types of model that have been proposed, but it is interesting to speculate about whether they really are mutually incompatible. The accepted yeast scheme is now far from being a simple linear sequence, and many of the events described are on parallel pathways; parallel pathways could also feature in a model that invokes a master clock mechanism, each path being independently regulated by the timer. It would be wrong to assume that both models are

the same - as has been described there are basic differences - but it is not clear that they are as far apart as they first seem. It may be that as the two experimental approaches, genetic and biochemical, converge into molecular biology, so the two interpretations equally come together. Alternatively, the differences may be real, and reflect the different regulatory needs of unicellular and multicellular organisms.

The study of the cell cycle in Drosophila may help to elucidate the mechanisms of mitosis in higher eukaryotes. The ease of isolating mutations allows a genetic approach to be used; the ability to carry out detailed cytology allows morphological changes in the cell cycle to be analysed; and sophisticated techniques have been developed for cloning genes that have been identified by mutations. All these factors make Drosophila an ideal system for such research. The Drosophila embryo is a syncytium for its first thirteen nuclear divisions, which occur at about ten minute intervals (Zalokar and Erk, 1976). The first nine of these divisions are within the interior of the embryo; most of the nuclei and their associated centrosomes then migrate out towards the cortex, until they are only about 10μm below the surface membrane. These nuclei divide in parasynchronous waves of mitosis that start at the two poles of the embryo and move towards the centre (Foe and Alberts, 1983). This allows several stages of mitosis to be seen in a single embryo. Cellularisation occurs at about 2.5 hours of development, at which stage there are

about 10,000 nuclei. This characteristic mitotic activity in early Drosophila embryos is easy to observe in fixed, whole-mount preparations, so any obvious defect in mitosis at this stage is likely to be readily detected. After cellularisation, the pattern of mitosis changes dramatically: the divisions are no longer synchronous, and the cell cycle time elongates - most nuclei divide three or four more times in the subsequent 20 hours before hatching. During the larval stages most cell growth occurs by an endomitotic mechanism, and the cells therefore become polyploid. The major exceptions to this are the imaginal discs, which are the primordia of adult structures and have no role in the larva, and the neuroblasts of the larval brain. Both of these tissues continue to undergo normal diploid cell division; most adult structures also grow by diploid mitosis. This characteristic and well studied pattern of cell division throughout the Drosophila life cycle allows mitosis to be analysed in several different ways. Firstly, it is easy to to examine the nuclei in the early embryo, as has already been described. Secondly, cytological preparations of larval neuroblasts can be used to examine mitotic figures later in development. Thirdly, recessive cuticle markers can be used to identify mitotic abnormalities in adult structures: in flies heterozygous for appropriate visible markers, somatic clones expressing the markers can arise as a consequence of mitotic recombination, chromosome loss, mutation, or chromosome

breakage that results in the loss of a fragment containing a wild type copy of the marker gene. These three different ways of studying Drosophila mitosis have been exploited to isolate many potential cell cycle mutations (Baker et al., 1976; Baker et al., 1978; Gatti et al., 1983; Gelbart, 1974; Ripoll et al., 1985; Smith et al., 1985).

Baker et al. (1976) examined a number of mutations that were known to be defective in meiosis, or to be hypersensitive to mutagens, and found that several also showed mitotic defects, as assayed by the existence of somatic clones of recessive cuticle markers. They concluded that many of the processes of DNA metabolism required during meiosis and DNA repair are also required during mitosis, and these mitotic mutations have since been studied in some detail. For example, Gatti et al. (1983) showed that the mutagen sensitive mutation, mus101, was involved in the condensation of heterochromatin. A different appproach has been to screen a collection of ts lethal mutations for mitotic defects at a semi-restrictive temperature (Smith et al., 1985). 15 out of 168 ts lethals showed mitotic defects, which fell into three main classes: mutations affecting chromosome condensation (like mus101), mutations causing chromosome breakages, and mutations which caused the defective transmission of chromosomes to daughter cells.

It has also been observed that a high proportion of lethal mutations that cause death at the larval-pupal

boundary show mitotic defects. This is presumably because mitotic lesions during larval growth will primarily cause the failure of imaginal disc growth; this will only become lethal when the imaginal discs become essential, that is, during pupariation. This observation implies that the mitosis that is required prior to these late larval stages, notably the nuclear divisions of cleavage, must either be dependent on a different set of zygotically expressed genes, of which none have been identified despite much study, or be dependent on maternally contributed products. This latter possibility is supported by the fact that little or no zygotic gene expression is detected prior to about two hours of embryogenesis (Anderson and Lengyel, 1979), at which stage 11 or 12 rapid nuclear divisions have already taken place. Clearly, for these initial divisions at least, the necessary components are provided maternally. These two observations, the high number of mitotic mutants amongst late larval lethals, and the lack of gene expression in cleavage embryos, provide the rationale for the project that is described in this thesis.

It was decided to look for potential mitotic defects amongst maternal effect lethal mutations in which embryos from homozygous females died early in embryogenesis. If, as has been described above, the components of early mitosis are laid down in the oocyte, a defect in one of these processes would be expected to cause mitotic abnormalities in the developing embryo. There is a major

assumption built into this experimental strategy, that of specificity of the defect to embryogenesis. There are three ways in which a female homozygous for a maternal effect lethal mutation that causes defects in embryonic mitosis can survive herself (as she must if the mutation is to be isolated at all). The first possibility is that the mutation lies in a gene whose product is only required very early in development; since the female must have come from a heterozygous mother, there would have been some wild type function in the egg to allow the homozygous female to complete embryogenesis. The second possibility is that a gene coding for a product important for all mitosis has stage specific promoters or exons; a mutation that affected part of the gene which was active only during oogenesis might be manifest as a maternal effect lethal. The third possibility is that the mutation is a leaky allele of a gene required for mitosis at all stages: if this is the case, it might be able to provide enough wild type function throughout the relatively slow mitotic cycle of larval and adult growth (of about 12 hours duration), but be unable to fulfil its role under the 'mitotic stress' of the extremely rapid nuclear divisions of early embryogenesis. A priori, it seems possible that products specific to early embryonic mitosis do exist, given the qualitative and quantitative differences between it and mitosis at other stages, including its high speed (at least 100-fold more rapid than at other times), the lack of cytokinesis, and the absence of significant levels

of transcription (Anderson and Lengyel, 1979). A mutation in a gene coding for this type of specific product would not be expected to cause defects at any stage other than early embryogenesis. In contrast, a weak allele of a gene required at other times during development as well, might show minor mitotic defects in tissues from larvae or adults homozygous for the allele. Thus the determination of the presence or absence of such zygotic defects can be used to indicate the nature of a gene affected by a mutation of interest.

There has been some debate over the relative merits of the terms 'female sterile' and 'maternal effect lethal'. I use the latter to mean mutations in which the lesions are not manifest until embryogenesis, and as such, maternal effect lethals are a subset of all female steriles, which also include mutations that act earlier in oogenesis. The isolation and characterisation of female sterile and maternal effect lethal mutations of Drosophila has been reported several times (Bakken, 1973; Rice and Garen, 1975; Gans et al., 1975; Mohler, 1977; Komitopoulou, 1983; Zalokar et al., 1975; Wieschaus et al., 1981; Perrimon and Gans, 1983), although none of these studies have had the primary aim of identifying mitotic mutations. Nevertheless, descriptions of the phenotypes that have been identified suggest that many of the mutations which result in the early death of embryos may show mitotic defects. However, it is impossible to know from preliminary analyses whether these defects occur

primarily in mitotic mechanisms, or whether they are secondary effects of other lesions. Bakken (1973) isolated 19 autosomal, recessive, maternal effect lethal mutations of this kind. By staining the chromatin of the eggs from homozygous females it was concluded that the majority of these mutations caused mitotic abnormalities, including tripolar spindles, amorphous chromatin bodies, polyploid nuclei, unevenly distributed cleavage nuclei, and anaphase bridges. Similar defects were observed by Zalokar et al. (1975) in many of the X-linked female sterile mutations isolated by Gans et al. (1975). Again, it should be stressed that the appearance of mitotic lesions does not prove the presence of a primary defect in a component of mitosis. Indeed, it has been shown that eggs which are treated with X-rays (Sonnenblick, 1940) or ultrasound (Counce and Selman, 1951) show abnormalities which frequently include regions of disorganised chromatin, and abnormal spindle and centrosome configurations very similar to those seen in mutants. This suggests that apparent mitotic lesions can be induced by agents that produce rather non-specific defects, and therefore that much caution should be exercised when interpreting mutant phenotypes and trying to deduce their cause.

We have examined a collection of maternal effect lethal mutations that were induced on the third chromosome by ethyl methanesulphonate (C. Nüsslein-Volhard, G. Jurgens, K. Anderson, R. Lehmann, pers. comm.). Several mutations

have been identified which show the same kinds of lesion as described above, and these are currently being analysed in detail (D. Glover, M. Leibowitz, J. Raff, C. Sunkel, pers. comm.). I have studied a mutation in which nuclear division, but not DNA replication, appears to be defective, leading to the formation of a small number of giant nuclei in the syncytial blastoderm. I have named this mutation gnu, as it results in giant nuclei, and refer to the embryos from females homozygous for the mutation as GNU embryos. The striking phenotype of GNU embryos strongly suggested that it affected a gene involved in nuclear division, and thus was the type of mutation in which we were interested. Using indirect immunofluorescent labelling, it has been shown that many parts of the mitotic apparatus are present and functionally adequate in GNU embryos, and it seems that the phenotype is not, in fact, likely to be caused by a lesion in one of the structural components of nuclear division. I have also shown that GNU embryos develop even when unfertilized, and that the primary defect of the gnu mutation appears actually to be in the repression of DNA synthesis that is normally observed in unfertilized eggs. The gnu gene has been mapped, both genetically and cytologically, and there exists a practicable cloning strategy that should allow its isolation. This will mean that the events involved in the activation of the unfertilized egg, a major developmental switch that is common to a very wide range of species, can be

characterised at the molecular level for the first time. It should also be possible to investigate the relationship between this activation process, and the initiation of the first cell cycle in the embryo.

CHAPTER 2

Materials and Methods

2.1 Origin of the gnu Mutation

The gnu mutation was recovered in a systematic screen for maternal effect lethals on ethyl methanesulphonate treated ru th st ri roe p^p e^s ca chromosomes (C. Nüsslein-Volhard, G. Jurgens, K. Anderson, and R. Lehmann, unpublished). The maternal effect lethal mutations lodestar, polo, and thule, were also recovered from this screen.

2.2 Drosophila Stocks Used

Unless otherwise referred to, all genetic loci are as described in Lindsley and Grell (1968). Wild type stocks used were Oregon R or Canton S (Lindsley and Grell, 1968) from cultures that are continuously maintained in this laboratory. The deficiency stocks used for the cytological mapping of gnu (Chapter 5) were those described in Table 5.2. The line of flies bearing the bacterial chloramphenicol acetyltransferase (CAT) gene (see Chapter 4), was the line T3.1 as described in Davies et al. (1986). Briefly, flies homozygous for the mutation ry^{42} were transformed, using standard techniques (Rubin and Spradling, 1982), with a construct containing the CAT gene, and 1.65kb from the 5' end of the Drosophila larval serum protein (LSP) 1α gene, in the transformation vector Carnegie 20. The transformants were made homozygous for the inserted DNA (on the 2nd chromosome). The P-strain

used in the hybrid dysgenic screen (Chapter 5) has been described by Engels (1985b). It has a recessive lethal chromosome balanced with the third chromosome balancer, TM6b, and has been shown to be a 'strong' P-strain. The M-strain females came from a stock homozygous for the markers red and ebony, and were shown by DNA hybridisation to be free of any P-element sequences (R. Karess, pers. comm.). The multiply balanced stock, Basc/Basc; Bw/CyO; Sb/TM2 (see Figure 4.3) is called H41, and was the gift of R. Karess.

2.3 Antibodies

I have used the following antibodies in this study: against nuclear lamin, T47 (H. Saumweber, pers. comm.); against centrosomes, Bx63 (Frasch et al., 1986); and against tubulin, Bx69 (Frasch, 1985). In the double labelling experiment a well characterised rat monoclonal anti-tubulin (from Sera-lab, UK), YL1/2, was used (Kilmartin et al., 1982); this has been shown to recognise specifically α-tubulin in Drosophila (Warn and Warn, 1985). Anti-actin was a mouse monoclonal from Amersham International (UK). I used dilutions of between 1 in 5 and 1 in 15 for hybridoma culture supernatant, and of 1 in 500 for ascites fluid, all in Buffer A (see Section 2.8). Rhodamine and fluoroscein conjugated second antibodies were bought from Jackson Immunoresearch Laboratories Inc. (USA), and were used at dilutions of 1 in 500 and 1 in 100

respectively .

2.4 DNA Hybridisation Probes

The DNA used for hybridisation probes was the kind gift of S. Delaney and D. Hayward. The 'LSP' plasmid was a 1.65kb BamHI fragment from the 5' end of the *Drosophila* larval serum protein 1α gene, cloned in to pEMBL8(+) (Dente et al., 1983). The 'CAT' plasmid was a 1.64kb BamHI - BglII fragment of pSV0CAT (Gorman et al., 1982) which contains the *E. coli* chloramphenicol acetyltransferase gene, cloned into pEMBL9(+) (Dente et al., 1983).

2.5 Drosophila Culture

Unless stated otherwise, all flies were kept at 24°C, on yeast, treacle, cornmeal food (45ml brewer's yeast, 62ml treacle, 110ml maize meal, 15g agar, per litre of water; 30ml 10% Nipagin and 7ml propionic acid added after boiling). Stocks were reared in plastic vials of 25mm x 80mm, or in 1/3 pint milk bottles, and the food was supplemented with dried yeast. Standard fly handling techniques were used (Roberts, 1986).

For the pair-matings and the sterility testing of the hybrid dysgenic screen, glass tubes of approximately 10mm x 100mm were used.

Embryos were collected on petri dishes containing grape

juice agar (80g sucrose, 8g yeast, 48ml concentrated grape juice, and 25g agar per litre of water; 15ml Nipagin and 7ml propionic acid added after boiling), supplemented with a yeast paste. These petri dishes were fitted to the bottom of 1/3 pint milk bottles that had had their base removed.

2.6 Cytological Mapping Crosses

Balanced stocks heterozygous for a known cytological deficiency on the third chromosome were crossed with gnu/TM3 Sb flies, and the resulting Df(3L)/gnu females were then tested for sterility by placing them with 2 or 3 males, and scoring the presence or absence of eggs and larvae after several days.

2.7 Cytological Determination of Deficiencies

Df(3L)/+ larvae were grown on rich yeast, glucose food (100g dried yeast, 100g glucose, 15g agar per litre; 15ml nipagin added after boiling) at 18°C. Third instar larvae that had recently crawled out of the food were selected for dissection. Salivary glands were rapidly dissected in phosphate buffered saline (140mM NaCl, 50mM phosphate buffer, pH 7.4), and transferred immediately to a 4ml glass vial containing ethanol:propionic acid (3:1, made fresh). After at least 30 seconds of fixing, the glands were pipetted onto a clean, dust-free slide, and all

fixative was carefully removed with absorbent tissue. A drop (approximately 0.1ml) of propionic orcein (1% synthetic orcein in 45% propionic acid; refluxed for 30 minutes, and filtered) was placed over the glands and then a clean 18mm^2 glass coverslip. The slide was then wrapped in a piece of filter paper, and tapped firmly, several times with a blunt instrument such as a pencil inside a rubber pipette bulb. The preparations were examined under phase contrast optics using a 63x or 100x objective. Photographs were taken on Kodak 2415 technical film.

2.8 Fixation and Devitellinisation of Embryos

Embryos were fixed and prepared for staining by a slightly modified version of the method of Dequin et al. (1984), which itself is based on the technique of Mitchison and Sedat (1983). Embryos were dechorionated in 60% hypochlorite bleach in 0.7% NaCl/ 0.1% Triton X-100 and then rinsed extensively in NaCl/Triton. Excess liquid was removed by blotting them on tissue paper and they were then 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 minutes. In some experiments 0.5µM taxol (1.25µl of a 1mM solution in DMSO) was included in the mixture to which the embryos were added; in these cases the embryos were shaken for 15-30 seconds

before the formaldehyde was added. In order for the results using taxol to be valid it is important that it only stabilises existing structures, and does not cause a change in the microtubule distribution, as it has been shown to do under certain conditions (Horwitz et al., 1981; De Brabander et al., 1981). De Brabander et al. have shown that in mammalian cells two kinds of microtubule artefact are found upon treating them with doses of taxol from 2.5μM to 100μM for periods of greater than 30 minutes: they see bundles of parallel microtubule fibres forming where none usually exist, and also asters which are not nucleated by centrosomes. Under the extremely mild conditions of taxol treatment in the present study (0.5μM for 15 to 30 seconds before fixing) I see no obvious artefacts in wild type embryos (Figure 3.6). This agrees with the results of Karr and Alberts (1986) who found no microtubule artefacts under identical conditions, nor using five times as much taxol, but started to see spurious microtubule arrays when they used 10μM taxol for five minutes prior to fixation. Furthermore, I have shown that all the asters in GNU embryos are nucleated by centrosomes (Figure 3.9). Taken together these arguments provide strong evidence that taxol is not forming spurious structures under these conditions, and that the microtubule distribution that is seen is a real phenomenon that occurs *in vivo*. After 10 minutes shaking, 2.5ml of methanol was added and the mixture shaken for a further 30 seconds to 2 minutes;

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 floating embryos were then aspirated away, and the devitellinised embryos rinsed twice in methanol, for a total of 10 minutes. They were then rinsed at least three times over an hour in Buffer A (15mM Tris-HCl pH7.4, 60mM KCl, 15mM NaCl, 1.5mM spermine, 0.5mM spermidine) plus 0.1% Triton X-100, and left in fresh Buffer A/Triton for at least 2 hours before staining.

2.9 Antibody Staining of Embryos

Between 50 and 200 embryos were transferred into staining nets that were made by cutting off the bottom 3cm of a 1.5ml plastic microfuge tube and replacing it with a stainless steel mesh of a fine enough gauge to retain embryos. All subsequent stages were carried out in these nets, placed in wells of a 24-well tissue culture plate. The embryos were incubated in Buffer A containing 10% foetal calf serum for 1 hour at room temperature, blotted dry, and then incubated in first antibody for 12 - 16 hours at 4°C. After this period excess liquid was removed by blotting and the embryos were washed in Buffer A for 1 - 2 hours with three changes, and then incubated in second antibody for 12 - 16 hours at 4°C. At the end of this incubation they were washed for at least 2 hours with four changes of Buffer A, and then for 10 minutes in Buffer A

containing 1µg/ml of Hoechst 33258 (Riedel De Haen AG, Hannover, FDR), finally being rinsed twice in Buffer A. The stained embryos were mounted in 85% glycerol containing 2.5% n-propyl gallate (Giloh and Sedat, 1982). Slides were viewed with a Zeiss Standard or Nikon Microphot FX microscope fitted with epifluorescent optics using appropriate filters for Hoechst, fluoroscein, and rhodamine. Embryos were photographed on Kodak 2415 Technical film and developed with Kodak D-19 developer.

The level of non-specific staining was determined by incubating embryos with second antibody alone. There was found to be a low level of uniform background staining using both rhodamine and fluoroscein conjugated antibodies, but no specific structures were detected.

2.10 Preparation of Larval Neuroblast Metaphase Chromosomes

Larval neuroblasts were cytologically prepared by the method of M. Gatti (pers. comm.). Third instar larvae that had crawled out of the food were selected, and the brain was dissected out in physiological saline (0.7% NaCl). As much as possible of the attached chitonous and fatty material was removed, as were the attached imaginal discs. The brain was then incubated in colchicine (Aldrich, approximately 10^{-5}M) for 90 minutes. After this period, the brains were transferred, using forceps, to a depression slide containing hypotonic solution (0.5%

sodium citrate) for 10 minutes. This treatment was followed by 15 - 30 seconds fixing in a small petri dish, and then each brain was placed in a small drop of 3.5% aceto-orcein on a clean, dust-free coverslip. The fixative was 9.25ml methanol, 9.25ml glacial acetic acid, 1.5ml distilled water, and the aceto-orcein was prepared by gently refluxing 0.35g of Natural orcein (Aldrich) in 45% glacial acetic acid for 45 minutes; the stain was filtered, and was cleared by centrifugation (2 minutes in an Eppendorf Microfuge) immediately before use. The coverslip with the brains was picked up with a clean microscope slide after the brains had incubated for 1 - 2 minutes in stain, and the preparation was squashed as hard as possible with the thumb. The edges of the coverslip were then sealed with nail varnish, and the chromosome spreads were viewed with phase contrast optics using a 63x or 100x objective.

2.11 Preparation of DNA from Drosophila Embryos

Between 50 and 500 embryos were placed in a glass 1ml homogeniser that had been precooled in a ethanol/dry ice bath, and were ground to a powder. 100µl of Solution 1 (10mM Tris-HCl, pH7.5, 60mM NaCl, 10mM EDTA, 0.15mM spermine, 0.15mM spermidine, 5% sucrose) was added to the powder, and as the mixture thawed, the tissue was homogenised. The homogenate was transferred to a 1.5ml polypropylene tube and the homogeniser rinsed with 50µl

more of Solution 1, which was also transferred to the 1.5ml tube. 150µl of Solution 2 (1.25% sodium dodecyl sulphate (SDS), 0.3M Tris-HCl, pH9.0, 0.1M EDTA, 5% sucrose) was added, the tube vortexed for a second, and then incubated at 65°C for 40 minutes. The tube was cooled and 45µl of Solution 3 (8M potassium acetate) was added. After 45 minutes on ice, the debris was pelleted in an Eppendorf Microfuge for 10 minutes, and the supernatant carefully removed to a clean tube. DNA was precipitated by incubation for 15 minutes at -70°C in the presence of 2 volumes of ethanol, and then pelleted for 10 minutes in an Eppendorf Microfuge. The pellet was resuspended in TE (10mM Tris-HCl, pH8; 1mM EDTA).

The DNA preparations were routinely treated with proteinase K (100µg/ml) for 1 hour at 45°C, followed by a phenol extraction (Maniatis et al., 1982), and a further ethanol precipitation step. Initially, samples were treated with RNAse (1µg, 37°C, 1 hour), however this was found to make no difference to the results of the experiments, and this procedure was not continued.

2.12 Immobilisation of DNA on Nitrocellulose Filters

DNA was immobilised onto nitrocellulose using a 'slot-blot' or a 'dot-blot' apparatus, which consists of a manifold of holes through which DNA solutions are drawn using a vacuum pump; a nitrocellulose filter is placed between the holes and the source of the vacuum, so that

DNA in solution is retained on a localised area of the filter. The DNA solutions were initially denatured by the addition of 0.1 volume of 3M NaOH followed by a ten minute incubation at room temperature. The solutions were neutralised with an equal volume of 2M ammonium acetate, and the samples loaded into the manifold; the nitrocellulose had been soaked in water for 5 minutes previously, and the slots were rinsed through with 1M ammonium acetate prior to loading the DNA, and again after loading. The filter was rinsed in 2x SSC (1x SSC is 150mM NaCl, 15mM sodium citrate), and baked for 2 hours at 80°C.

2.13 DNA Hybridisation

Filters were prehybridised for 4 hours at 65°C in 5x SSC, 10x Denhardt's solution (0.2% polyvinylpyrolidine, 0.2% bovine serum albumin, 0.2% ficoll), 0.1% SDS, and 50µg/ml sonicated salmon sperm DNA which had been denatured by boiling for 5 minutes and then quenched on ice. Hybridisation was carried out in a solution of the same composition for 16-18 hours, with the radioactive probe being present at 10-50ng/ml. The filters were washed over 2 hours at 65°C in two changes of 2x SSC, 0.1% SDS, followed by two changes of 0.2x SSC, 0.1% SDS, and then allowed to air dry. Before autoradiography, the filters were wrapped in cellophane (Saran Wrap). Autoradiography was carried out at -70°C using an intensifying screen.

2.14 Radiolabelling of DNA

DNA was labelled with the isotope ^{32}P using nick translation (Rigby et al., 1977) of a whole plasmid, or by 'oligo-labelling' (Feinberg and Vogelstein, 1983, and addendum, 1984) a plasmid insert that had been previously isolated on an agarose gel.

A standard nick translation protocol was used. A typical reaction contained in a total of 10µl: 250ng of DNA, 20pg DNAse I, 5 units of *E. coli* DNA polymerase I, 50mM Tris HCl, pH7.5, 5mM $MgCl_2$, 50µg/ml bovine serum albumin (BSA), unlabelled dNTPs at 25µM, and 50µCi of ^{32}P αdNTP (specific activity >3000Ci/mmole, Amersham International). The reaction was carried out at 14°C for 60 minutes.

The oligo-labelling protocol was exactly that of Feinberg and Vogelstein (1984). Briefly, the fragment of interest was cut out of a 1% low gelling temperature agarose gel, and dissolved in water (3ml/g of gel) by heating to 100°C for 7 minutes. This solution was then stored at -20°C until used. Before setting up the reaction, this solution was boiled for 7 minutes, and then incubated at 37°C for 10 minutes. The labelling reaction was carried out in 50µl and contained: 10µl oligolabelling buffer, 2µl BSA (10mg/ml), 32.5µl DNA fragment in gel solution (approximately 250ng DNA), 50µCi ^{32}PαdCTP (>3000Ci/mmole, Amersham International), and 2 units of Klenow fragment of *E. coli* DNA polymerase I.

Oligolabelling buffer is: 125μM unlabelled dNTPs (usally A, G, and T), 260mM Tris HCl, pH8.0, 750mM HEPES, pH6.6, 320mM $MgCl_2$, 62mM 2-mercaptoethanol, and 34 optical density units/ml of random 6-mer oligonucleotide (P. L. Biochemicals). The reaction was carried out at room temperature for 12 - 16 hours.

In both cases, the efficiency of the reaction was assayed by measuring the incorporation of the labelled nucleotide into acid precipitable counts. The DNA from an aliquot of the reaction was precipitated with 1ml of acid wash (1M HCl, 1% NaH_2PO_4, 1% $Na_4P_2O_7$) in the presence of 50μg of carrier salmon sperm DNA. After 5 minutes on ice the mix was filtered through a 1cm GF/C disc (Whatman), and the precipitated counts measured by Cherenkov radiation. Nick translations showed typical incorporations of between 30% and 60%, and oligo-labelling efficiency was usually between 60% and 90%, giving probes with specific activities of between 1×10^8 and 1×10^9 dpm/μg.

Labelled DNA was separated from unincorporated nucleotides by passing the reaction mixture over a Sephadex G50 (medium) column which had been previously equilibrated with 10mM Tris-HCl, pH7.5, 20mM NaCl, 2mM EDTA, 0.1% SDS, and collecting the first radioactive peak to be eluted from the column. In the case of oligo-labelling reactions, the reaction mix was heated to 65°C for 5 minutes prior to loading it onto the Sephadex column.

CHAPTER 3

gnu: A Nuclear Division Mutation of Drosophila That Causes the Dissociation of Nuclear and Centrosomal Division

3.1 INTRODUCTION

Some aspects of the phenotype of a maternal effect lethal mutation of *Drosophila* that is defective in nuclear division, but apparently not DNA replication, thus causing the formation of giant nuclei in the syncytial blastoderm, are described in this chapter. This mutation was isolated during an ethyl methane sulphonate mutagenesis screen for maternal effect lethal mutations on the third chromosome (C. Nüsslein-Volhard, pers. comm.; see Materials and Methods for more details). I have named this mutation *gnu*, as it results in giant nuclei, and use the term 'GNU embryos' to describe the embryos from homozygous females.

Because nuclear division is so dramatically disrupted in GNU embryos, I decided to examine the components of the cell cycle apparatus in order to determine whether the giant nucleus phenotype could be correlated with a particular defect in mitosis. To this end, a series of monoclonal antibodies against different components of the mitotic apparatus have been used to stain these embryos with indirect immunofluorescent techniques. The results of this analysis have indicated that whilst the nuclear cycle of GNU embryos is disrupted, many aspects of the cytoplasmic cycle are unaffected. Specifically, the centrosomes replicate independently of nuclei in mutant embryos and are functionally capable of nucleating microtubule growth.

Centrosomes are the organelles that organise the spindle poles and thus must divide once each cell cycle in order to maintain their ratio with nuclei; their replication is a major event in the early stages of mitosis. A number of groups have reported that under some conditions a limited amount of centrosomal replication can occur in the absence of nuclear division (for example see Mazia et al., 1960; Sluder and Begg, 1985; Wolf, 1980). There has, however, been some speculation about the extent to which this occurs. It is important to know whether these major events of the cell cycle are regulated independently, or whether they are part of a cascade, each event dependent on the previous one in the pathway. In the former case one would expect to be able to uncouple the events, whereas in the latter the disruption of one would necessarily lead to disruption of the other. My observation that the centrosomes in GNU embryos replicate independently of the nuclei, suggests that at least these two major processes are regulated independently during the cell cycle, rather than one being a necessary preliminary to the other.

Since the gnu mutation appears to affect nuclear division, a process common to all mitotically dividing cells, the fact that it is a maternal effect lethal, and that adults homozygous for the mutation are apparently unaffected is surprising. A cytological analysis of larval neuroblasts of homozygotes has failed to

demonstrate any zygotic effects of gnu. This suggests that the process in which the gnu gene product is involved is limited to oogenesis and early embryogenesis. Since mitosis occurs at other stages of the Drosophila life cycle as well as at these times, it would appear that the gnu gene product is not necessary for all mitosis, but instead mediates a process unique to these very early stages.

3.2 DNA Replication Without Nuclear Division Causes Giant Nuclei in GNU Embryos

The most striking characteristic of embryos from a homozygous *gnu* mother can be seen by staining their DNA with the fluorescent dye Hoechst 33258. Two such embryos are a shown in Figure 3.1. It is clear from the size of the nuclei, and the intensity of the fluorescence that DNA replication occurs within these embryos, but there is little or no nuclear division, resulting in the formation of a small number of giant nuclei. This contrasts with the appearance of the three wild type embryos in Figure 3.1. These are at division cycles 9, 10, and 13 and contain progressively greater numbers of nuclei with a diploid DNA content. *gnu* maps to the left arm of the third chromosome, just to the left of *thread*, at map position 42.5, and several deletions of segments of Chromosome 3 that uncover the *gnu* mutation have been identified (see Chapter 5). Embryos from mothers heterozygous for *gnu* and these deficiencies exhibit the GNU phenotype. This suggests that the *gnu* mutation described here produces very little or no wild type gene product and thus can be considered an extreme *gnu* allele.

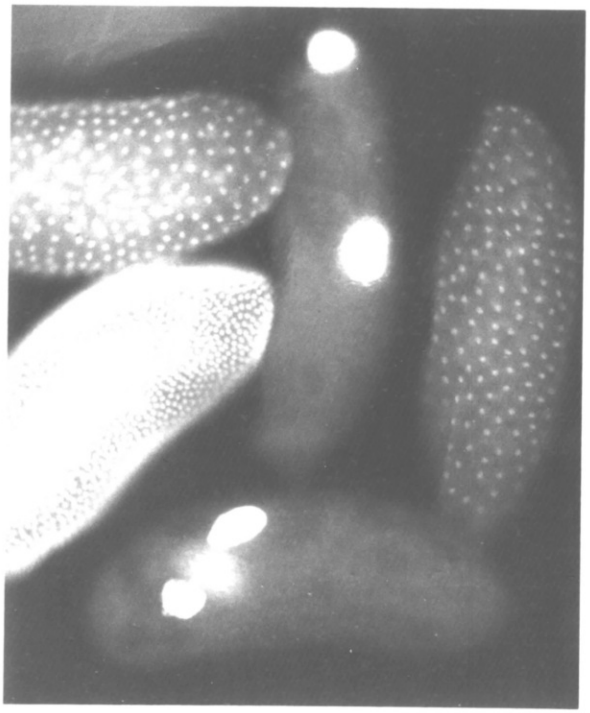
I have followed the development of the nuclei in GNU embryos. Ten minutes after egg deposition apparently

Figure 3.1

Nuclei of Wild Type and GNU Embryos

Embryos were prepared as described in Materials and Methods (Section 2.8), and their DNA stained with Hoechst. In this field three wild type embryos of stages 9, 10, and 13 with approximately 500, 1000, and 3000 nuclei are lying alongside two GNU embryos with giant nuclei.

Figure 3.1



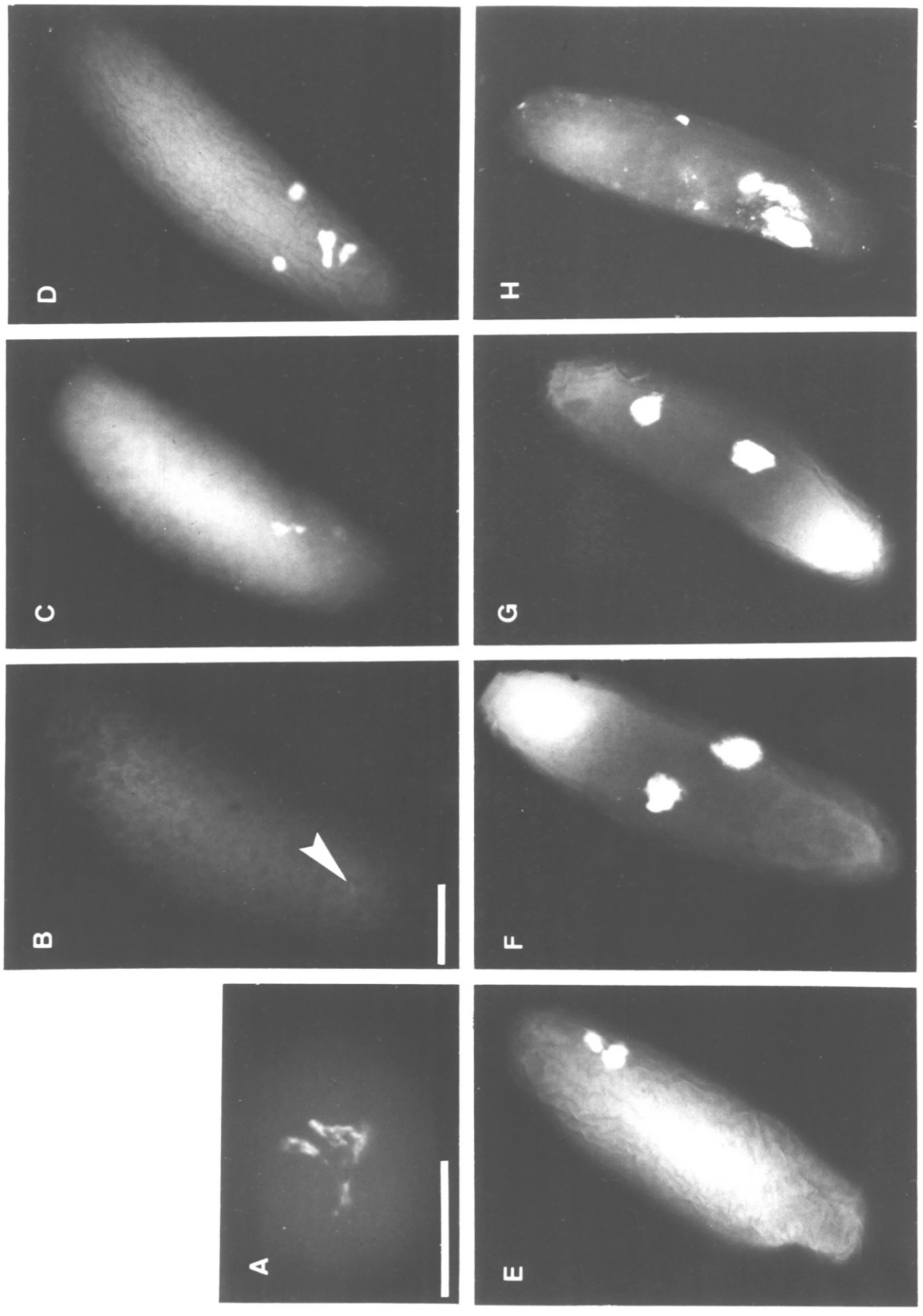
normal meiotic products, three polar bodies and the female pronucleus can be seen, together with the male pronucleus (data not shown). There is a considerable variation of the morphology of these haploid nuclei in both wild type and GNU embryos, which makes it difficult to determine whether there are any minor abnormalities in the GNU embryos at this stage. Figure 3.2 shows representative embryos that have been allowed to develop for increasing amounts of time before fixing and staining. At 25-40 minutes the embryos are already quite distinct from wild type embryos (Figure 3.2; panels A & B), being larger and having a more diffuse appearance. Most embryos have between one and three nuclei and the number of nuclei per embryo does not appear to increase with time: instead the it seems to be set at an early stage and those nuclei then continue to grow without further division (Figure 3.2; panels C-F). The nuclei do not appear to undergo a significant increase in size after 8 hours (Figure 3.2; panel F), by which stage in wild type embryos gastrulation and germ-band elongation have occurred with considerable differentiation of gut and neural tissues. By 24 hours the giant nuclei begin to break down and release pieces of chromatin throughout the embryo (Figure 3.2; panel H). By studying time courses like the one shown in Figure 3.2, it is clear that giant nuclei are frequently derived from the polar bodies of GNU eggs, as well as from the pronuclei. In contrast, the polar bodies of wild type embryos never

Figure 3.2

The Development of GNU Embryos

Embryos were collected for a period of 15 minutes and then allowed to develop at 25°C until they reached 30-45 minutes, panel B; 60-75 minutes, panel C; 90-105 minutes, panel D; 120-135 minutes, panel E; 8 hours, panel F; 16 hours, panel G; and 24 hours, panel H. They were then fixed and stained with Hoechst. A representative embryo from each age group is shown. Panels B-H are at the same magnification, and the bar in panel represents 80μm. Panel A is a high power view of the nucleus which is indicated by an arrow in panel B, with the bar representing 20μm.

Figure 3.2



replicate, and can often still be seen in their original haploid form after many rounds of cleavage have occurred (Sonnenblick, 1950). This phenomenon is examined in more detail in Chapter 4. Examination of a similar series of GNU embryos by phase contrast microscopy has shown that the pole cell buds never form (as characteristically occurs at cycle 10 of wild type embryos), and that there is no cellularisation of the embryo as would occur at cycle 14 in wild type embryos. The observations that have been described suggest that while nuclear division is almost completely absent in GNU embryos, DNA replication occurs in a relatively unregulated manner, leading to the formation of giant nuclei derived from the pronuclei and polar bodies.

3.3 The Giant Nuclei Have Envelopes Containing Lamin

The lamins are a group of proteins associated with the nuclear membrane of eukaryotic cells. The staining given by antibodies directed against nuclear lamins in wild type *Drosophila* embryos has been examined by Fuchs et al. (1983). These antibodies stain the nuclear envelope strongly at interphase. At metaphase, staining by anti-lamin antibodies is greatly reduced and the antigen appears to be dispersed. To determine whether the giant nuclei in GNU embryos are surrounded by envelope proteins

with similar characteristics, I stained them with an antibody that recognises Drosophila nuclear lamin (T47, H. Saumweber, pers. comm.). The staining pattern obtained suggests that the giant nuclei are enveloped by a lamin-containing membrane (Figure 3.3). It should be noted that not all the nuclei do stain with anti-lamin (compare the DNA and lamin staining in Figure 3.3a), although it is not possible to say whether this is due to the absence of a nuclear envelope around some giant nuclei, or whether the envelope remains but the antigen recognised by T47 is absent or modified. Alternatively, the variation may be an artefact of staining or fixing, although this seems rather unlikely since it has been observed that while staining efficiency of most antibodies varies between embryos, it is usually consistent within an embryo (data not shown).

3.4 Centrosomes Continue Independent Replication and Division

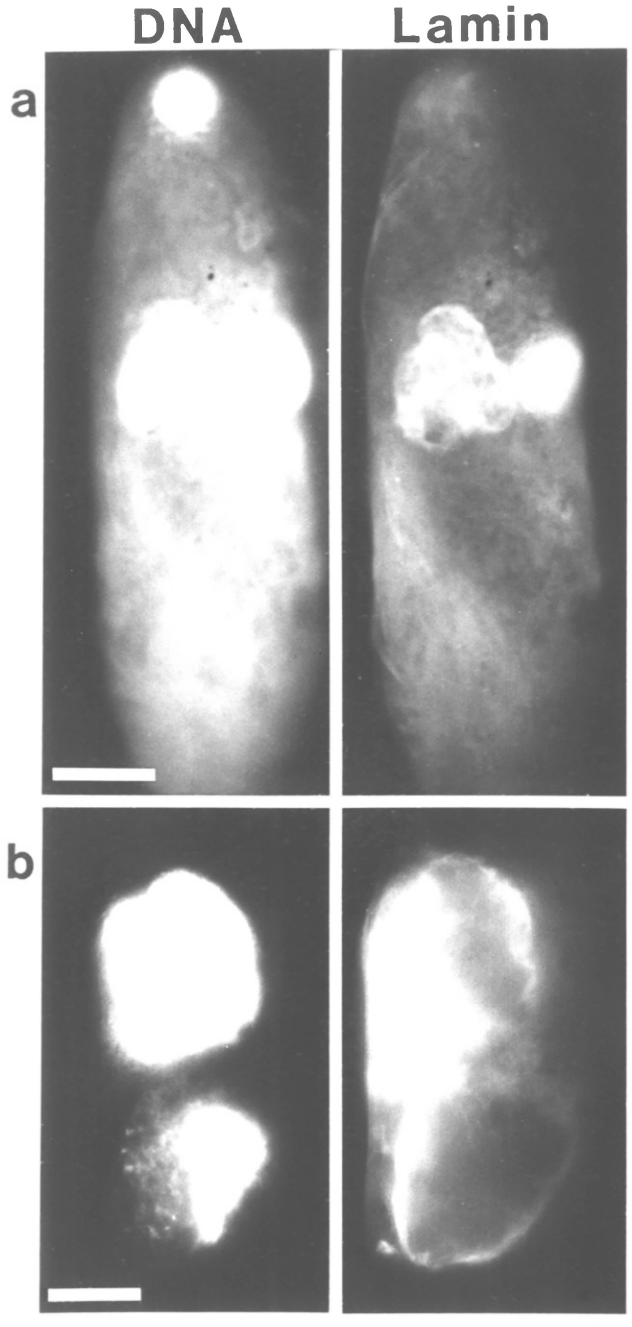
In addition to the nuclear division cycle, cyclical mitotic activities occur within the cytoplasm of normal cells, one of which is the division of the centrosome and the subsequent movement of the daughter centrosomes to the pole positions of what will become the mitotic spindle. In fertilized sea urchin eggs one round of centrosomal division can be induced independently of nuclear division

Figure 3.3

The Nuclear Lamin Staining of GNU Embryos

GNU embryos were incubated with an antibody against *Drosophila* nuclear lamin, T47, and then with rhodamine conjugated anti-mouse IgG, and Hoechst (see Materials and Methods). Panel 'a' shows a GNU embryo containing three giant nuclei; it can be seen that two of these are surrounded by a membrane-like structure that is stained by T47, whereas the top nucleus in the embryo shows no T47 staining. Panel 'b' is a higher magnification view of another giant nucleus. The bar in Panel 'a' represents 80μm, and that in 'b', 20μm.

Figure 3.3



under certain conditions (Mazia et al., 1960). The extreme disruption of nuclear division in GNU embryos raises questions about the presence, and distribution, of centrosomes and related components of the normal mitotic cycle. The antibody Bx63, which recognises Drosophila centrosomes (Frasch et al., 1986), has been used to discover whether GNU embryos have any recognisable centrosomes. A detailed description of the staining of wild type embryos with this antibody has been given by Frasch et al. (1986). Briefly, there are two components to the staining pattern: centrosomes are stained strongly, and there is also a weaker nuclear staining that persists into metaphase and anaphase in an area over the spindle. The upper panel of Figure 3.4 shows a field of nuclei in late anaphase in the cortex of a blastoderm embryo of wild type Drosophila; centrosome staining is seen as distinct dots at the spindle poles.

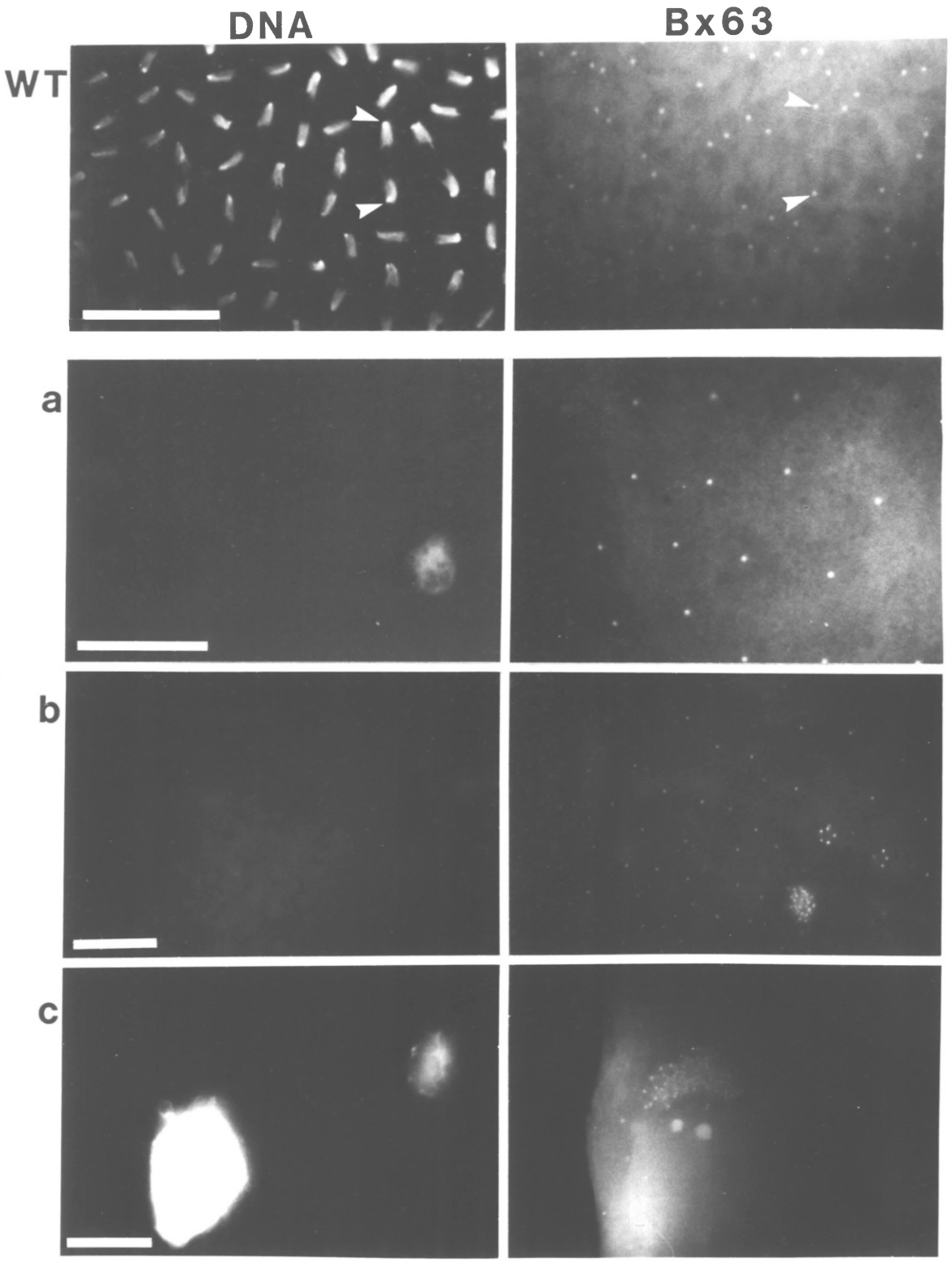
As there has been no report of anything other than very limited independent centrosomal replication, it was surprising to find that GNU embryos do contain centrosomes that are apparently completely dissociated from the nuclei. Since eggs that have been recently laid contain no visible centrosomes, their presence in GNU embryos implies that centrosomes continue to replicate and migrate to the cortex in a manner unaffected by the absence of nuclear division. Their distribution varies between embryos and between areas within the same embryo. In some

Figure 3.4

Centrosome Staining of Wild Type and GNU Embryos

Wild type (WT) and GNU (a-c) embryos were incubated with an antibody directed against centrosomes, Bx63, and then with rhodamine conjugated anti-mouse IgG, and Hoechst (see Materials and Methods). The upper panel (WT) shows a field of anaphase chromosomes from a wild type embryo in which centrosomes are found at the spindle poles. The poles of one anaphase figure and their corresponding centrosomes are indicated by arrows. In GNU embryos there is no correspondence between the positions of centrosomes and nuclei. Weak nuclear staining is also sometimes apparent with the Bx63 antibody (panel 'c'). The bars represent 20μm.

Figure 3.4



regions they form a rather regular array just below the surface of the embryo (see Figure 3.4a). They often form clusters, ranging in number from 3 or 4 to more than 20, which are frequently, but not exclusively, in the proximity of a giant nucleus (Figures 3.4b and 3.4c). In some embryos a weak staining over giant nuclei is also found with Bx63 (Figure 3.4c). Because embryos are rather large and opaque structures, the centrosomes are most easily seen close to the surface. However, in a few cases in which the embryos have been damaged, centrosomes may be seen deeper within the cytoplasm.

3.5 The Distribution of Microtubules in GNU embryos

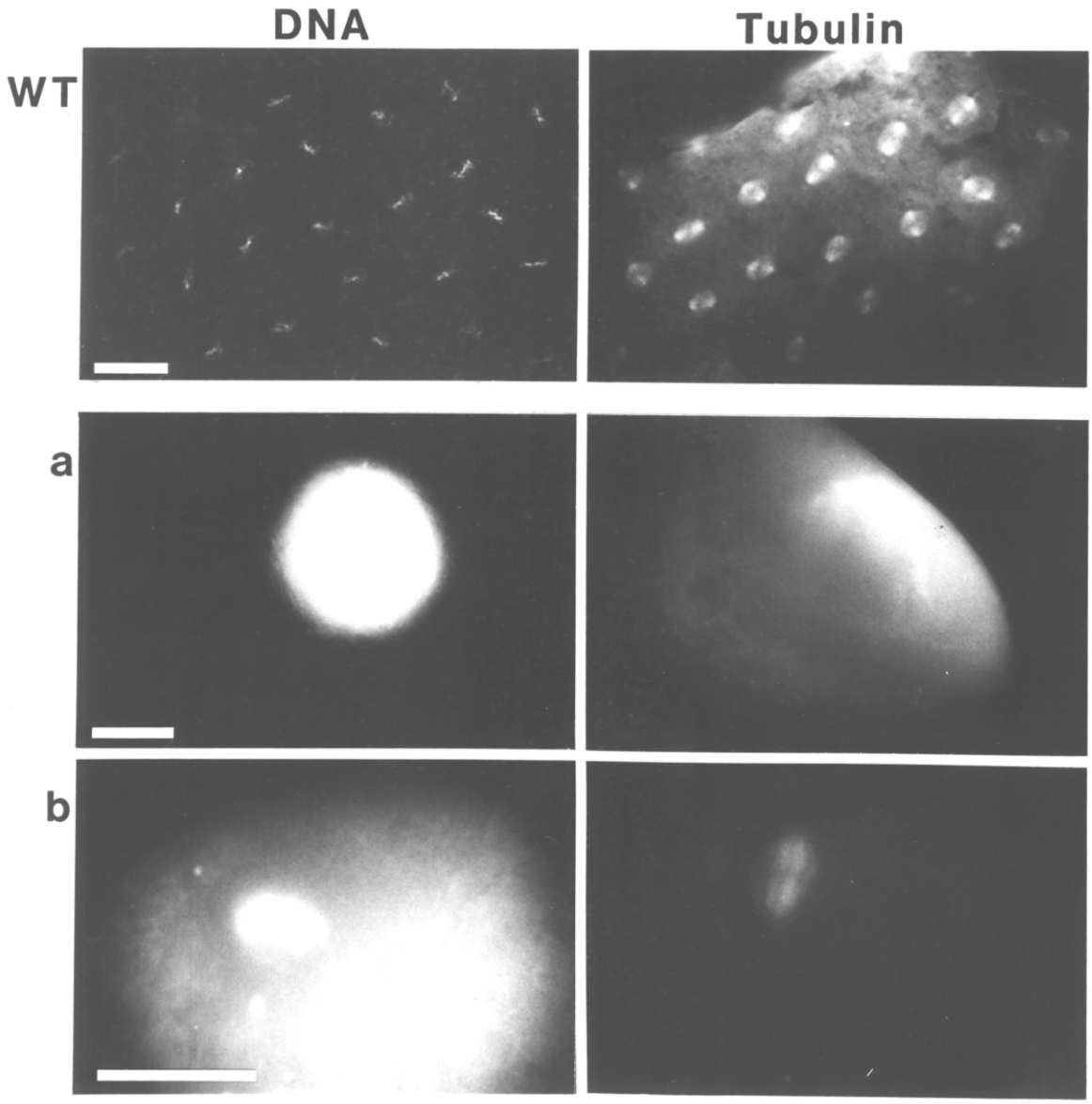
Microtubules are nucleated by centrosomes into asters, and during metaphase become attached to the kinetochores of chromosomes on the metaphase plate to form the mitotic spindle; they are thus a crucial component of the mitotic apparatus. Figure 3.5 shows the staining of wild type and GNU embryos with an antibody against *Drosophila* tubulin, Bx69 (Frasch, 1985). Microtubules are fairly labile in the presence of formaldehyde, in which these embryos are fixed, but nevertheless the metaphase spindles are preserved and stained in the wild type embryo (Figure 3.5, WT). In GNU embryos two forms of staining were observed. Figure 3.5a shows a rather weak staining that lies over a giant nucleus, whereas 5b shows the staining of an

Figure 3.5

Tubulin Staining of Wild Type and GNU Embryos Without Taxol

Wild type (WT) and GNU (a and b) embryos were incubated with Bx69, an antibody against Drosophila tubulin, then with rhodamine conjugated anti-mouse IgG, and Hoechst (see Materials and Methods). In the wild type embryo spindle microtubules are associated with each set of metaphase chromosomes. In panel 'a' weak anti-tubulin staining is apparent over a giant nucleus. In panel 'b' a single spindle can be seen to lie over a small piece of chromatin in a late GNU embryo. The bars represent 20μm in panels 'WT' and 'a', and 12μm in panel 'b'.

Figure 3.5



apparently normal spindle. Spindles like this are rather rare, but when found, are invariably associated with small pieces of chromatin released from the breakdown of a giant nucleus, of the type shown in Figure 3.2H. The presence of spindles with apparently normal morphology suggests that there is no major abnormality of the spindle components caused by the gnu mutation.

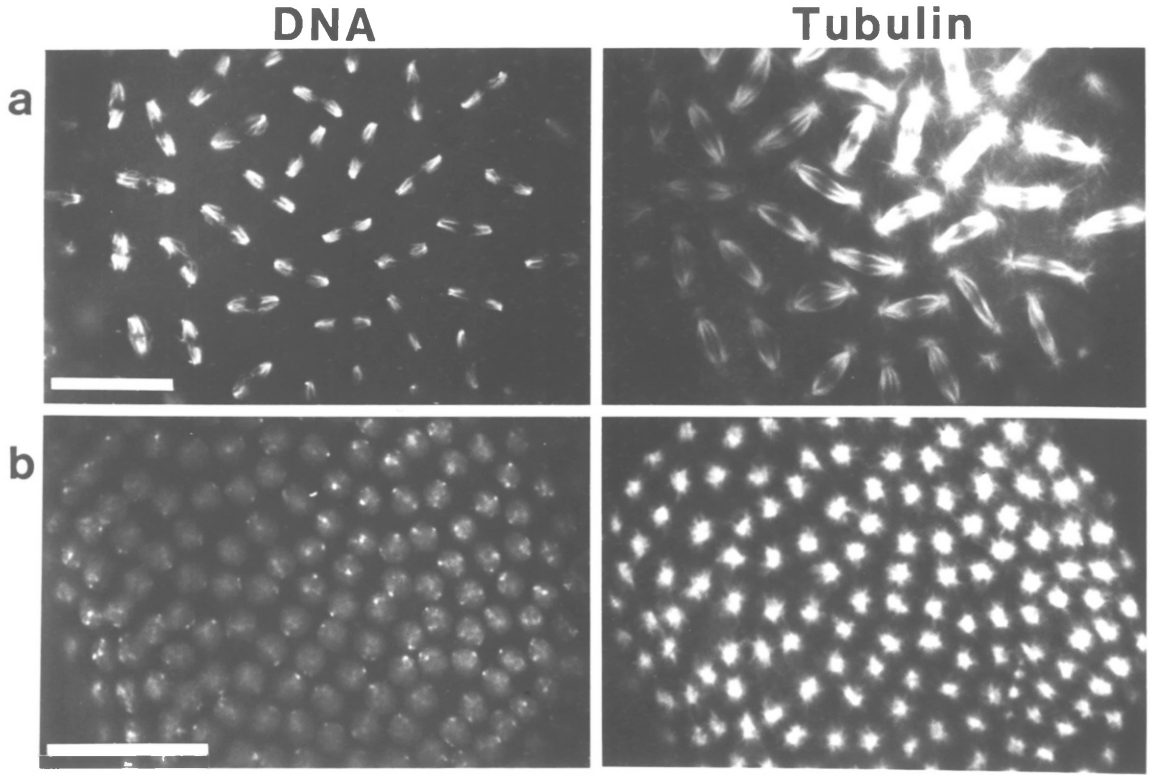
The distribution of microtubules by indirect immunofluorescence after a very brief exposure to taxol, a microtubule stabilising toxin (Wani et al. 1971), has also been examined. The pattern of microtubules throughout mitosis, under these fixation conditions has been described in detail by Karr and Alberts (1986). I was able to repeat their observations, and in Figure 3.6 show just two stages of mitosis in the cortex of the wild type embryo. At anaphase there are spindles with small asters also growing from the centrosomes (Figure 3.6a), and at interphase we observe a single aster per nucleus (Figure 3.6b). In taxol treated GNU embryos a variety of microtubule staining patterns are seen. In early embryos (those with small nuclei) a cortical layer of tubulin with the appearance of a fine network is stained (data not shown); this is similar to the cortical staining of early wild type embryos (Karr and Alberts, 1986). Later GNU embryos show structures that are like asters except that they grow to be very large (Figure 3.7): Figure 3.7b shows one aster that is about twenty times the diameter of the

Figure 3.6

Tubulin Staining of Wild Type Embryos Fixed in the Presence of Taxol

Embryos were fixed in the presence of 0.5μM taxol (see Materials and Methods, Section 2.8), and were then immunostained with Bx69 as in Figure 3.5. Two phases of the mitotic cycle are shown. Panel 'a' shows anaphase chromosomes with corresponding spindles, and small asters that project from the centrosomes in the opposite direction from the spindles. Panel 'b' shows interphase nuclei, each associated with a single aster. The bars represent 20μm.

Figure 3.6



wild type interphase asters in Figure 3.6b. These asters invariably occur near the surface of the embryo, and their distribution appears to be independent of the location of giant nuclei. When the giant nuclei begin to break down two new types of staining appear. Figure 3.7c shows the presence of spindle-like structures (marked with arrows) which are frequently found in the presence of taxol. They are formed over small pieces of chromatin and correspond to the structures that were observed in embryos that had not been treated with taxol (Figure 3.5b). Finally, Figure 3.7d shows the irregular mesh of microtubules that can often be found on the surface of old embryos whose nuclei are breaking down

3.6 The Asters in GNU Embryos Are Nucleated by Centrosomes

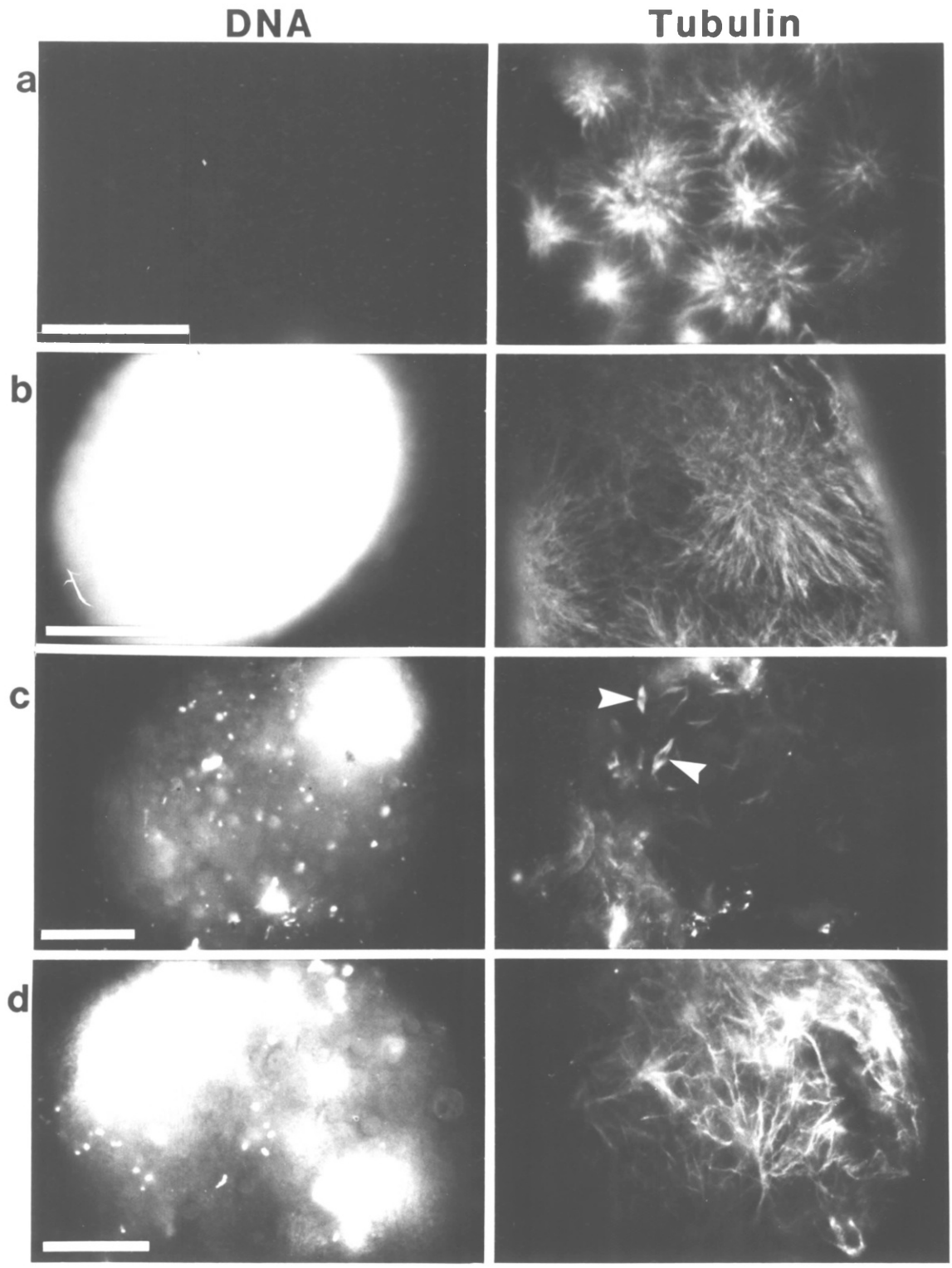
In order to try and understand the unusual distribution of microtubules found in GNU embryos I was interested to discover whether centrosomes in GNU embryos are able to nucleate microtubules into asters as in wild type embryos. Using well characterised antibodies against tubulin, YL1/2, and against centrosomes, Bx63 (Frasch et al., 1986), I stained both sets of structures simultaneously by indirect immunofluorescence. Figure 3.8 shows two fields, one of which contains three centrosomes in a field in which there are no nuclei, the other showing many

Figure 3.7

Tubulin Staining of GNU Embryos Fixed in the Presence of Taxol

Embryos were fixed and stained as in Figure 3.6. The plane of focus is just below the surface of the embryo in each case. In panel 'a' asters can be seen in a region where there is no giant nucleus. Panel 'b' shows a large aster with a giant nucleus lying under it. In panel 'c' the giant nucleus of an embryo is breaking down, and two spindles (indicated by arrows) can be seen associated with small discrete pieces of chromatin. Panel 'd' shows another old embryo with a disintegrating nucleus, in which there is an irregular network of microtubules. Bars represent 20μm.

Figure 3.7



centrosomes in the vicinity of a giant nucleus. In all cases a centrosome is seen at the centre of each aster, however large. This indicates that centrosomes can nucleate microtubules in GNU embryos, so the structures seen are genuinely analagous to asters and not spurious microtubule arrays. Interestingly, large clusters of centrosomes appear unable to nucleate microtubules effectively (Figure 3.8b).

3.7 The Distribution of Cortical Actin

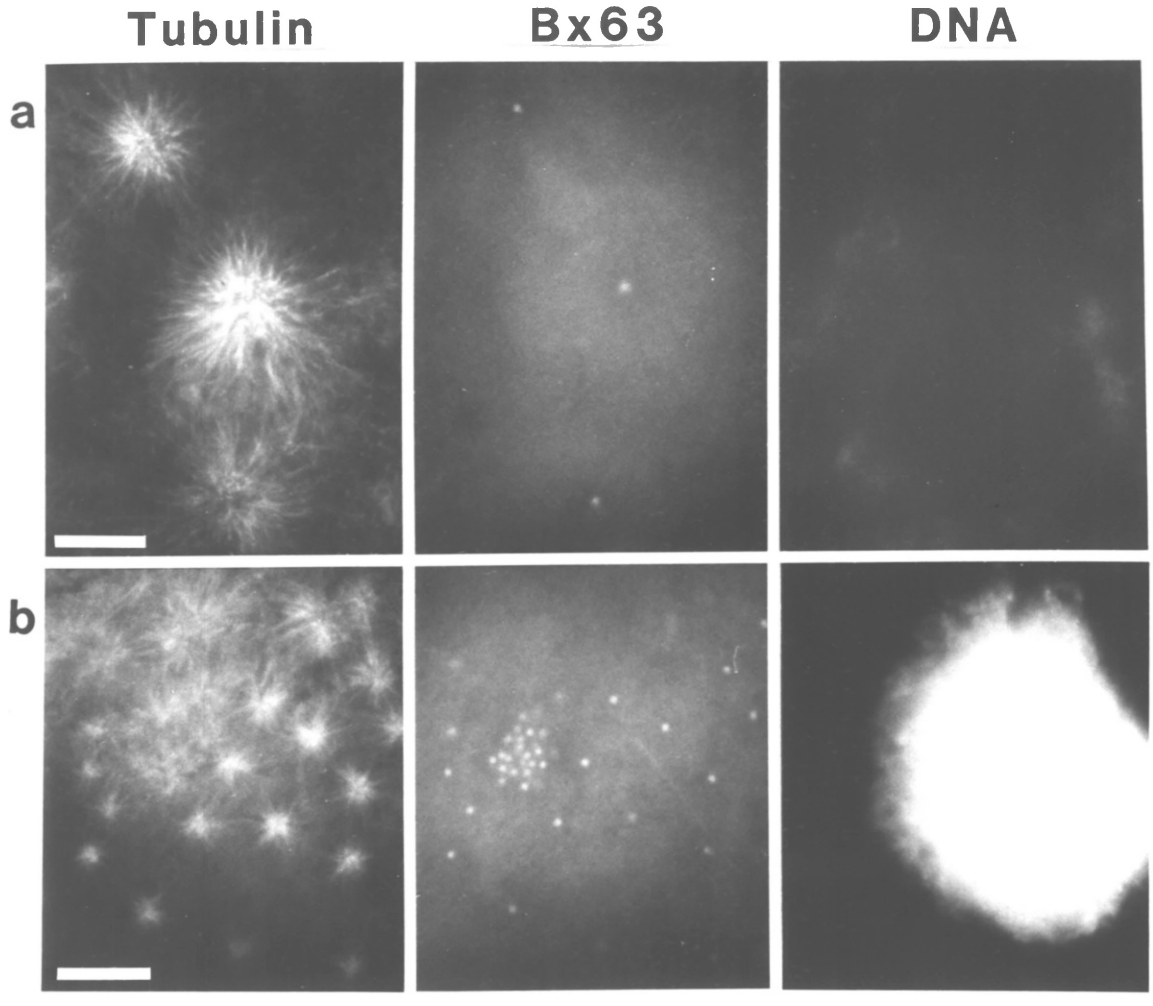
The surface of the early wild type *Drosophila* embryo exhibits a characteristic pattern of actin rich 'caps' which divide roughly in unison with the underlying nuclei (Warn and Magrath, 1982; Warn et al., 1985; Karr and Alberts, 1986). Consequently there is a close association between the centrosomes associated with each nucleus and the actin cap also associated with that nucleus. During the last two or three divisions of the syncytial blastoderm, these caps of actin are closely abutted, thus giving the appearance of actin-rich furrows between each nucleus. Figure 3.9a shows such a wild type embryo with the pole cells in the bottom right-hand corner. The unusual pattern of staining observed in GNU embryos is shown in Figure 3.9b. The surface of late embryos is covered with an irregular array of actin-sparse holes in a background of cortical actin staining. Early GNU embryos,

Figure 3.8

Double Immunostaining of GNU Embryos with Anti-Tubulin and Anti-Centrosome Antibodies

Embryos were incubated sequentially with Bx63 antibody (mouse anti-centrosome); then with rhodamine conjugated anti-mouse IgG; then with YL1/2 (rat anti-tubulin); then with fluoroscein conjugated anti-rat Ig, and finally with Hoechst (see Materials and Methods). Panel 'a' shows three large asters nucleated by single centrosomes. Panel 'b' shows a field of centrosomes in the vicinity of a giant nucleus. Asters of microtubules radiate from the well separated centrosomes, whereas the cluster of centrosomes appears not to nucleate microtubules efficiently. Bars represent 10μm.

Figure 3.8



on the other hand, show continuous cortical actin staining much like early wild type embryos (data not shown). This network of holes in the surface actin does not correspond to any type of actin staining in the wild type embryo, so its cause is not obvious. However it is clear that the surface actin distribution in GNU embryos does change with time, in the absence of nuclear division.

Double-label indirect immunofluorescence to monitor the distribution of both centrosomes and actin has also been carried out. These data (not shown) demonstrate that there is no correspondence between the distribution of actin holes and the positions of centrosomes in GNU embryos.

3.8 gnu Has No Detectable Zygotic Effects

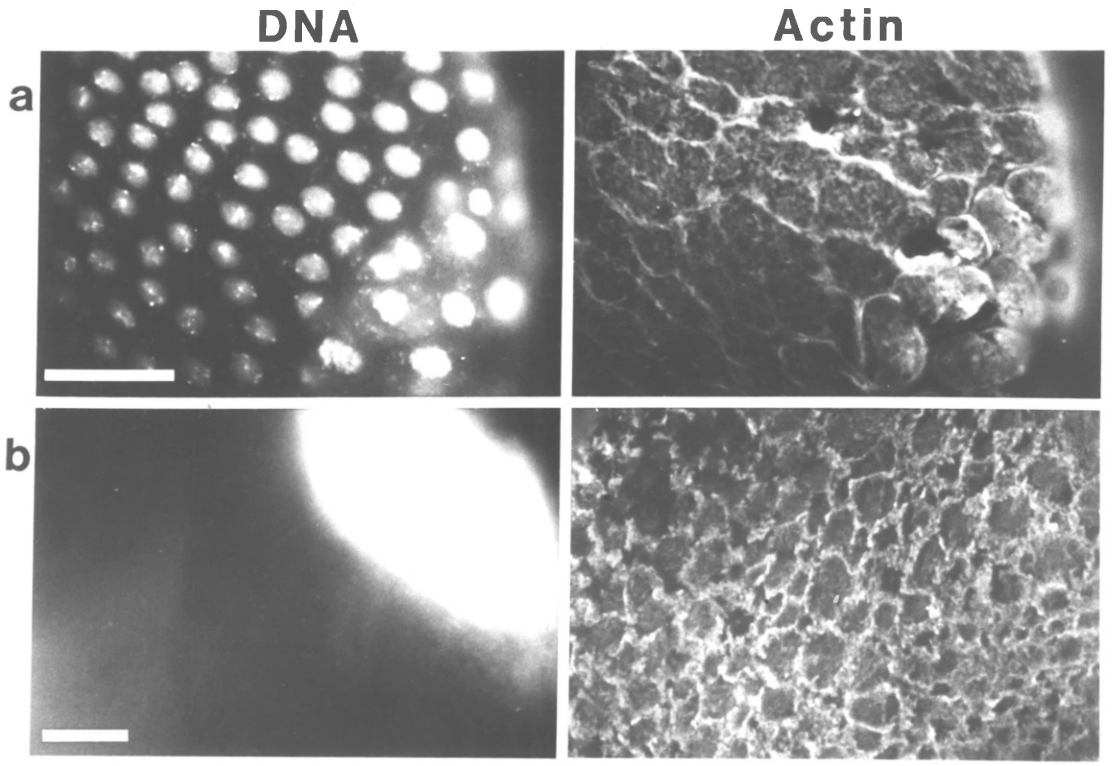
Homozygous gnu progeny of heterozygous females are apparently quite healthy and have no obvious abnormalities. If the gnu gene participates in a process essential to all mitosis, mitotic defects might be expected in these homozygotes. An easy way of looking for such defects in these individuals is to prepare cytological squashes of the larval brains which are one of the few larval tissues to grow by diploid cell division. Figure 3.10 shows three examples each of wild type neuroblast squashes and squashes from larvae homozygous for the gnu mutation. The frequency of metaphases has

Figure 3.9

Actin Staining of Wild Type and GNU Embryos

Embryos were incubated with an antibody against actin, then with rhodamine conjugated anti-mouse IgG, and finally with Hoechst (see Materials and Methods). Panel 'a' shows the posterior end of a wild type blastoderm embryo. The actin caps are closely abutted, giving actin rich furrows between each nucleus. Panel 'b' shows the anti-actin staining pattern of GNU embryos. A cortical layer of actin is seen, in which there is a rather irregular network of actin-sparse holes. The actin staining pattern is not altered in the vicinity of a giant nucleus. The bars represent 20μm.

Figure 3.9



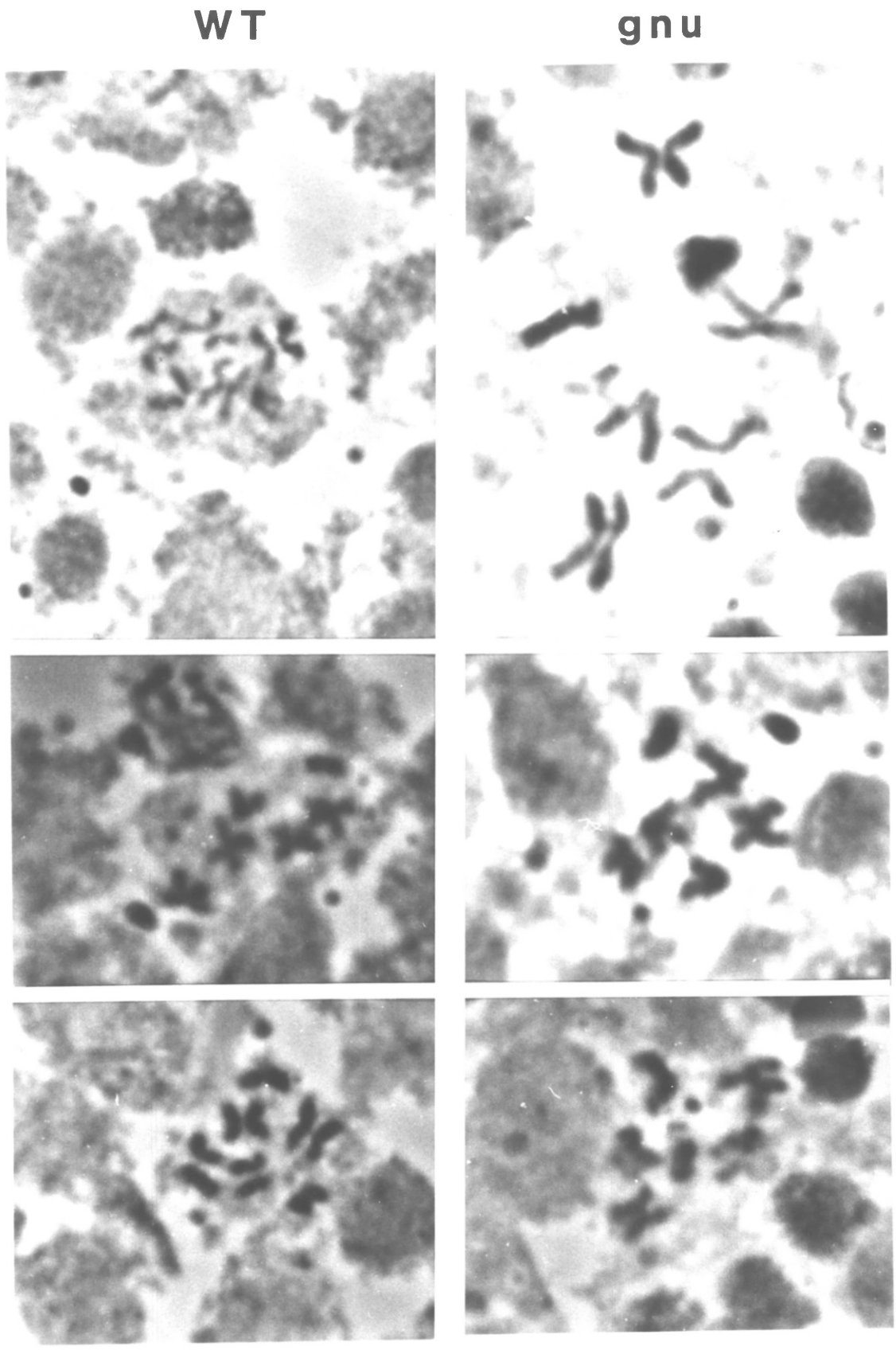
been estimated to be normal, and no obvious cytological defects have been identified. However, since it is not feasible to study mitosis in all tissues, the possibility that the gnu mutation does cause some minor mitotic defects in homozygous flies cannot be ruled out. Nevertheless, it does seem that the effects of gnu are largely, if not completely, limited to early embryogenesis.

Figure 3.10

Larval Neuroblast Metaphase Chromosomes of Wild Type and *gnu/gnu* Larvae

Larval neuroblast squashes were prepared as described in Materials and Methods (Section 2.10). Three panels of wild type metaphase chromosomes, and three of metaphase chromosomes from *gnu/gnu* homozygotes are shown. No significant differences, either in chromosome number, or in chromosome morphology are seen between the metaphase figures of wild type and *gnu/gnu* larvae.

Figure 3.10



3.9 DISCUSSION

The first 13 nuclear divisions in the wild type Drosophila embryo occur within a syncytium, at approximately 10 minute intervals. During cycle 14 the embryo becomes cellularised, at which stage there are approximately 10,000 nuclei. I have described a maternal effect lethal mutation, gnu, in which DNA replication continues while nuclear division is almost completely absent in embryos from homozygous females. This causes the formation of embryos with a small number of giant nuclei (which I refer to as GNU embryos). The envelopes of these nuclei stain to a variable degree with an antibody against lamin. Although there is a possibility that this variability in staining is an artefact, this result suggests that the giant nuclei may go through phases in which the nuclear membrane undergoes considerable structural change, as occurs during prometaphase in wild type embryos. The observations presented here demonstrate that whilst nuclear division is disrupted, the centrosomes continue to divide in the syncytial blastoderm. Furthermore, the centrosomes still migrate from the interior to the cortex of the syncytium where they nucleate the formation of giant asters. Later the giant nuclei appear to break down, and some spindle-like structures, formed about small fragments of chromatin, are then seen. Finally, it has also been shown

that the cortical actin in GNU embryos forms an unusual network of holes that is unlike any wild type phase of actin staining.

In some way the gnu mutation uncouples nuclear division from cytoplasmic events of the mitotic cycle in Drosophila embryos. The autonomy of cytoplasmic events has been demonstrated by the elegant experiments of Hara et al. (1980) who showed that the enucleated egg of Xenopus laevis continues to undergo periodic surface contractions. These cortical waves have exactly the same frequency as those that occur normally at the metaphase/anaphase transition in control fertilised eggs. This led to the suggestion that there is an autonomous cytoplasmic oscillator which is responsible for timing the mitotic events of each cell cycle. My observations suggest that centrosome replication continues independently of nuclear divisions in GNU embryos, consistent with there being cytoplasmic cycles which are maintained in the absence of a normal nuclear cycle. I have not, however, determined whether the timing of such cycles is affected in GNU embryos.

The observation that the division of centrosomes is not dependent on the presence of nuclear division or the formation of spindles is intriguing since these processes are normally so tightly coupled. Rattner and Phillips (1973) showed that when mouse L cells were treated with arabinosyl cytosine to inhibit DNA synthesis, centrioles

(the microtubular structures within centrosomes) divide independently of DNA replication. In this case the centriole cycle subsequently arrested in a configuration normally found at prophase and the centrioles did not undergo further division. Sluder and Begg (1985) have analysed in detail the phenomenon first described by Mazia et al. (1960) in which centrosome division is induced in sea urchin eggs by mercaptoethanol. In this system centrosomes split rapidly upon mercaptoethanol treatment but the newly formed centrosomes quickly associate with chromosomes via spindle fibres. Wolf (1980) has studied the distribution of asters (which appear to be nucleated by centrosomes) during development of cleavage embryos of the gall midge, Wachtliella persicariae. He describes a 'pseudocleavage' process of unfertilised eggs in which the asters sometimes undergo up to four divisions in the absence of nuclear division. GNU embryos are, to my knowledge, the first reported case in which multiple rounds of independent centrosome division occur. Since this phenomenon was observed in GNU embryos, independent centrosome replication has been found to occur in a number of maternal effect lethal mutations in which nuclear division is defective (M. Leibowitz and C. Sunkel, pers. comm.). This supports the hypothesis that the processes of centrosomal and nuclear division are independently regulated, and confirms the value of these type of mutations in genetically dissecting the mitotic cycle.

The results presented here suggest a possible course of events for what occurs in GNU embryos during their abnormal development. The centrosomes divide in the absence of nuclear division, and at some stage migrate from the interior of the embryo to the cortical region. On reaching the cortex, the centrosomes recruit the layer of surface microtubules into forming asters (as has been proposed in wild type embryos by Karr and Alberts, 1986). The asters are originally small but continue to grow, perhaps indefinitely, to sizes twenty or more times larger than wild type asters. When the giant nuclei begin to disintegrate (at about 24 hours), small pieces of chromatin are released and these provide functional kinetochores that capture free microtubule ends. The microtubules are stabilised and this leads to the formation of some spindles morphologically similar to those in wild type embryos. Later still, the embryo begins to decay, and the microtubules start to form an irregular network. This interpretation is consistent with the model proposed by Mitchison and Kirschner (1985) from their studies of microtubule nucleation in vitro. They suggest that microtubules grow in random directions from the centrosome. Those growing towards the chromosome are captured by kinetochores and thus stabilised against the eventual depolymerisation that happens to uncaptured microtubules on the loss of their GTP caps. The feature of this model is that it requires no directionality of

fibre growth: microtubules do not 'know' in which direction the chromosome lies. This would certainly appear to be the case in GNU embryos in which the huge, round asters show no sign of growth directed toward the giant nuclei. On the other hand, there appears to be a requirement for the nuclei of GNU embryos to be fragmented before the chromatin will participate in forming spindle-like structures. This suggests that the microtubule organising centres of the chromosomes within the giant nuclei are not available, either because of the barrier of the nuclear membrane or because the chromatin is decondensed or in an equally inappropriate state. Karsenti et al. (1984) have shown that preparations of nuclei devoid of centrosomes can mediate the formation of anastral microtubule arrays when microinjected into Xenopus eggs. It would be interesting to follow the behaviour of such preparations in the GNU embryo. Karsenti et al. (1984) have also concluded that in the Xenopus egg it is the association with the nucleus that renders an otherwise inactive centrosome capable of nucleating microtubules during mitosis. In contrast to their result, centrosomes are clearly capable of nucleating microtubules independently of nuclei in the GNU embryo.

The network of holes observed in the cortical actin of GNU embryos is difficult to explain. The pattern is unlike any that occurs in the wild type syncytial

blastoderm. However, it is apparent that the distribution does change with time, from the smooth actin staining of early embryos to the network that we see later (Figure 9b). In the wild type embryo caps of actin divide in unison with the nuclei close under the surface, and it could be the absence of nuclei in GNU embryos which is instrumental in giving rise to the abnormal actin staining pattern. It is interesting to note that Euteneuer and Schliwa (1985) have shown that in a neuroblastoma cell line, the movement of centrosomes is dependent on actin microfilaments. It is not known whether the centrosome movement in GNU embryos is due to diffusion or to some kind of directed movement, but the rather regular array of centrosomes that is often seen (for example Figure 3.4a) suggests that at least there may be some mechanism for positioning the centrosomes at the cortex. It is possible that actin filaments play a role in this process. However, the fact that there is not a simple correspondence between holes in the actin network and the position of centrosomes implies that if such a mechanism does occur, it is not simple.

My observations point to the conclusion that the defects caused by the gnu mutation are limited to the early embryo. Both homozygous gnu larvae and adults are apparently healthy and unaffected. Furthermore larval neuroblasts from homozygous flies have mitotic figures that appear normal. The gnu mutation appears to be an

extreme allele of the gnu gene since flies heterozygous for gnu and a deficiency that includes the gnu gene show the same GNU phenotype as gnu homozygotes. For this reason it is unlikely that gnu is a weak allele of a gene that is necessary for all mitosis and that a stronger allele would show zygotic defects in mitosis. This is an important result as several female sterile mutations have been isolated in which although the homozygous adults are viable, mitotic abnormalities are found in the larval neuroblasts (for example, Ripoll et al., 1985). Since the gnu mutation so drastically affects nuclear division, the fact that its effects seem to be limited to embryogenesis requires some explanation. The nuclear division in the cleavage embryo is quite distinct from normal mitotic division. There is an average cycle time of ten minutes, which is about 100 times faster than that in most diploid tissue. The embryo is at this stage a syncytium, and thus there is no requirement for cytokinesis and, furthermore, there is no detectable RNA synthesis (Anderson and Lengyel, 1979) until cellularisation occurs at cycle 14. If there are gene products specifically required for the unusual nuclear division that occurs at this stage of development, a mutation in one of them would be expected to give a maternal effect lethal phenotype. Alternatively, it may be that the gnu gene product plays a role not in mitosis itself, but in the processes required at the earliest times to convert the egg from a quiescent state,

to a state in which the DNA must replicate extremely rapidly so that the the ten minute mitotic cycle can be achieved. A defect in these processes might lead to abnormalities in early nuclear division. Indeed, there is now evidence, presented in Chapter 4, that this latter is the case, and that *gnu* is involved in the activation of DNA replication that normally occurs upon fertilization.

CHAPTER 4

The gnu Mutation Causes Inappropriate Replication in Fertilized and Unfertilized Eggs

4.1 INTRODUCTION

GNU embryos appear to be defective in nuclear division, but not DNA replication, and this leads to the formation of a small number of giant nuclei in the syncytial blastoderm (see Figure 3.1). The polar bodies of GNU eggs, as well as the pronuclei, give rise to giant nuclei. In contrast the polar bodies in wild type eggs show no sign of replication even though they often persist throughout the cleavage stages, when DNA synthesis is being initiated every ten minutes in the surrounding cleavage nuclei. Although the gnu phenotype is so striking, it is not easy to identify the primary defect caused by this mutation. The gnu mutation has no apparent zygotic effects, which makes it likely that it mediates a process unique to oogenesis or early embryogenesis.

The first indication that the gnu mutation affects the block to DNA synthesis that exists in unfertilized egg was the surprising discovery, described in this chapter, that GNU eggs develop even when unfertilized. This led to the idea that that the GNU cytoplasm may provide some type of 'replicative environment', in which some, or all, of the normal repression of DNA replication is lifted. To test this idea further it was decided to experiment with introducing into GNU embryos nuclei that would not be expected to replicate in wild type embryos, and to test for their inappropriate replication. There have been many reports of nuclear transplantation experiments in

Drosophila (for example, Zalokar, 1971; Illmensee, 1972) and this approach could be used for the proposed experiment. In order to test for inappropriate replication of nuclei injected into GNU eggs, it would be necessary to use donor nuclei that were not undergoing rapid DNA synthesis, and they would have to be marked with a DNA sequence that could be used to distinguish them from nuclei of the recipient egg when total DNA was extracted from injected eggs. Appropriate nuclei might include those from a blastoderm of a line of flies that contains foreign DNA sequences inserted into its genome using a P-element transformation vector (Rubin and Spradling, 1982): blastoderm nuclei have completed the rapid divisions, and thus DNA replication, of cleavage, and will only progress through three or four more rounds of division during embryogenesis. Although this method is feasible, I was not experienced in such microinjection techniques, and a genetic approach was used instead.

The female sterile haploid mutation, mh (Gans et al., 1975) causes any sperm pronucleus entering the embryo from a homozygous female to fail in syngamy. Consequently the egg is activated, but only the female pronucleus initiates cleavage and the embryo develops with haploid nuclei, until dying later in embryogenesis. I have asked whether the sperm pronucleus DNA, marked with a foreign DNA sequence to distinguish it from the female meiotic products, is able to replicate in eggs from females homozygous for gnu and mh.

These experiments have allowed me to propose a model in which it is suggested that the gnu gene product participates in a process that blocks all DNA synthesis in the unfertilized egg. It is proposed that this block is not normally removed until a pronucleus achieves syngamy. Whether or not this model proves to be correct, the block to DNA replication that occurs in unfertilized eggs is a very general phenomenon, occurring in many species (Went, 1982) although very little is known about its mechanism. Thus the molecular characterisation of the gnu gene and its product will be of significant interest.

Finally, the fact that GNU eggs can develop in the absence of a sperm, has allowed me to address the question of whether centrosomes in the cleavage embryo are contributed maternally or paternally. The fact that unfertilized GNU eggs are found to contain functional centrosomes provides further information about a problem that has occupied biologists for many years (see, for example, Wilson, 1928).

4.2 GNU Embryos Develop From Unfertilized Eggs

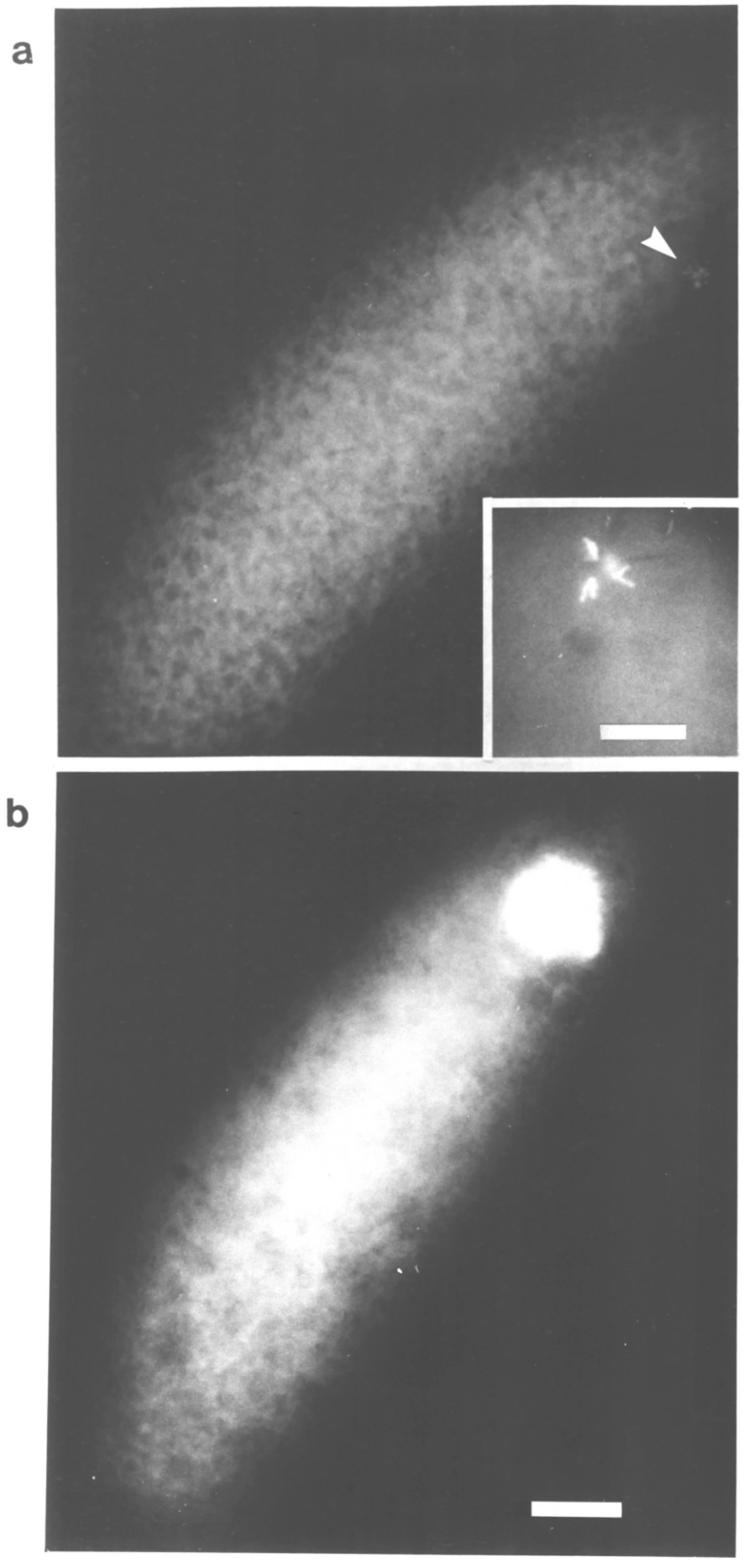
Wild type virgin females can be induced to lay eggs by keeping them on yeast for a few days in the absence of males. These unfertilized eggs are arrested at the end of meiosis II so that the four haploid products of meiosis - the three polar bodies and the pronucleus - are present and can be seen in fixed eggs stained with the DNA binding fluorochrome, Hoechst 33258. Frequently two or more of these haploid nuclei fuse, so that in practice what is usually observed is between one and four nuclei (Figure 4.1a). The state of the chromatin in these eggs is variable: in some cases it is decondensed and diffuse, and in others the chromosomes are highly condensed (as in the inset of Figure 4.1a). Embryos laid by virgin females homozygous for gnu show a strikingly different behaviour. Instead of being developmentally arrested, they develop giant nuclei and are cytologically indistinguishable from GNU embryos laid by females allowed to mate with males (Figure 4.1b). When this phenomenon was first observed it was assumed that the developed embryos came from a few females that had already mated and thus would have been wrongly classified as virgin. However the same result is obtained even when females that have just eclosed (with their wings still tightly folded) are selected. Furthermore, all the eggs from a collection that has been

Figure 4.1

The Nuclei of Unfertilized Wild Type and GNU Eggs

Eggs laid overnight by virgin females were collected and stained with Hoechst (see Materials and Methods). Panel 'a' shows a wild type egg: there are no signs of development and an unreplicated meiotic nucleus is marked with an arrow. The inset panel shows a typical meiotic nucleus at a higher magnification. In contrast, the unfertilized GNU egg shown in Panel 'b' has undergone considerable DNA replication, giving rise to a giant nucleus. The bar represents 50μm, and 10μm in the inset of Panel 'a'.

Figure 4.1



allowed to age for a few hours after removing the females from the vial are found to have giant nuclei. Again this argues against there being a small proportion of contaminating females that have already mated, out of the group selected as virgins. This result suggests that the gnu mutation in some way lifts the block to DNA replication that exists in the wild type unfertilized egg.

4.3 The Sperm Pronucleus Does Enter GNU Eggs, and is Replicated

A question that arises immediately from the observation that unfertilized GNU eggs can develop giant nuclei and resemble GNU embryos from females kept with males is whether, in the presence of males, the eggs are fertilized and, if this is the case, whether the sperm pronucleus is then replicated. Courtship behaviour is seen in a vial containing males and homozygous gnu females, as is copulation. However this does not address the question of whether fertilization is successful. In order to examine this, homozygous gnu virgin females were mated with males from a transformed line which is homozygous for a hybrid P-element construct that contains, among other things, the bacterial chloramphenicol acetyl transferase (CAT) gene (Line T3.1; Davies et al., 1986). The CAT gene therefore acts as a molecular marker for for the male genome. DNA was prepared from embryos collected from this cross and hybridised with both a 1.6kb fragment of the Drosophila

larval serum protein 1α gene (LSP probe), and with a 1.6kb fragment of the CAT gene (CAT probe). The LSP probe will hybridise with DNA from both male and female pronuclei, whereas the CAT probe will only hybridise with DNA derived from the male pronucleus. This allows the unambiguous determination of whether the male pronucleus replicates in GNU embryos.

Figure 4.2 shows a 'slot blot' experiment in which DNA is isolated from embryos and loaded onto a nitrocellulose filter by applying it to a manifold of slot-shaped holes attached to a vacuum pump. The DNA, immobilised on the nitrocellulose, is then hybridised with a radiolabelled DNA probe and the sequences homologous to the probe are quantified by the intensity of signal on an autoradiograph of the filter. In order to determine that there are no DNA sequences homologous to the CAT gene in Drosophila, DNA from wild type and GNU eggs (laid by females fertilized with males not carrying the CAT gene) was probed with the LSP probe and the CAT probe (Figure 4.2a). It can be seen that while DNA from these embryos hybridises with the LSP probe, there is no hybridisation with the CAT probe. 5ng of CAT DNA on the same filter hybridises strongly with the CAT probe, confirming that the probe was radiolabelled to a specific activity approximately equal to that of the LSP probe; this indicates that any CAT sequences that were present in the DNA would have been detected. Figure 4.2b shows that when wild type females are mated with CAT-bearing males, the

CAT gene replicates as expected. In this case half the DNA in the developing embryo is derived from the male pronucleus and similar hybridisation signals can be seen with the LSP and CAT probes. A similar signal is also seen with LSP and CAT when homozygous gnu females are mated with CAT-bearing males, indicating that the male pronucleus does replicate. This provides strong evidence that GNU eggs can be fertilized, and that when they are, the sperm pronucleus does replicate in the embryo. On the basis of this result it can be concluded that GNU eggs do not require fertilization for DNA replication to be activated, but are able to be fertilized, in which case the male and female genomes both replicate.

Figure 4.2 also shows that the total amount of DNA in these GNU embryos is much less than that in the the wild type embryos. In each case the embryos were aged between 19 and 24 hours. This result, repeated in all such blots (see, for example, Figure 4.5), implies that GNU embryos arrest with a reduced DNA content when compared with the amount of DNA in wild type embryos at the end of embryogenesis. The course of GNU development suggests that nuclei reach maximum size at about 8 hours post-laying (described in Chapter 3). By eight hours of wild type development, all rapid nuclear division has been completed for several hours, but the nuclei are destined to divide three or four more times during embryogenesis, and it may be these extra rounds of replication that account for the greater amount of DNA found in wild type

Figure 4.2

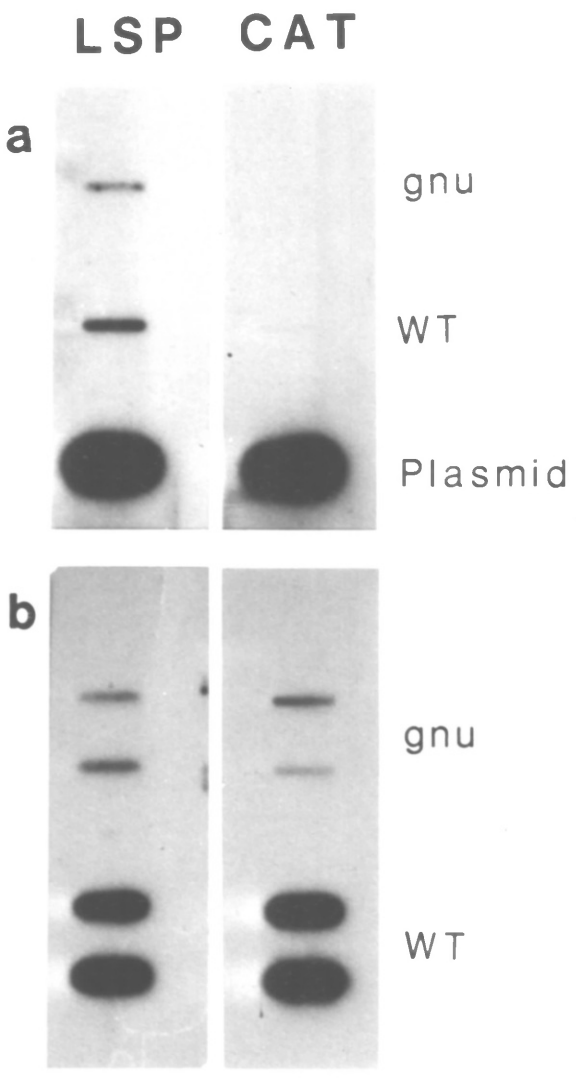
The Male Pronucleus Does Replicate in GNU Eggs

DNA was isolated from embryos, immobilised onto nitrocellulose filters, and hybridised with oligo-labelled fragments from plasmids containing the *Drosophila* larval serum protein 1α gene (LSP) or the bacterial chloramphenicol acetyltransferase gene (CAT) (see Materials and Methods).

a. DNA isolated from embryos from a cross between wild type (WT) or *gnu/gnu* (gnu) females and males not carrying the CAT gene. The DNA from 250 embryos was divided into two equal parts and probed with LSP or CAT. There is no hybridisation of either embryo DNA with the CAT probe, demonstrating the absence of CAT sequences in the genome of wild type or GNU embryos. The 'Plasmid' control is 5ng of the parental plasmid from which each probe was isolated. The approximately equal degrees of hybridisation of the LSP and CAT probes shows that both probes were of equivalent specific activity.

b. DNA isolated from embryos from a cross between wild type (WT) or *gnu/gnu* (gnu) females and males bearing the CAT gene (see Materials and Methods, Section 2.2). The DNA from 200 embryos (upper band in each pair) and 300 embryos (lower band in each pair), was divided into two equal parts and probed with the LSP and CAT probes. The CAT probe hybridises with DNA from both types of embryo, indicating that DNA derived from the male is replicated in both wild type embryos and GNU embryos.

Figure 4.2



embryos than in GNU embryos.

4.4 gnu Restores Diploidy to Some Embryos Expected to be Haploid

The observation described above, that unfertilized GNU eggs develop giant nuclei, leads to the idea that the cytoplasm of the GNU embryo may provide a 'replicative environment' in which normal repression of DNA replication is lifted. In Chapter 3 it was described how the polar bodies of GNU embryos, as well as the pronucleus, replicate to form giant nuclei, whereas in wild type embryos the polar bodies never show any signs of replication even when the cleavage nuclei have been through many rounds of division. This observation clearly fits well with the idea of a replicative environment. In order to test this idea further the gnu mutation was combined in the maternal genome with the X-linked female sterile mutation mh (Gans et al., 1975; Santamaria and Gans, 1980: also called fs(1)1182). Embryos from females homozygous for mh (referred to here as MH embryos) are haploid even though they do require fertilization in order to begin cleavage. Cytological and genetic analysis has shown that the male pronucleus does not participate in syngamy, and that the female pronucleus alone is the source of the haploid cleavage nuclei (Zalokar et al., 1975; Santamaria and Gans, 1980). Females were constructed that were homozygous for both gnu and mh (see

Figure 4.3 for the genetic scheme) in order to test whether the male pronucleus, unable to develop in the cytoplasm of an MH embryo, would replicate when the gnu gene is also mutated. I have confirmed that the MH embryos are cytologically haploid. Figure 4.4 shows mitoses in a wild type cleavage embryo, and those from an MH embryo. A comparison of these mitoses clearly indicates that the nuclei in mutant embryos have fewer chromosomes, although the plane of focus is too narrow to allow the exact number of chromosomes to be counted from a photograph. However, by focussing up and down with the microscope, it can be confirmed that these embryos are haploid during cleavage. MH embryos invariably die later during embryogenesis.

Figure 4.5 shows the result of an experiment in which females homozygous for both mh and gnu were crossed with CAT-bearing males and, in the same way as with the experiment described in Section 4.3, DNA from the resulting embryos was hybridised with an LSP probe and a CAT probe. Figure 4.5a demonstrates that, as expected, the male genome (specifically, the CAT gene, present only in the male genome) is significantly under-replicated in embryos resulting from a cross between females homozygous for mh alone and CAT-bearing males. However when CAT-bearing males are mated to females homozygous for mh and gnu, the resulting embryos, which resemble normal GNU embryos (data not shown), replicate male derived DNA to approximately the same extent as female derived DNA

Figure 4.3

Genetic Scheme for the Production of Females homozygous for both mh and gnu

All genetic markers and balancer chromosomes are as described in Lindsley and Grell (1968). The multiply balanced stock, Basc/Basc; Bw/CyO; Sb/TM2, was the gift of K. Karess (see Materials and Methods, Section 2.2).

Figure 4.3

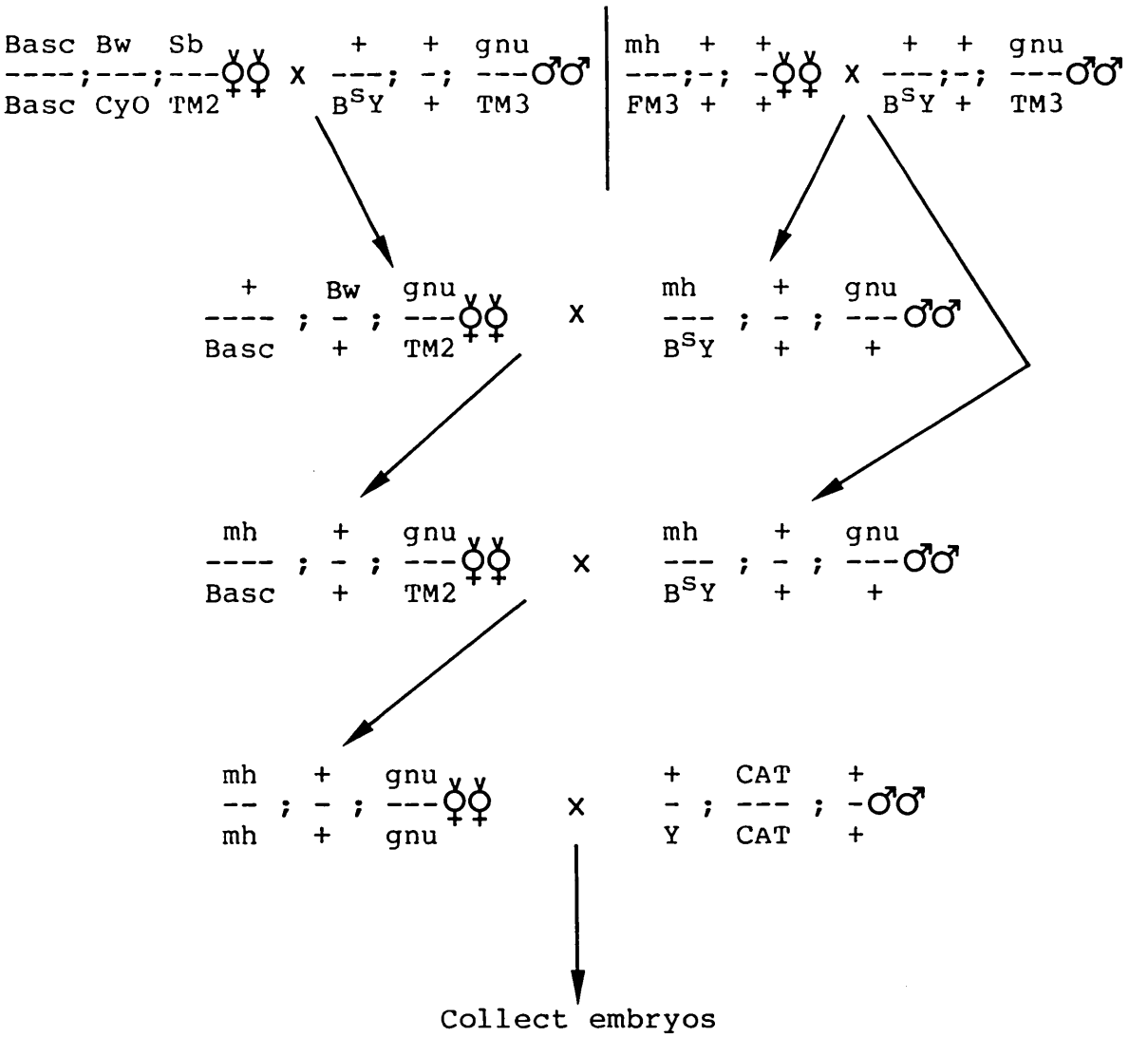
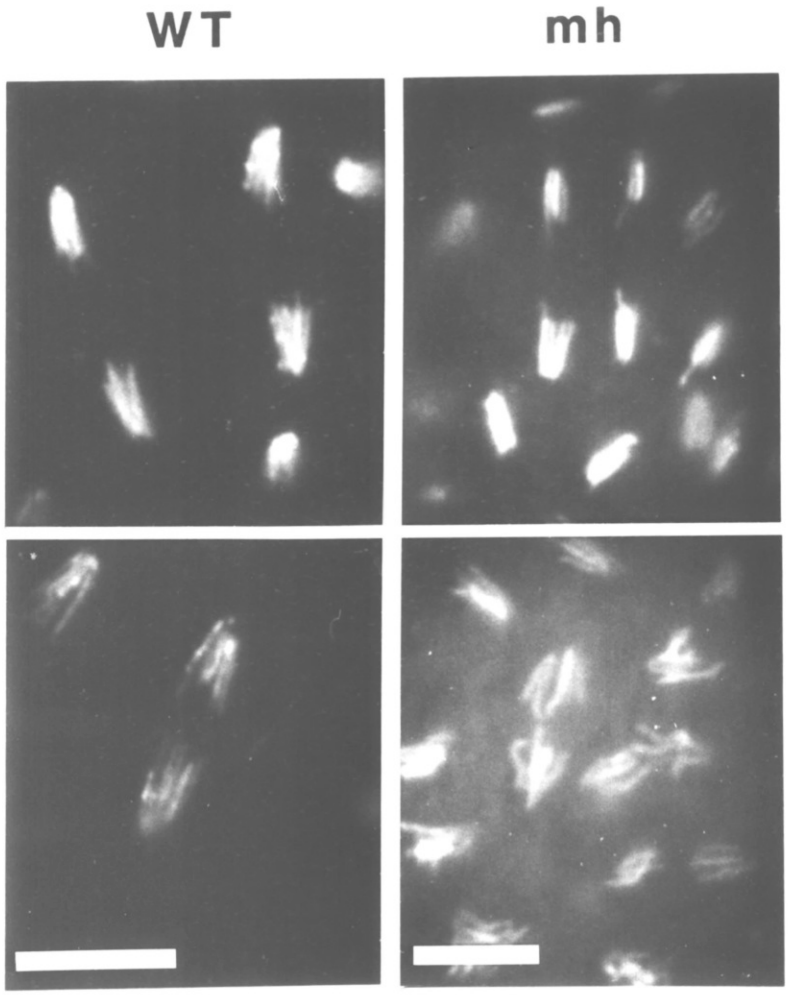

Basc Bw Sb
----;---;---♀♀ X
Basc CyO TM2
+ + gnu
---; -; ---♂♂
B^SY + TM3
mh + +
---;-; -♀♀ X
FM3 + +
+ + gnu
---;-; ---♂♂
B^SY + TM3
+ Bw gnu
---- ; - ; ---♀♀ X
Basc + TM2
mh + gnu
--- ; - ; ---♂♂
B^SY + +
mh + gnu
---- ; - ; ---♀♀ X
Basc + TM2
mh + gnu
--- ; - ; ---♂♂
B^SY + +
mh + gnu
-- ; - ; ---♀♀ X
mh + gnu
+ CAT +
- ; --- ; -♂♂
Y CAT +
Collect embryos

Figure 4.4

Anaphase Chromosomes from Wild Type and MH Embryos

Wild type (WT) and MH (mh) embryos were stained with Hoechst (see Materials and Methods). Although the focal plane is too narrow to count the number of chromosome arms in the anaphase figures shown, it is seen that the figures from the MH embryos have fewer than those in wild type embryos. Careful observation of similar figures results in the conclusion that MH embryos are haploid. The bars represent 10μm.

Figure 4.4



(Figure 4.5b). It should be noted that there is some weak hybridisation with the CAT probe in Figure 4.5a which is unexpected as the male pronucleus is known not to undergo cleavage in MH embryos. This could be due to background non-specific hybridisation; however such background has been found to be very low over a number of similar blots (see, for example, Figure 4.2a). Another possible explanation is that this method is sensitive enough to detect the single haploid nucleus per embryo that is expected to carry the CAT gene (the male pronucleus). However, when DNA prepared from unfertilized wild type eggs (containing only the four haploid meiotic products) is probed with similar probes, the signal obtained is much weaker that that seen in Figure 4.5a (see Figure 4.5c), which makes this explanation seem unlikely. Perhaps the most probable explanation for the small amount of CAT hybridisation in Figure 5a, is that the male pronucleus does replicate to a small extent in MH embryos, even though it almost certainly does not divide. The cytological and genetic techniques used to determine the haploidy in these embryos (Zalokar et al.,1975; Santamaria and Gans, 1980) are unable to address the question of whether the male pronucleus replicates, only demonstrating that it cannot participate in cleavage. The important point to note in this experiment is the clear difference in the ratio of LSP:CAT hybridisation between MH embryos and MH;GNU embryos. This, then, is a further example where a nucleus that would not normally replicate (or

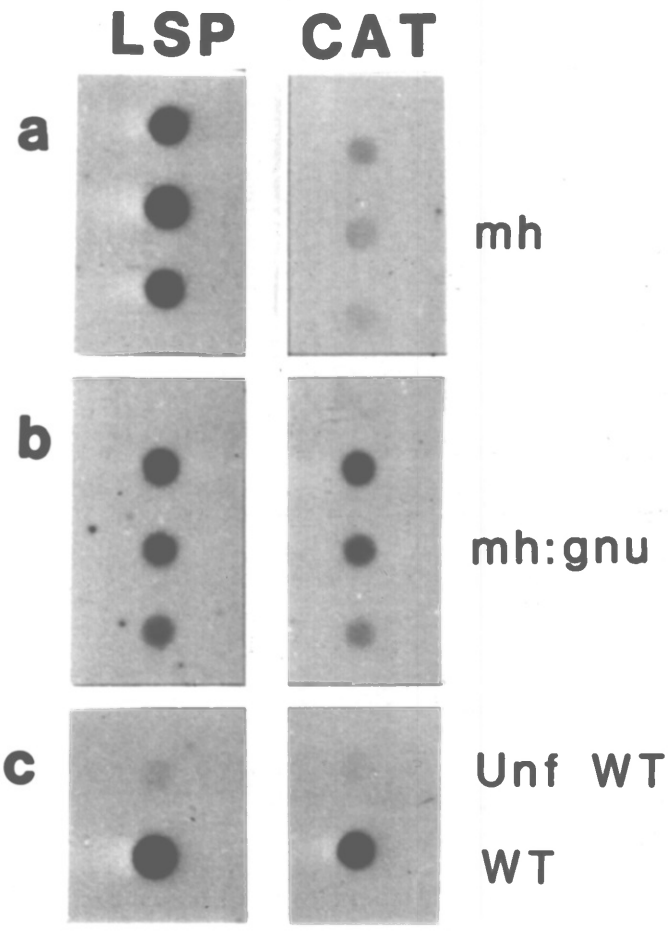
Figure 4.5

The Male Pronucleus Replicates in MH;GNU Eggs

a. DNA was isolated from 3 batches of embryos (0-24 hours old) from a cross between mh/mh females and males bearing the CAT gene (400, 400, and 300 embryos, from top to bottom). The DNA samples were divided into two equal parts, and probed with LSP and CAT. Only a small amount of hybridisation is seen with the CAT gene compared to the LSP gene, indicating that the male derived DNA is considerably less replicated than female derived DNA.
b. DNA was isolated from 3 batches of embryos (0-24 hours old) from a cross between mh/mh;gnu/gnu females and males bearing the CAT gene (230, 160, and 160 embryos from top to bottom). The DNA samples were divided into two equal parts, and probed with LSP and CAT. Equivalent amounts of hybridisation are seen with both probes, indicating that male derived DNA is replicated.
c. DNA was isolated from 100 eggs laid by virgin wild type females (Unf WT) and from 100 embryos from a cross between wild type females and males bearing the CAT gene. The DNA was divided into two equal parts, and probed with LSP and CAT. The unfertilized wild type eggs are expected only to contain the four products of female meiosis, and the hybridisation is correspondingly low. The CAT gene is replicated in embryos from wild type females crossed with male bearing the CAT gene, as expected, since the male pronucleus participates in mitotic cleavage.

(See Materials and Methods for protocols and stocks.)

Figure 4.5



perhaps would under-replicate) in an embryo - in this case the male pronucleus introduced into an embryo from a female homozygous for the mh mutation - does when the gnu gene is mutated. This result adds weight to the hypothesis that the gnu mutation causes normal repression of DNA replication to be lifted in GNU embryos.

4.5 Unfertilized GNU Embryos Contain Centrosomes

The fact that uninseminated GNU eggs develop allows them to be used to address the question of what is the source of centrosomes in the developing embryo - a question that has been of interest to biologists for many years. Boveri formulated a 'Theory of Fertilization' in which he postulated that the mature egg had all the components needed for early development except the centrosome, which was the crucial contribution of the sperm, allowing the formation of the first mitotic spindle and therefore the initiation of development (reviewed in Wilson, 1928). This theory has largely been accepted since then, although there are enough examples that break Boveri's rule to demonstrate that the answer to this problem is not straightforward, and that there are almost certainly not invariant mechanisms. In Drosophila, electron microscopic studies have shown that the centrioles disappear from the oocyte during oogenesis and that the meiotic divisions are acentriolar (Mahowald and Strassheim, 1970). The fate of these centrioles is not

known, although it seems likely that their components disperse rather than breakdown. Centrioles are found in Drosophila sperm and therefore are probably transported into the egg upon fertilization (Sonnenblick, 1950). Thus Drosophila would seem to comply with Boveri's rule.

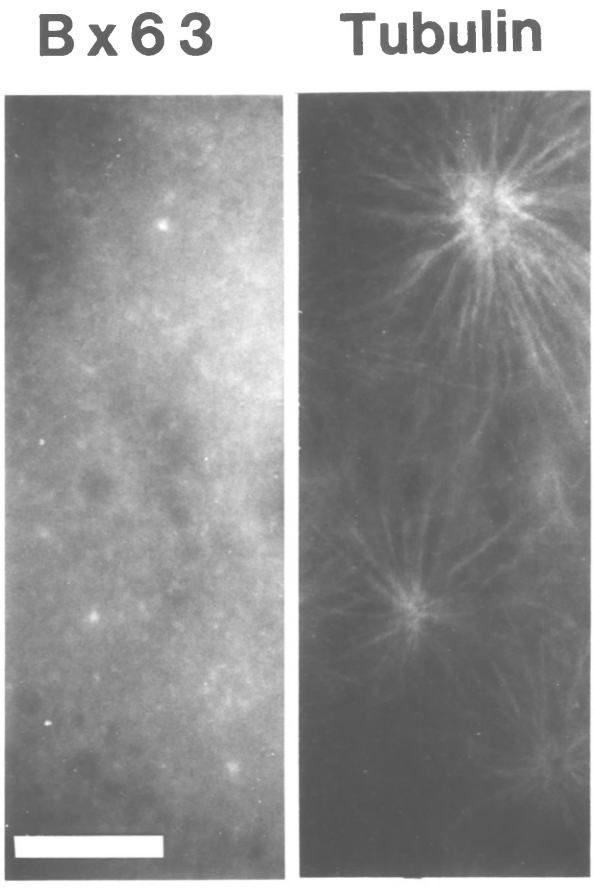
Using the antibody against Drosophila centrosomes, Bx63 (described in Chapter 3), to immunostain unfertilized GNU eggs, it is possible to determine whether they have centrosomes. Figure 4.6 shows that there are indeed centrosomes in these embryos, and that they are capable of nucleating asters in a manner indistinguishable from fertilized GNU embryos (compare Figures 4.6 and 3.9). Centrosomes are never found in unfertilized wild type embryos (data not shown), but it can be concluded from this result that the unfertilized Drosophila embryo does have the ability to produce a functionally and morphologically normal centrosome and therefore that the provision of a centriole by the sperm is not obligatory.

Figure 4.6

Unfertilized GNU Eggs Contain Centrosomes and Asters

Eggs from virgin gnu/gnu females were collected and stained first with Bx63 against centrosomes; second, rhodamine conjugated anti-mouse IgG; third, YL1/2, against tubulin; and fourth, fluoroscein conjugated anti-rat IgG (see Materials and Methods). The centrosome and tubulin staining in one of these eggs is shown. Three centrosomes can be seen in the field, and they all lie at the centre of a microtubule aster. This indicates that the unfertilized GNU egg is able to form functional centrosomes. The bar represents 10μm.

Figure 4.6



4.6 DISCUSSION

The experiments described in this chapter originate from the unexpected observation that unfertilized eggs from females homozygous for the gnu mutation (GNU eggs) develop giant nuclei which are indistinguishable from those seen in fertilized GNU eggs. This is in sharp contrast to unfertilized wild type eggs, in which DNA synthesis is arrested, and the four haploid products of meiosis show no sign of development. It has also been shown that although there is no requirement for fertilization in GNU eggs, insemination can occur, in which case the sperm nucleus does replicate. Furthermore the male pronucleus replicates in eggs from females that are homozygous for both the female sterile haploid mutation, mh, and gnu; the male pronucleus in eggs from females homozygous for mh alone (MH eggs) never participates in cleavage (Santamaria and Gans, 1980), and is significantly under-replicated (Figure 4.5). Finally, it has been possible to exploit the development of unfertilized GNU eggs to show that functional centrosomes can be formed in the absence of any contribution from a sperm.

These results lead to the idea that the cytoplasm in GNU eggs is able to sustain DNA synthesis in inappropriate circumstances. The observation that the polar bodies in GNU eggs frequently give rise to giant nuclei is further evidence for this notion. In wild type embryos there is

no trace of replication of the polar bodies, even though they often persist throughout the cleavage stages of embryogenesis, and can be clearly seen until cellularisation occurs after 14 nuclear divisions (data not shown). In this case there must be some specific block to polar body replication as there are presumably signals in the cytoplasm which are causing the cleavage nuclei to undergo replication parasynchronously (Foe and Alberts, 1983). The mutant gnu gene in some way overrides this repression. Similarly, the observation that the pronucleus (as well as the polar bodies) can replicate in unfertilized GNU eggs implies that the gnu mutation must remove the signals that prevent the meiotic products from replicating in wild type unfertilized eggs. Again, the same kind of derepression appears to occur when the male pronucleus is able to replicate in eggs from females homozygous for both mh and gnu, when it cannot in eggs from females homozygous for mh alone. Thus, with three different examples of blocked DNA synthesis, in which there is no reason to believe that the mechanism is the same, the gnu mutation can override the repression.

The developmental arrest that occurs in unfertilized wild type eggs is common to most species of sexually reproducing organisms (Went, 1982). Under normal circumstances the egg is activated upon fertilization, at which time a variety of biochemical and morphological changes allow development to begin, but there are examples of activation occurring by other mechanisms. The most

striking case is that of parthenogenesis, which can be either natural or artificially induced, in which it is clear that the development of the egg must be triggered by some signal other than the sperm. Parthenogenesis occurs in a wide range of species although its rate and the significance in populations varies enormously. Moreover, many unfertilized eggs can be artificially activated using a variety of treatments that put the egg under some form of stress (Went, 1982); some of these activated eggs form viable mature organisms, and others die later during development, but they all seem to overcome the blocks to DNA synthesis that normally exist prior to fertilization. In a detailed study of the Hymenopteran, Pimpla turionellae, which is facultatively parthenogenetic, Went (1982) has shown that activation is not dependent upon fertilization, but instead on the mechanical compression to which the egg is subjected as it passes through the oviduct. This compression can be mimicked artificially and eggs treated in this way develop into mature organisms.

There is only one species of Drosophila known in which parthenogenesis appears to be the sole method of reproduction (D. mangabeirai, Templeton, 1983), but tychoparthenogenesis - low rates of 'accidental' parthenogenesis - occurs in all species of Drosophila examined (Stalker, 1954; Templeton, 1983). In D. melanogaster, the rate is very low (2 larvae developed from more that 500,000 unfertilized eggs; Stalker, 1954).

Notwithstanding this low rate of parthenogenesis it is clear that the activation of the Drosophila egg is not a simple one stage process, and that the first part of this activation is not in fact dependent upon the sperm. Doane (1960) showed that the previously accepted belief that the unfertilized egg is blocked at meiosis I (like the developing oocyte) is wrong: instead the egg has clearly completed both rounds of meiosis. Mahowald et al. (1981) confirmed this observation, but also showed that several other changes previously believed to be dependent upon fertilization, also occur in unfertilized eggs. These changes do not occur in mature oocytes, even when they are retained by the female for 15 days, but can be artificially triggered in these oocytes if they are explanted and treated in a hypotonic solution. Thus it appears that many of the processes of activation, even in Drosophila which shows very little parthenogenesis, are triggered by oviposition rather than by insemination. However the fact that the four haploid products of meiosis II are then arrested in unfertilized eggs, demonstrates that there remain at least some blocks to DNA synthesis. It appears to be these blocks that the gnu mutation affects.

The gnu mutation is able to remove the block that exists to the replication of the male pronucleus in MH embryos. The nature of the mh mutation is not clear. It has been shown that diploid nuclei injected into the unfertilized MH egg cause the egg to develop to at least

larval stages (Santamaria, 1983), demonstrating that the cytoplasm of these eggs have all the components required for normal development from a diploid nucleus. The mutation shows no zygotic effects (Gans et al., 1975; Zalokar et al., 1975): the genotype of the sperm does not effect the development of the embryo, and homozygous adults from heterozygous mothers show no defects other than female sterility. These observations suggest that the primary lesion in the mh mutation concerns the ability of the egg to form the first mitotic spindle with the incoming sperm, and that it is only the haploidy of the cleavage nuclei that causes the embryos to die. The results described in this chapter show that the sperm pronucleus, having been left out of the first mitotic division, does not replicate (or significantly under-replicates), which implies that as well as failing to allow the sperm to participate in syngamy, the MH egg is unable to derepress the sperm DNA synthesis.

The results discussed above are consistent with a model in which all the haploid nuclei in the unfertilized egg are blocked in DNA replication, after the egg has been initially activated by oviposition, and that this block is only lifted from a nucleus which participates in the first mitotic spindle. If this was the case, the polar bodies would never replicate, nor would any nuclei in unfertilized eggs, nor would the male pronucleus that has failed in syngamy in an MH egg. A defect in this process would be expected to give the same pattern of derepression

of replication that occurs in the gnu mutation. Clearly this model is hypothetical, but it does fit the observations described, as well as being consistent with what little knowledge there is about egg activation. It does not explain why the gnu mutation should cause the formation of giant nuclei, rather than just activating cleavage, and this point will be further discussed in Chapter 6.

The model proposed can be tested further by looking at the interactions between gnu and haploid mutations other than mh (described in Gans et al., 1975; Zalokar et al., 1975), in the same way as has been described here. These interactions will be tested in the future. There also exists a male sterile mutation ms(3)K81 (Fuyama, 1984), which will be similarly examined. Sperm from males homozygous for ms(3)K81 are able to activate eggs, but unable to take part in syngamy, thereby inducing cleavage of the female pronucleus and subsequent haploid development. The genotype of the female does not affect the phenotype of embryos fertilized by a mutant sperm, so the situation can be seen as complementary to the female sterile haploid mutations such as mh. It will be interesting to determine whether the DNA from this sperm, defective in syngamy, is capable of replication in GNU eggs.

The observation that functional centrosomes can develop in unfertilized GNU embryos is intriguing as it provides evidence about a very general problem in biology.

Since Boveri formulated his 'Theory of Fertilization' (see Wilson, 1928) it has largely been accepted that the centriole in the cleavage embryo is contributed by the sperm. However there is much evidence to show that this is not the case in all species, and even that a single species does not always use the same mechanism (see, for example Wilson, 1928; Schatten et al., 1986). An example of this latter case occurs in organisms in which tychoparthenogenesis occurs, including Drosophila. The meiotic divisions in Drosophila are acentriolar (Sonnenblick, 1950; Mahowald and Strassheim, 1970), and a centriole, which is believed to enter the egg upon fertilization, can be detected in the sperm (Sonnenblick, 1950). However, in the rare cases of parthenogenesis that do occur, the centriole is obviously not provided by a sperm. Similarly, centrosomes were found in unfertilized sea urchin eggs that had been artificially activated (Dirksen, 1961) - another organism that generally adheres to Boveri's rule.

One possible source of confusion about this issue is the difference between the centriole and the centrosome. The centriole is a characteristically-structured organelle made of tubulin which is usually found at the centre of centrosomes, but which is not obligatory for spindle pole formation, and whose role in microtubule nucleation during mitosis is unclear. The centrosome is the larger, less ordered body that is seen as an amorphous, electron-dense region at the spindle pole in electron micrographs (Evans

et al., 1985). Some cells, such as higher plant cells, lack centrioles, but apparently always have centrosomal material at the spindle poles (Callarco-Gillam et al., 1983), and electron microscopy has indicated that nucleated microtubules actually derive from the centrosomal material rather than the centriole (Evans et al., 1985).

Mouse embryos are one of the examples that do not adhere to Boveri's rule; they also provide an example of the type of confusion that exists about the requirement for centrioles or centrosomes at fertilization. Using an anti-centrosomal antibody, Schatten et al. (1986) have shown that the mouse sperm lacks a centrosome (though it does have a centriole), whereas the meiotic divisions of the oocyte do have centrosomal material at their poles, although they have no centrioles (Szollosi et al., 1972). The first five cleavages of the embryo are carried out using the centrosomal material that was present in the egg before fertilization and no centrioles are found at the spindle poles (Schatten et al., 1986). Later divisions do have centrioles, as well as centrosomes, at their spindle poles, although the source of these centrioles is not clear: presumably they are either assembled from materials present in the egg, or they are descendants of the centriole that was present in the sperm. The observation that the sperm centriole is not required in the early stages of mouse embryogenesis may explain the fact that parthenogenesis is relatively easy to induce in mice

(Graham, 1974), when compared with species such sea urchins, in which the centrosome is paternally contributed (Went, 1982).

In this chapter I have demonstrated that the unfertilized Drosophila egg has all the components required to produce functional centrosomes. The antibody, Bx63, used to immunostain these embryos recognises a component of the centrosome, not the centriole (W. Whitfield, pers. comm.), but the exact location or function of the antigen is not known so it is not possible to state exactly which part of the centrosome it is that is present in these eggs. However, the fact that these centrosomes are capable of nucleating morphologically normal asters, indicates that they must be functionally adequate. Although this result does not prove that the centrosomes are normally maternally contributed in wild type development, there are two other lines of evidence that do lend weight to the possibility. Firstly, it has been shown that large amounts of Bx63 antigen accumulate in the wild type oocyte nucleus late in oogenesis (M. Frasch, PhD thesis, 1985; S. Millar, pers. comm.); and secondly, the amount of Bx63 antigen, as detected on western blots, does not increase during embryogenesis after a short period post-oviposition (M. Frasch, PhD thesis, 1985), implying that by very early stages of embryogenesis there is a large pool of the antigen ready to be assembled into centrosomes. However, since unfertilized wild type eggs are never found to have

centrosomes (data not shown), it appears that the egg cytoplasm needs to be 'activated' in some way - in this case by the presence of a mutated gnu gene - before centrosomes can form, or more precisely, before they can be detected by these immunostaining experiments. The centre of the egg is opaque and it is not usually possible to see centrosomes in this position in whole mounts even of fertilized eggs (unless the egg is damaged, allowing its contents to be seen more clearly). Thus the possibility cannot be ruled out that even unfertilized wild type embryos can produce a small number of centrosomes that do not replicate in the developmentally arrested cytoplasm, and thus never reach sufficient number to be seen.

The experiments described in this chapter have led to a clearer understanding of the possible primary defect caused by the gnu mutation. It seems that this defect lies in the processes that are involved in the regulation of the synthesis of DNA replication in the unfertilized egg prior to final activation by the incoming sperm. Since the arrested state of the unfertilized egg is a general phenomenon, occurring in widely divergent species, the function of the gnu gene product is of considerable interest. Chapter 5 describes initial experiments directed toward the cloning, and ultimately the molecular characterisation, of this gene.

CHAPTER 5

The Genetic and Cytological Mapping of gnu

5.1 INTRODUCTION

The *gnu* gene appears to be involved in the mechanism of the repression of DNA replication that occurs in the unfertilized *Drosophila* egg. This phenomenon is one that is found in a very wide range of organisms (Went, 1982), but about which very little is known, so the molecular characterisation of the *gnu* gene and its product is likely to be of considerable interest. For this reason, one of the long-term aims of this project is to clone the gene, thereby allowing the kind of molecular analysis that has been used to elucidate other complex biological processes in *Drosophila* following the cloning of the relevant genes. As a preliminary to choosing a cloning strategy for the *gnu* gene, it is necessary to map its position in the genome. The very extensive list of identified genetic loci in *Drosophila* (Lindsley and Grell, 1968) means that fairly accurate genetic mapping can be performed with any new mutation, wherever it is located. There have also been isolated a great number of chromosome rearrangements which cause cytologically defined abnormalities in the chromosome structure of *Drosophila*. These can be used to identify the cytological location of a gene of interest (see, for example, Hilliker et al., 1980). The left arm of the third chromosome, where *gnu* maps, is not one of the best studied regions, so the list of available rearrangements is not as extensive as in some other regions of the genome. However, several relevant

deficiencies have been traced, and, in conjunction with the meiotic recombination mapping, the gnu gene has been located.

The rather precise cytological mapping of gnu means that several potential cloning methods may be considered. Scalenghe et al. (1981), have pioneered the microdissection technique, in which the region of interest can be physically dissected out of a polytene chromosome squash and, using nanolitre reaction volumes in droplets suspended in an oil chamber, the DNA can be cloned. This technique has been used to clone several genes (for example, tra (Butler et al., 1986)), but the technical skill required is formidable, and only a small number of laboratories have been successful. An alternative cloning strategy is to use the method of chromosome walking and jumping (Bender et al., 1983a). In this case, the cloning is fairly straightforward, but it requires a piece of DNA near the gene of interest that has already been cloned as an 'entry point' into the walk. This requirement limits the number of genes that can easily be isolated with this method. However, there are cloned regions in the vicinity of gnu (P. Cherbas, pers. comm.), and the possibility of cloning the gnu gene will be discussed in some detail.

A different strategy for cloning Drosophila genes involves 'transposon tagging' the gene of interest, by producing a mutant allele of the gene which contains a P-element (Kidwell, 1986). The gene can then be cloned by isolating the DNA sequences that flank the P-element in a

genomic library of the DNA from the mutant stock of flies. A P-element mutagenesis screen was designed in order to obtain P-induced alleles of gnu and three other maternal effect lethal mutations (thule, lodestar, and polo). However this screen was not successful, and no mutant alleles of any of the four genes was isolated. Therefore it seems likely that the strategy that will eventually be used to clone the gnu gene will be a chromosomal walk.

5.2 The gnu Mutation Maps at 42.5 Map Units on Chromosome 3

The gnu mutation was induced on a chromosome carrying the visible markers ru th st ri roe p^p e^s ca (for descriptions of all the genetic markers described in this chapter, see Lindsley and Grell, 1968), and a small-scale recombination mapping experiment had previously failed to separate it from scarlet (st), which maps at 44.0 map units (mu) on Chromosome 3 (C. Nüsslein-Volhard, pers. comm.). It was therefore decided to map the mutation more precisely by measuring the rate of meiotic recombination between gnu and the markers in that region of the chromosome, namely thread (th), scarlet, and radius incompletus (ri). The genetic scheme for producing recombinants is shown in Figure 5.1. A total of 2267 recombinant chromosomes were scored for the visible markers, and a proportion of these was then tested for sterility, to identify the presence or absence of the gnu mutation on the recombined chromosome. The results of this experiment are given in Table 5.1.

From this table it can be concluded that gnu lies to the left of th; it was separated from th (ie gnu^+ th^- or gnu^- th^+) on 2/256 chromosomes. This gives the genetic distance of gnu from th as being 0.78 mu. The rate of recombination between the markers was also calculated, and

this corresponded well with the known positions of these genes (0.75 mu measured between th and st: 0.7 mu accepted value; 3.3 mu measured between st and ri: 3.0 mu accepted value). This implies that there is no gross chromosomal rearrangement in this region of the gnu chromosome, a result confirmed by the cytological appearance of this chromosome in preparations of salivary gland polytene chromosomes (data not shown).

The recombination experiment was repeated, but because of the observed proximity of th and gnu, the only recombination measured this second time was that between these two loci. Furthermore, the only recombinant chromosomes tested for sterility were those that were th^- st^- ri^-. Measuring the rate of fertile flies of this genotype was believed to be more accurate than measuring sterility amongst the class th^+ st^+ ri^+, as there is a low rate of spontaneous sterility that occurs in single females, and this would tend to produce an overestimate of recombination. 294 th^- st^- ri^- recombinant chromosomes were tested, of which two were fertile. This figure agrees well with that obtained in the first mapping experiment, so the combined value of 4/550 would appear to be the best estimate of the rate of recombination between th and gnu. This figure corresponds to a genetic separation of 0.73 mu between these two mutations and, as th maps at 43.2 mu (Lindsley and Grell, 1968), it provides a map position of approximately 42.5 mu for gnu.

Figure 5.1

Genetic Scheme for Recombination Mapping of *gnu*

Genetic markers are as described in Lindsley and Grell (1968). Individual females to be tested were scored for the presence or absence of the markers *th*, *st*, and *ri*, and then individual females from the different recombinant classes were tested for sterility with 2 or 3 males. Only females that produced eggs that never hatched were counted as sterile: females that did not lay any eggs were not included in the numbers given in Table 5.1. The position of *gnu* shown is that deduced from this experiment.

Figure 5.1

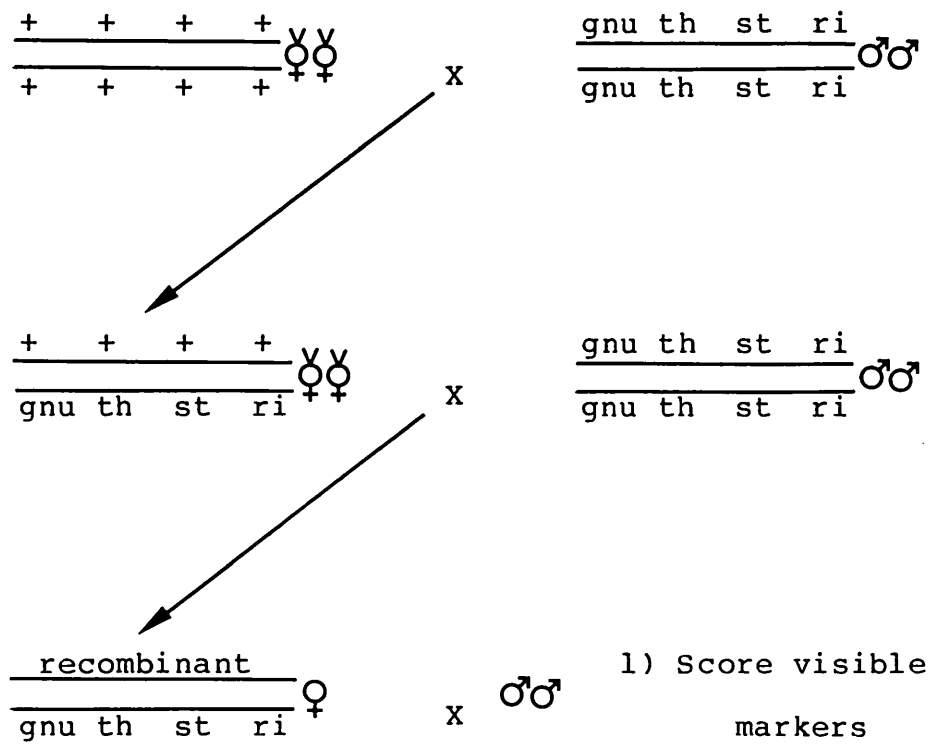

+ + + +
+ + + +
X
gnu th st ri
gnu th st ri
+ + + +
gnu th st ri
X
gnu th st ri
gnu th st ri
recombinant
gnu th st ri
X
1) Score visible markers
2) Test for female sterility

Table 5.1

Recombination Mapping Data for gnu

Recombinant class	Number identified	Number tested for sterility	Number sterile
+ + +	1241	48	1
th + +	10	9	9
th st +	41	39	39
+ st +	0	0	0
th st ri	934	123	122
th + ri	0	0	0
+ st ri	7	7	0
+ + ri	34	30	0

5.3 The gnu Gene Maps Cytologically at 70E4-6 to 70F4-6

Using deficiencies with known cytological breakpoints to define the position of the th gene, it is possible to localise it to the interval 72A5;72C1,2 (Lindsley and Zimm, 1985). In order to try and cytologically map the gnu gene, a series of deficiencies that remove regions of the chromosomes to the left of the th interval were examined for their ability to uncover the gnu mutation. The test used was to produce the gnu/Df(3L) heterozygote and test this female for sterility. The results obtained are given in Figure 5.2, and the breakpoints of the deficiencies shown are listed in Table 5.2. It can be concluded that the gnu gene lies in the interval defined by the proximal breakpoint of $Df(3L)fz^{D21}$, 70E4-6 (C. Vinson, pers. comm.), and the distal breakpoint of $Df(3L)Brd^{R15}$, 70F4-6.

Most of the deficiencies listed were characterised cytologically by the laboratories that produced them, but two sets of breakpoints were determined by me: $Df(3L)fz^{GP3b}$ had not been previously mapped and $Df(3L)Brd^{R15}$ had only been mapped approximately. Since the latter defines the proximal end of the interval in which gnu must lie, it was decided to determine the breakpoints more precisely. The relevant portion of the polytene chromosome arm 3L of a $Df(3L)Brd^{R15}/+$ heterozygote is shown in Figure 5.3. Comparing the wild type chromosome (Figure 5.3a) with the deficiency shown in

Figures 5.3b and 5.3c, it can be seen that the deficiency removes material between 70F4,6 and the doublet 71C1,2.

Flies heterozygous for Df3LBrd^{R12} (which removes the gnu gene) and gnu show a phenotype that is no more extreme than gnu/gnu homozygotes (data not shown). The implications of this result have been discussed in Chapter 3.

Figure 5.2

The Cytological Localisation of the *gnu* Gene Using Chromosomal Deficiencies

The deficiencies tested for their ability to uncover the *gnu* mutation are shown. The top four are those that do uncover *gnu*, and the bottom seven do not. The details of the breakpoints of these deficiencies are given in Table 5.2. From these results, it has been concluded that *gnu* lies in the indicated interval, that is, 70E4,6;70F4,6.

Figure 5.2

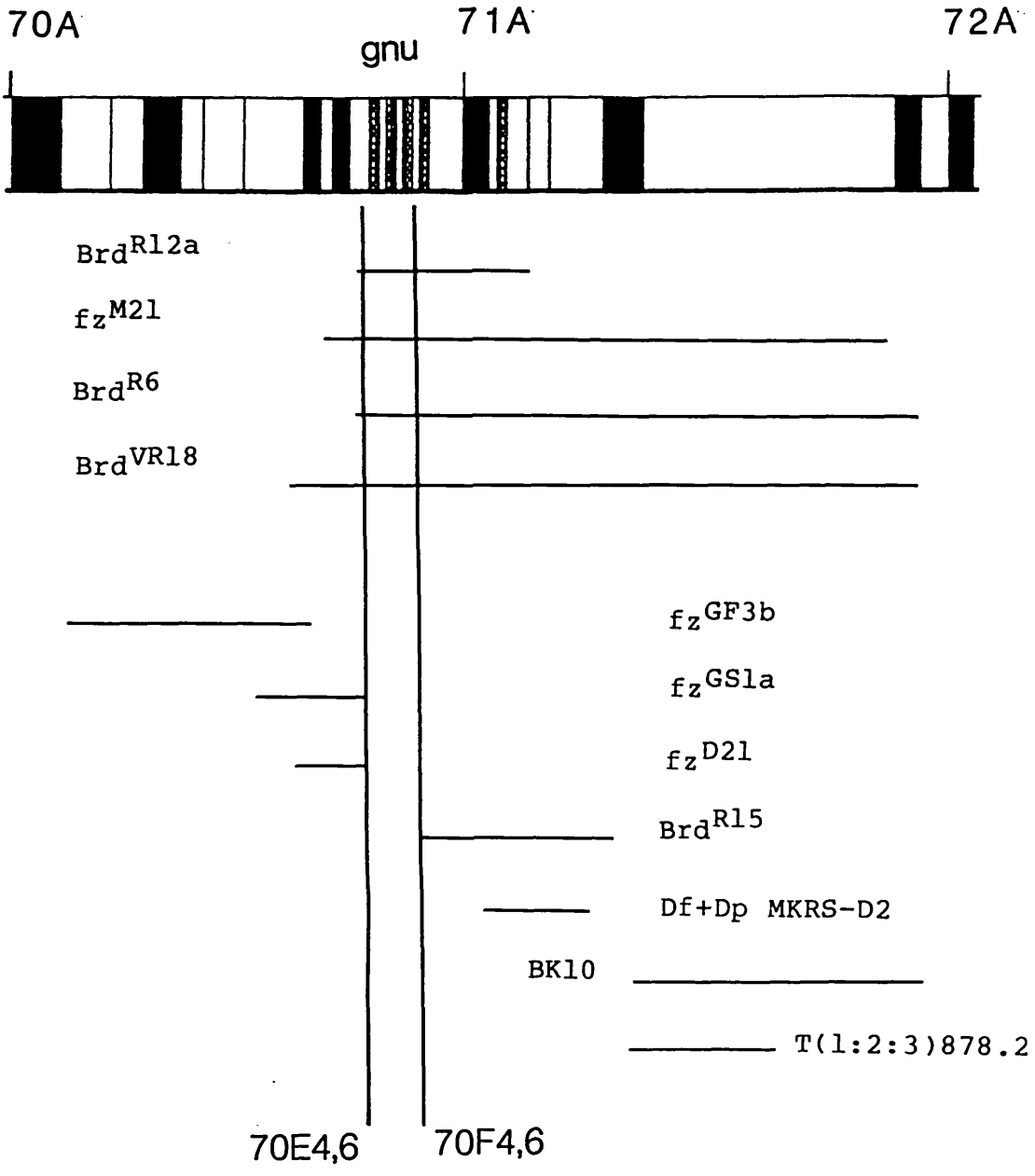

70A
gnu
71A
72A
Brd^R12a
fz^M21
Brd^R6
Brd^VR18
fz^GF3b
fz^GS1a
fz^D21
Brd^R15
Df+Dp MKRS-D2
BK10
T(1:2:3)878.2
70E4,6
70F4,6

Table 5.2

Stocks Used for Cytological Mapping of gnu

Stock	Cytology	Donor
Df(3L):		
fz^{GP3b}	70B2-3;70D6	1
fz^{GS1a}	70C6-15;70E4-6	1
fz^{M21}	70D2-3;71E4-5	1
fz^{D21}	70D3;70E3-8	1
Brd^{VR18}	70D;71F	2
Brd^{R12a}	70E;71B	2
Brd^{R6}	70E;71F	2
Brd^{R15}	70F4-6;71C1-2	2
Df+Dp MKRS-D2	71A2-B1;71B4-8	3
T(1:2:3)878.2	71C1-2;71D4-E1	3
BR10	71C2;71F4	4

The donors of these stocks, to all of whom I am grateful, were:

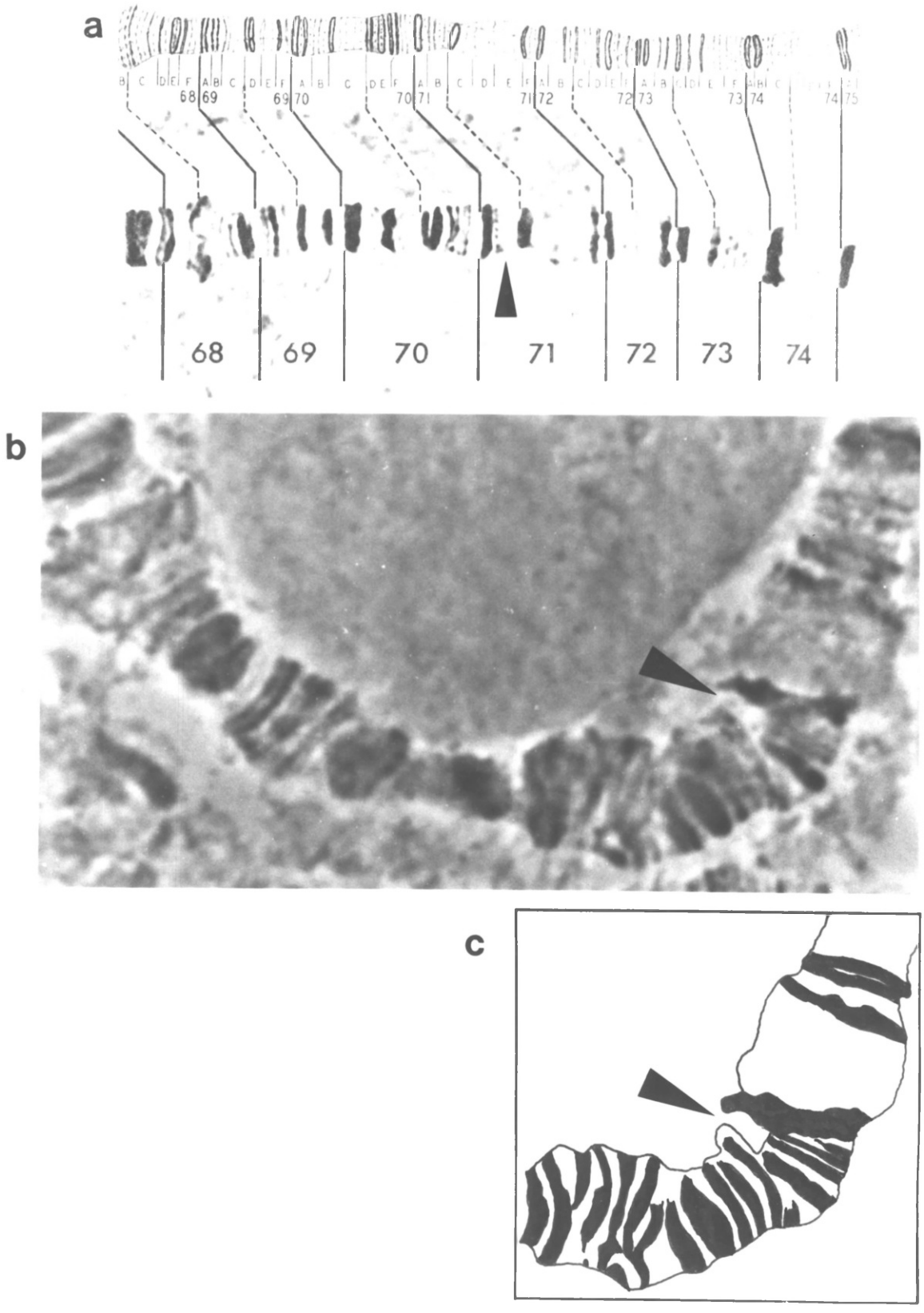
1. Dr Charles Vinson, University of Virginia.
2. Dr Jim Posakony, University of California, San Diego.
3. Prof William Gelbart, Harvard University.
4. Dr Brenda Leight, Indiana University.

Figure 5.3

Cytology of the Deficiency Df(3L)Brd^{R15}

Panel 'a' shows the relevant region of a wild type chromosome (taken from Lefevre, 1976). Panel 'b' shows a photograph, and Panel 'c' a freehand interpretation, of part of 3L of a polytene chromosome from a deficiency/+ heterozygote (see Materials and Methods, Section 2.7). The region of the deficiency is indicated by an arrow in each of the three panels. It is seen as a short unpaired region, where the chromosome is half the normal width. Careful examination allows the assignment of the breakpoints of this deficiency as 70F4,6 proximally, and 71C1,2 distally.

Figure 5.3



5.4 A P-Element Mutagenesis Screen to Isolate Further Alleles of gnu

The phenomenon of hybrid dysgenesis is caused by the mobilisation of specific transposable elements (P-elements) in the germ line of F1 hybrids when males carrying P-elements (P males) are crossed with females without P-elements (M females) (Kidwell et al., 1977; Rubin et al., 1982; Bingham et al., 1982). The exploitation of this high rate of transposition has led to a general protocol for the production of mutant alleles of Drosophila genes (Engels, 1985a; Kidwell, 1986), and this strategy has been used to produce mutations in many different genes (see, for example, Adler, 1984; Yamamoto and Mason, 1985). One of the main advantages of this method of mutagenesis is the high probability that a mutation induced by hybrid dysgenesis will be caused by the insertion of a P-element in, or around, the DNA sequence of the gene in question. This provides a molecular marker for the DNA of the mutated gene, which enables it to be cloned relatively straightforwardly. This 'transposon tagging' approach has been successfully used to clone several Drosophila genes (see, for example, Searles et al., 1982; Kidwell, 1986). The isolation of new alleles of gnu would allow further genetic and developmental analysis of the mutation. As the eventual aim of this work is to clone the gnu gene in order to carry out its molecular characterisation, a P-element

mutagenesis screen was designed.

The screen was conceived as a continuous 'rolling screen' in which a different mutation could be tested in each generation for non-complementation with the same dysgenically mutated chromosomes. Using this approach, it was hoped that P-induced alleles of four different maternal effect lethal mutations (gnu, thule, lodestar, and polo) might be isolated. This screen was carried out in collaboration with C. Sunkel and M. Leibowitz, and the genetic scheme used is shown in Figure 5.4. The males used in the hybrid cross were a strong P-strain (π2, Engels, 1985b), and the red e/red e females were confirmed to be M-strain by the absence of P-element sequences in their DNA (R. Karess, pers. comm.). It will be seen that the main feature of this screen is that at each generation two different classes of progeny were selected, females to be tested for sterility, and sibling males to be used for the next stage of the screen.

The search for alleles of maternal effect lethal mutations, such as the ones used in the present study, is inherently more time consuming than screening for zygotic mutations, as the phenotype (female sterility) is not manifest until the F2 generation. This means that two generations of pair matings are required for each mutant tested, and this severely limits the number of chromosomes that can realistically be screened. In this experiment we started the first round (Cross 3 in Figure 5.4) with 6700 potentially mutated chromosomes (red e^*). One

problem with the 'rolling screen' was a high rate of loss of test chromosomes at each stage and consequently, by the time we were testing the fourth mutant, the number of chromosomes remaining was very small. The cumulative figures for the number of chromosomes tested for each mutation in this experiment, and in a repeat of the same scheme, are given in Table 5.3. It will be seen that no P-element induced alleles of any of the four mutations were isolated. This is presumably because not enough chromosomes were screened, although we cannot rule out the possibility that some, or all, of these mutations are 'cold-spots' of P-element insertion (Adler, 1984; Kidwell, 1986).

Figure 5.4

Genetic Scheme for a Hybrid Dysgenic Mutagenesis Screen for Alleles of *gnu*, *thule*, *lodestar*, and *polo*

All markers are as described in Lindsley and Grell (1968). The initial P males and M females are described in Materials and Methods (Section 2.2). The females to be tested for sterility were put singly into a tube with 2 or 3 males, and scored for the absence of hatched larvae after 5 or 6 days. Any females that appeared sterile were retested with new males. The sibling *red e*/Balancer males of any females that remained sterile after the second test were used to establish lines containing the mutated chromosome of interest. (MEL - maternal effect lethal).

Figure 5.4

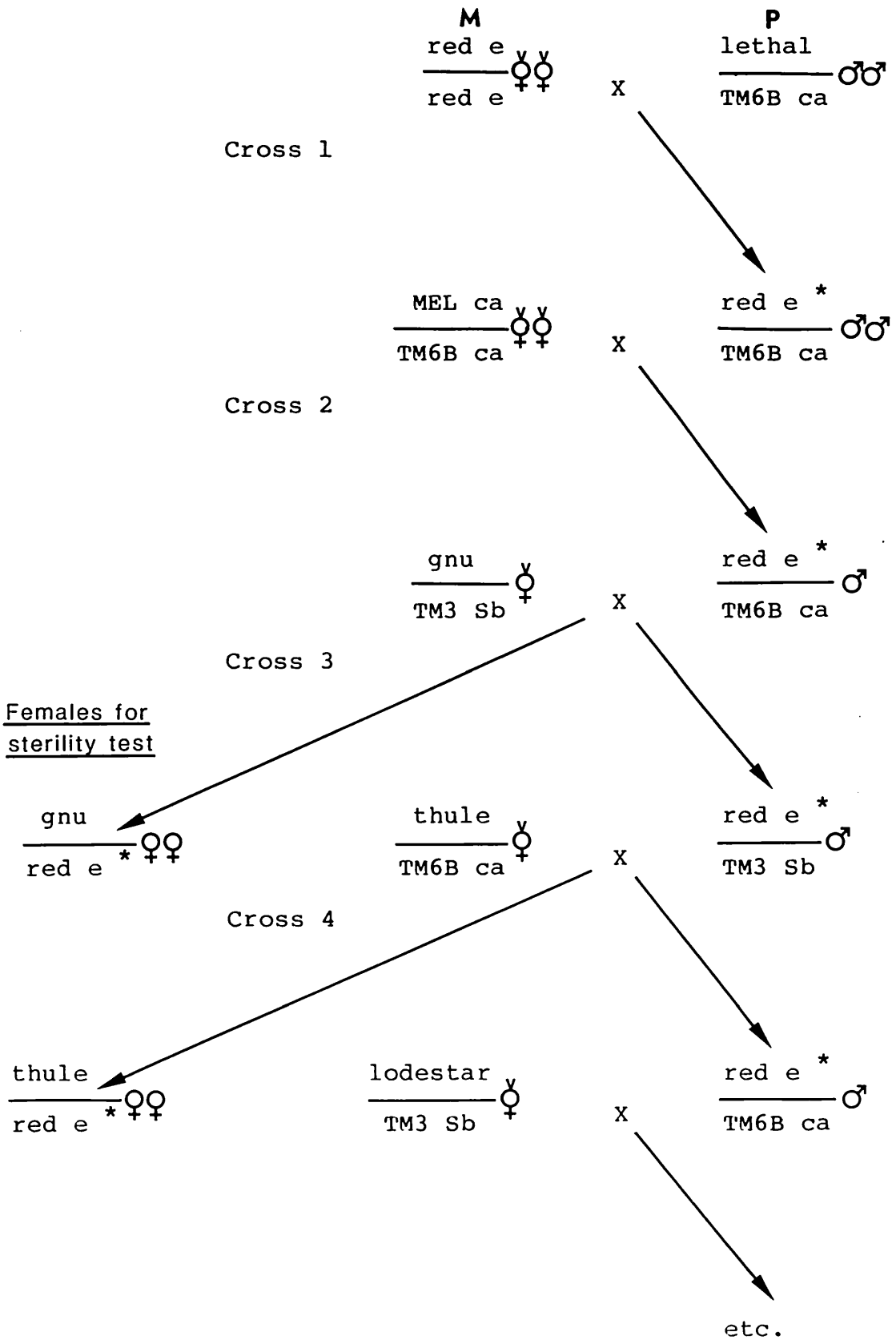

M
red e
red e
X
P
lethal
TM6B ca
Cross 1
MEL ca
TM6B ca
X
red e *
TM6B ca
Cross 2
gnu
TM3 Sb
X
red e *
TM6B ca
Cross 3
Females for sterility test
gnu
red e *
thule
TM6B ca
X
red e *
TM3 Sb
Cross 4
thule
red e *
lodestar
TM3 Sb
X
red e *
TM6B ca
etc.

Table 5.3

Data from P-Element Mutagenesis Screen

Mutant	Number of red e^*/mat(3) tested for sterility	Number sterile
gnu	6187	0
thule	3720	0
lodestar	2437	0
polo	1055	0

5.5 DISCUSSION

The genetic and cytological mapping of the gnu gene are described in this chapter. gnu lies at approximately 42.5 mu on the left arm of the third chromosome, and is located between 70E4-6 and 70F4-6 on the polytene chromosome banding pattern. An attempt to produce alleles of gnu by P-element insertion mutagenesis has not been successful, so an approach other than transposon tagging will have to be used in order to clone the gene. The fairly precise cytological location makes a chromosome walking and jumping strategy a possibility, and indeed, there does exist an entry point into the region around the gnu gene (P. Cherbas, pers. comm.).

Chromosome walking is a technique in which, having obtained a segment of DNA from a known region of the genome, the DNA flanking that clone can be isolated by using end fragments of the original clone as probes to screen a genomic library. Similarly, end fragments from these next clones are used to isolate the next segments of flanking DNA, so that after many rounds of this process, considerable lengths of continuous stretches of DNA can be cloned (Bender et al., 1983b). The size of each 'step' of the walk is determined by how much DNA can be contained in the vector used (about 18kb in bacteriophage vectors, and 40kb in cosmid vectors), and by the degree of overlap between adjacent clones, and in practice averages about 10kb for a phage vector. A modification of this

technique, which allows a jump from one region of the chromosome to another, without the need to clone the intervening DNA, is to take advantage of existing chromosomal rearrangements that bring together DNA sequences that are normally separated by hundreds or thousands of kilobases. There have been several reported examples of the cloning of genes using chromosome walking and jumping (for example, Bender et al., 1983a; Bender et al., 1983b; Kidd et al., 1983; Garber et al., 1983; Butler et al., 1986; Kilcherr et al., 1986; McKeown et al., 1987). The two critical features that influence the success of chromosome walking appear to be the availability of a convenient entry point into the region of interest, and the total length of walk required: up to about 300kb is feasible, more than that is very time consuming. These two factors will now be considered with respect to the possibility of cloning the gnu gene using such an approach.

An entry point into a walk consists of a piece of cloned DNA from the region to be studied, and can be obtained either by using already existing clones whose cytological position is known, or by microdissection of the relevant bands from the polytene chromosomes (Scalenghe et al., 1981). To the best of my knowledge, there is no existing cloned DNA in the 70EF region, but there is a 220kb walk between 71C1,2 and 71F1,2, which was used to clone the the ecdysone inducible Eip28/29 gene from the 71C region (P. Cherbas, pers. comm.). The

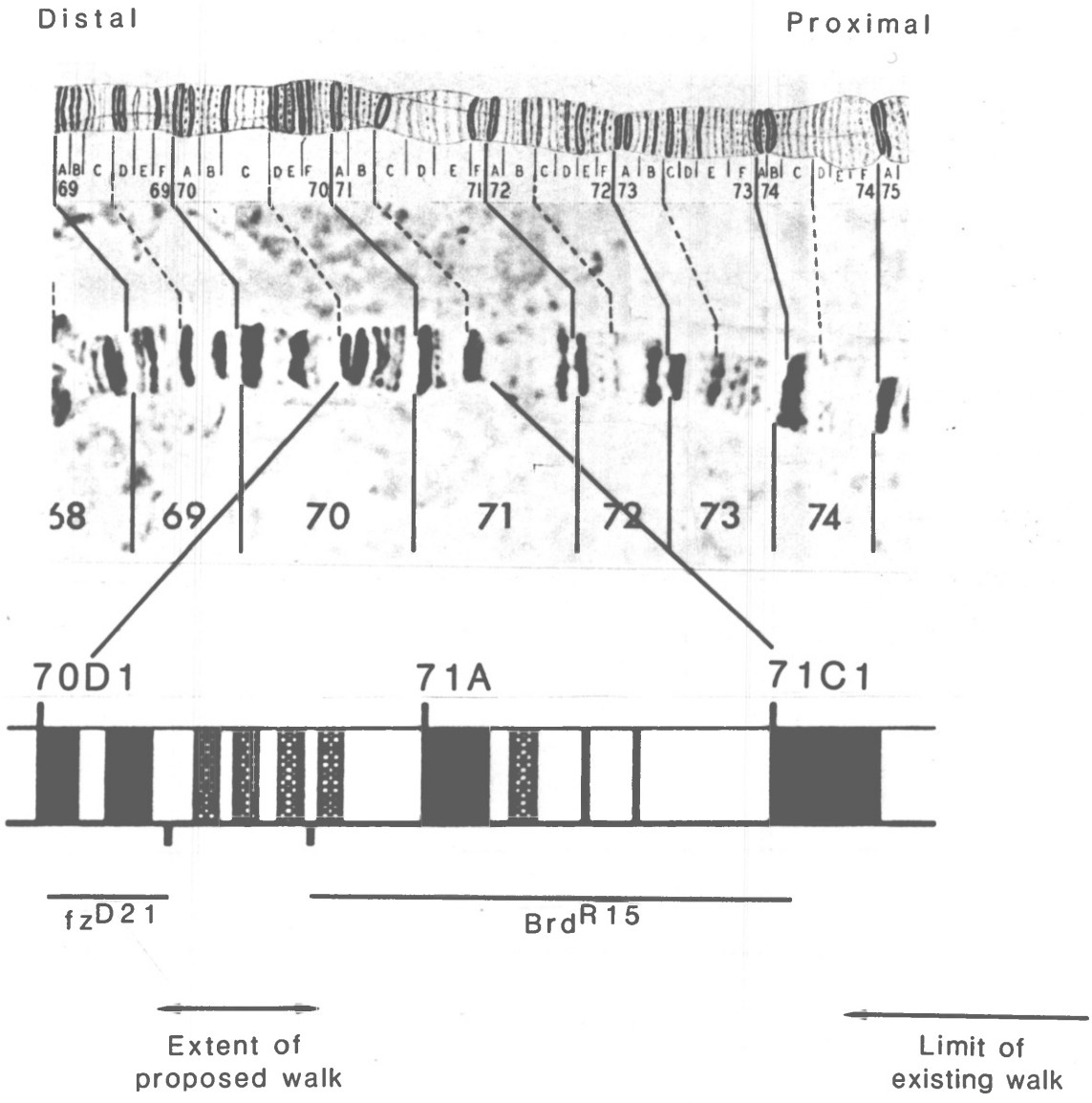
deficiency Df(3L)Brd^{R15} removes DNA between 71C1,2 and 70F4,6, the proximal limit of the gnu interval, and therefore brings sequences from these positions adjacent to each other. Therefore, if the proximal end point of this deficiency can be identified at the DNA level, the clone that spans it can be used to probe a library of DNA made from the deficiency stock and hence obtain sequences from 70F4,6. These 70F4,6 sequences can then be used as a starting point from which to walk through the gnu interval until the distal breakpoint (70E4,6) is reached, at which stage the gnu gene will necessarily have been isolated (see Figure 5.5). This end-point can be identified by using a library of DNA from flies carrying the Df(3L)fz^{D21} chromosome (which defines the distal breakpoint) from which to clone the steps of the walk, and by hybridising successive clones in situ to wild type chromosomes. When the breakpoint is reached the clone that spans it will hybridise to two distinct places on the wild type chromosomes, corresponding to the two breakpoints of the deficiency used. In practice, this will not be straightforward, as the Df(3L)fz^{D21} stock is lethal when homozygous, which means that the library of DNA from this stock will also contain DNA from a balancer chromosome, which will not be deleted for the region. This will mean that there will be two classes of clone that span the breakpoint, one from the deficiency chromosome, and one from the balancer. This problem can be overcome by selecting, at each stage of the walk, a group of clones,

Figure 5.5

Potential Cloning Strategy for the *gnu* Gene

The significant deficiencies and existing cloned DNA are shown against a diagram of the relevant part of 3L, and against a photograph and drawing of the chromosome taken from Lefevre (1976). It is not clear (see text) whether the existing walk which terminates in the 71C1,2 doublet overlaps with the proximal breakpoint of *Df(3L)Brd*^{R15}, which also lies in the 71C1,2 doublet. The extent of the proposed walk is estimated to be between 80kb and 130kb (see text).

Figure 5.5


Distal
Proximal
68
69
70
71
72
73
74
70D1
71A
71C1
fzD21
BrdR15
Extent of proposed walk
Limit of existing walk

and mapping them in a preliminary manner. When the breakpoint is reached, two distinct classes will be found, corresponding to the two chromosomes. This *in situ* hybridisation technique can be complemented by comparing Southern blot patterns obtained when clones from the walk are hybridised with restriction enzyme digested DNA from either wild type or deficiency stocks. These techniques have been successfully used to delimit chromosome walks used to clone *Notch* (Kidd et al., 1983) and the *rosy-Ace* locus (Bender et al., 1983b).

The initial part of this walking strategy involves identifying a clone which spans the proximal breakpoint of *Df(3L)Brd*^{R15}. The breakpoint lies within the heavy doublet 71C1,2, and Figure 5.3a shows that some of the doublet appears to be removed by the deficiency. The distal limit of the existing walk is also within this doublet, but is estimated to be within the proximal half of the band (P. Cherbas, pers. comm.). It is not known whether the breakpoint lies within sequences already included in the walk, or whether the walk will have to be extended distally to reach this position. If the breakpoint is already cloned, then the necessary chromosome walk can begin at 70F4,6; if not, then this first walk will have to be included in the estimate of total walking distance required.

In some of the long chromosome walks that have been reported an attempt has been made to estimate the amount of DNA in each band (Spierer et al., 1983; Pirotta et al.,

1983; Garber et al., 1983; Butler et al., 1986). The main conclusion of such studies is that the figure is extremely variable, ranging from about 3kb to nearly 200kb, and that, on average, interband regions contain 10-20 times less DNA than bands. This dramatic variation means that it is not possible to determine precisely how much DNA spans a given region of polytene chromosome, but there is a definite correlation between density (and width) of bands, and their DNA content (Spierer et al., 1983). Therefore a comparison of the appearance of the bands that have been characterised with those in the gnu interval does produce an estimate of the length of this interval, which is probably accurate enough for this to be a worthwhile preliminary exercise before attempting a chromosome walk.

If the 71C1,2 band is considered first (although it is not clear that this region will need to walked across at all), it is found to be comparable with heavy bands that have been cloned already such as 3C1-3 (Pirotta et al., 1983), 73A3-4 (Butler et al, 1986), and 87E1-2 (Spierer et al., 1983). (All comparisons of bands are made from the photographic representation of the polytene chromosomes by Lefevre (1976)). These doublets have been found to contain approximately 150kb, 100kb and 160kb respectively. Therefore an estimate of 150kb for the 71C1,2 doublet seems reasonable. If it is assumed that the one third of the distal edge of the band is removed by $Df(3L)Brd^{R15}$, and that the walk extends 20kb into the proximal edge of

the band, the estimated distance needed to walk to the breakpoint is 80kb.

In the actual gnu interval (70E4,6;70F4,6) there are 6 - 9 bands drawn by Bridges (revised drawings as shown in Lindsley and Grell, 1968), but, as has been discussed about several other regions of the genome (Berendes, 1970; Lefevre, 1976), not all the Bridges' bands are usually seen in chromosome preparations. In fact, I can consistently only see three faint bands in this region (which agrees with the Lefevre photograph, 1976), of which one, 70F1,2, is given as a doublet by Bridges. The calculation can be done for both estimates of band number, which will probably give an upper and lower limit for the length of DNA in the gnu interval. Taking Bridges' account, there are four faint bands and five very faint bands in the maximum interval (70E4;70F6). Using estimates of DNA per band from the references given above, it is reasonable to assign 20kb to each faint band, and 10kb to each very faint band. These values give a total of 130kb for the interval. Using the photographically visible bands as an estimate, there are two faint bands and one moderate band, and if 40kb is allowed for the moderate band (the 70F1,2 doublet of Bridges), then a value of 80kb for the entire interval is obtained. Taken together, these calculations provide an upper limit of the length of the proposed walk of 210kb (including the potential 71C1,2 walk), and a lower limit of 80kb (excluding the 71C1,2 region). These calculations are

only given to demonstrate the feasibility of a walking strategy to clone gnu, and are not intended to be a precise estimate of the length of the walk, since there is clearly considerable variation in the amount of DNA in bands even of approximately similar density and width (Spierer et al., 1983).

The attempt to induce hybrid dysgenic alleles of gnu, and three other maternal effect lethals (thule, lodestar, and polo), thereby tagging the genes with P-elements, was unsuccessful. It is not possible to determine why no alleles were obtained, but several comments can be made about the strategy used. The primary reason for failure is likely to have been that too few chromosomes were screened. 6187 chromosomes were tested for non-complementation with gnu, and considerably fewer with the other three mutants. The reasons for these relatively small numbers were twofold. Firstly, the fact that a screen for female sterility has to be tested in the F2 generation means that two rounds of pair matings are required for each mutation, and the time needed to set up these pair matings limits the number of chromosomes that can be tested. Furthermore, in this 'rolling screen', two tubes of flies were set up from each tube from the previous generation, again multiplying the amount of work involved. Thus the original 6700 pair matings in the third generation of the screen (see Figure 5.4) occupied about 13,000 tubes at the next generation, of which half were tested for sterility after one week, and the other

half had to be sorted into test females and red e*/Balancer males for the next round of the screen, two weeks after setting them up. We still believe that it would not be practicable to make the screen much larger without a greater investment of manpower.

The second reason that the number of chromosomes tested was so low was the unexpectedly high rate of loss of chromosomes at each stage of the screen. About 15-20% of all tubes of pair matings set up never produced any progeny (or too few to collect the necessary genotypes), and this meant that by the final stages of the screen only a very few test chromosomes remained (compare 6187 chromosomes tested with gnu, with 1055 tested with polo). The reasons for this high rate of loss are not clear, but probably relate mainly to the practical problems of handling such great numbers of pair matings. Flies tended to stick to the food at a high frequency, partly because the deep, narrow tubes used collected condensation on their insides, and therefore did not dry efficiently. Other reasons must include some natural sterility, and some dysgenically-induced gonadal sterility (Kidwell et al., 1977), although at 24°C the rate of such sterility is not expected to be high. In retrospect, a larger screen for alleles of a single mutation would have been more likely to have succeeded, although the significant probability that any given gene is a 'cold spot' for P-element insertion (Kidwell, 1986), means that such an approach carries risks of its own.

Rates of success of inducing P-element alleles in given genes vary enormously, from 0 out of more than 10^6 chromosomes for the alcohol dehydrogenase gene (Kidwell, 1986) to 0.4% for Beadex (Simmons et al., 1984). Other rates achieved include 9/11,250 for claret (Kidwell, 1986); 3/42,266 for mei-41, 2/42,266 for mei-9, and 1/42,266 for mus(1)102 (Yamamoto and Mason, 1985); 7/20,100 for hairy (Ingham et al., 1985); and 2/250,000 for Aristapedioid (Adler, 1984). It can be concluded from these figures that gnu (0/6187) is not a particularly hot spot for P-element insertion, but that there is no evidence that it is a genuinely cold spot. It is difficult to estimate how many genes are truly refractory to P-element insertion, especially as failed attempts are unlikely to be reported as often as successful ones, but it has been suggested that the figure might be as high as half of all genes (Searles et al., 1982; Simmons and Lim, 1980). For this reason P-element mutagenesis is probably most efficiently used to generate classes of mutation, rather than to produce alleles of specific genes. For example, about 4% of P-mutated chromosomes gave zygotic lethal phenotypes (R Karess, pers. comm.), and 2/2942 chromosomes gave female steriles (Kidwell, 1986).

CHAPTER 6

Discussion

6.1 Summary of Results

The results that have been described in the preceding chapters have analysed aspects of the phenotype of the gnu mutation of Drosophila. gnu is a maternal effect lethal which results in the embryos of homozygous females being defective in nuclear division, but not in DNA replication, and this leads to the formation of a small number of giant nuclei in the syncytial blastoderm. The experiments that I have carried out have led to the following conclusions about the gnu mutation. First, much of the structural apparatus of mitosis is present and functionally adequate in GNU embryos; specifically centrosomes are able to replicate, and their ability to nucleate microtubules appears unaffected. Second, GNU eggs develop even if unfertilized, although there is no block to their insemination, and the male pronucleus can replicate. Third, polar bodies replicate in GNU embryos. Fourth, the male pronucleus is able to replicate in embryos from females that are homozygous for the female sterile haploid mutation, mh (Gans et al., 1975; Santamaria and Gans, 1980), and gnu; the male pronucleus is not replicated in the embryo of a female homozygous for mh alone. Fifth, the unfertilized GNU egg contains centrosomes which can nucleate microtubules, demonstrating that the sperm is not the only possible source of centrosomes in the Drosophila embryo. Sixth, the gnu gene maps at 42.5mu on Chromosome 3, and lies between 70E4,6 and 70F4,6 on the banding

pattern of salivary gland polytene chromosomes.

6.2 The Centrosome Cycle

Perhaps the most intriguing result obtained from the immunostaining of GNU embryos is the uncoupling of nuclear and centrosomal division. As has been discussed in Chapter 3, although there have been reports of some limited independent centrosomal division, this is the first case where the centrosome's capacity for division in the absence of nuclear division is so extensive. It clearly implies that there is no requirement for association with the nucleus in order for centrosomes to replicate. Since mitosis can be seen as a series of necessary events, which presumably must occur in a specific temporal order, it might be expected that these events are linked in a linear pathway, each one being dependent upon the action of the previous one. Indeed, as was discussed in Chapter 1, an extensive genetic analysis of the yeast cell cycle, has led to a model which is based on a linear pathway of this type (Pringle and Hartwell, 1981). A priori, this might seem to be the most efficient method of ensuring that the complex series of processes which comprise mitosis are carried out in the required order. However, the results presented here argue that in Drosophila, nuclear and centrosomal division, at least, are not linked in such a relationship. Instead they appear to be independent, and can be imagined to be on

parallel pathways, so that one does not rely upon the other. Presumably these pathways are regulated in normal mitosis, in such a way that ensures their coordination, perhaps by some 'master oscillator' mechanism to which both can respond. The cytopasmic oscillations that are known to exist in Xenopus eggs, and which continue even when the egg is enucleated (Hara et al., 1980), might occur in response to an analagous oscillatory mechanism. It is interesting to speculate that while such a system of parallel pathways might superficially appear less efficient than a single linear chain of events, it would be expected to allow a greater degree of flexibility in the detailed mechanisms of cell division, a mitotic flexibility that might be essential in higher eukaryotes in which patterns of cell growth change during development. The most obvious change that does occur in the type of cell growth in Drosophila is the switch between diploid cell division and endomitosis, resulting in polytenisation, which occurs in most larval tissues. Another change that the mitotic mechanisms must accommodate is the switch between the rapid, synchronous nuclear divisions that are characteristic of the cleavage stages, and the much slower, asynchronous divisions - including cytokinesis - found after cellularisation. The potential of uncoupling different parts of the cell cycle might facilitate these developmental changes.

6.3 Aster Formation in GNU Embryos

Information about another phenomenon of mitosis, that of microtubule growth and stabilisation, has also been derived from the antibody staining experiments that have been described. There has been much discussion about the dynamics of microtubule growth during different phases of the cell cycle (see, for example, Williams et al., 1986). Briefly, Mitchison and Kirschner (1985) have proposed a model in which, once nucleated from the centrosome, microtubules continue to grow outwards in all directions until either they lose their GTP 'caps', in which case they rapidly depolymerise completely, or until they attach to a kinetochore, in which case they are stabilised and a mitotic spindle is initiated. However, Horio and Hotani (1986) have used video microscopy to observe aster microtubule growth and shrinkage in real time, and the kinetics of this process seem more complex than allowed for in the Mitchison and Kirschner model. The observation that I have made, that asters, generally believed to be the most ephemeral of microtubule structures, can grow until they reach at least twenty times their normal diameter, implies that any model to explain the kinetics of microtubule growth must incorporate sufficient stability of polymerising microtubules to allow the existence of such giant asters.

6.4 The Mitotic Apparatus in GNU embryos

It can be seen that the analysis of gnu has led to some insights into the processes of centrosomal replication and the dynamics of microtubule growth. Other observations of GNU embryos, and of embryos of similar maternal effect lethals (D. Glover, C. Sunkel, M. Leibowitz, and J. Raff, pers. comm.), have led to some more general characteristics of the mitotic apparatus in Drosophila becoming apparent. For example, it is clear that a lamin-containing structure can envelope giant nuclei and other abnormal nuclei in a manner that is very like the nuclear membrane of wild type embryos; this is similar to the result of Forbes et al. (1983) in which nuclear envelopes were found to form around bacteriophage λ DNA injected into Xenopus eggs. Also, it appears to be a characteristic ability of centrosomes to nucleate microtubules into asters, even when completely dissociated from nuclei, as was described in Chapter 3. This contrasts with the conclusion that centrosomes are only able to nucleate asters when associated with nuclei, which was reached by Karsenti et al. (1984) studying the injection of purified centrosomes into sea urchin eggs. Another phenomenon, observed sometimes in GNU embryos, but frequently in the embryos of other maternal effect lethals, is the clustering of centrosomes on, or around, the nuclear envelope. This suggests that there is a general affinity of centrosomes for the nuclear envelope,

which could be useful in positioning centrosomes adjacent to the nucleus in wild type, interphase cells.

6.5 The Maternal Origin of Centrosomes

A consequence of the observation that GNU eggs can develop even when uninseminated is that it allows one to address the question of the source of the centrosomes in the Drosophila embryo. I have shown that the unfertilized GNU egg contains centrosomes, and that these centrosomes are functional, as judged by their ability to nucleate microtubules into asters. It is not possible to conclude from this result that the centrosomes are maternally contributed in wild type development, but the capacity of the unfertilized egg to form centrosomes is clear, even if this only happens when the egg is activated in some way. It is interesting that, while the block to the development of the unfertilized egg is one that is common to a very wide range of species (Went, 1982), two different strategies have evolved for obtaining the centrosomes required for the early cleavage divisions of embryogenesis. Thus, during normal mouse development the centrosomes are maternally derived (Schatten at al., 1986; Callarco-Gillam et al., 1983), whereas sea urchin centrosomes are paternally derived (Schatten et al., 1986). It has generally been accepted that centrosomes are also paternally derived in Drosophila (Sonnenblick, 1950). However, I have shown that unfertilized Drosophila

eggs can produce centrosomes, and this has also been reported to be the case in artificially activated sea urchin eggs (Dirksen, 1961). Therefore, the overall picture is still unclear but it seems that although there are some species in which the centrosome is maternally derived, and others in which it comes from the sperm, there is some ability to override the paternal requirement in at least two of this latter class of species. It is clear that Boveri's generally accepted rule, that the centrosome is paternally derived, is not universally true and, in fact, he himself was aware of several exceptions (Wilson, 1928).

6.6 The Primary Lesion of the gnu mutation

The mitotic apparatus that has been examined by antibody staining in GNU embryos appears relatively unaffected by the gnu lesion. Therefore, although the study of gnu has provided considerable amounts of information about mitosis - both about specific mechanisms, and about more general properties of the various parts of the mitotic apparatus - the primary defect of the gnu mutation appears not to be in the structural elements of nuclear division. This illustrates the point that superficial analysis of the phenotype of a mutation does not necessarily reveal its underlying defect, even when the phenotype is so striking as to make the lesion seem obvious: the giant nuclei of GNU embryos

would seem to be clearly a result of defective mitosis, but it turns out that the primary defect appears actually to be in the repression of DNA synthesis in the unfertilized egg - a process only indirectly related to mitotic nuclear division.

There is also more direct evidence that gnu affects something other than the structural apparatus of mitosis. One indication is the fact that the gnu mutation is a maternal effect lethal, and shows no detectable zygotic effects; since some mitosis occurs throughout the Drosophila life cycle, a defect in an essential structural component might be expected to be zygotically lethal. A second indication is the observed replication of DNA from polar bodies in GNU embryos; a third, the discovery that GNU eggs can develop without fertilization. These observations have now led to the hypothesis that the wild type product of the gnu gene participates in the block to DNA synthesis which is found in unfertilized eggs, and that the gnu mutation is responsible for setting up a 'replicative environment' in the GNU egg. This model is supported by the fact that if a GNU egg is inseminated, the sperm pronucleus does replicate. Furthermore, if the maternal genome is homozygous for mh and gnu, the male pronucleus replicates, whereas the MH cytoplasm normally prevents it from doing so. This latter result allows one to begin to postulate a scheme of genetic interactions between gnu and other genes. Thus, the gnu gene is epistatic over the mh gene, indicating that it operates at

a higher level in the pathway which regulates the initial development of the egg. It is hoped that the analysis of the interaction between gnu and other mutations will lead to further knowledge about the processes of activation of DNA synthesis in the egg.

Most of the information that currently exists about the mechanism of the repression of DNA synthesis in the unfertilized egg has come from the study of maturation promoting factor (MPF) in Xenopus (Maller and Krebs, 1980). This factor was first identified as being an activity in the unfertilized egg that, when injected into the oocyte, is able to induce maturation (Masui and Markert, 1971; Smith and Ecker, 1971). MPF also appears to be involved in the later activation of DNA synthesis in the egg. The unfertilized Xenopus egg is arrested in metaphase of meiosis II, and contains a large amount of MPF; immediately after fertilization, a strong anti-MPF activity is found, and DNA synthesis is initiated. This result leads to the idea that it is the fixed high level of MPF that keeps the unfertilized egg in an arrested state, and that the MPF-inhibiting factor is responsible for allowing the S-phase of the first cleavage division to begin (Gerhart et al., 1984). There is a less well defined factor, cytostatic factor (CSF), also found in unfertilized eggs, which is able to induce metaphase arrest when injected into cleavage embryos (Newport and Kirschner, 1984). These experiments have demonstrated that there is a complex set of interactions between

various factors which keep the unfertilized egg in developmental arrest and, although the details of the mechanism are still far from clear, it does seem likely that this line of research will lead to insights into the activation of the Xenopus egg. To my knowledge, MPF activity has not been demonstrated in Drosophila, but its apparent ubiquity (see, for example, Nelkin et al., 1980; Kishimoto et al., 1982) makes it likely that it does exist. In this case it would be interesting to investigate the MPF activity of GNU cytoplasm. If the current models are correct, it might be expected that GNU embryos contain no MPF activity, thereby never allowing the nuclei to pass into metaphase and subsequent division. However, it is also possible that the gnu lesion occurs below MPF in the hierarchy of events that are involved in activation, and that the MPF activity of GNU eggs is normal.

6.7 gnu and Parthenogenesis

Since gnu appears to lift the repression of DNA synthesis that stops unfertilized eggs from replicating their DNA, it is interesting to consider what relationship the gene has with parthenogenesis. Most Drosophila species are tychoparthenogenetic, that is they show a low rate of 'accidental' parthenogenesis (Stalker, 1954). Therefore, in the rare individuals that do develop parthenogenetically, the developmental blocks, including

the block to DNA synthesis, must be overcome in the absence of a sperm. How these barriers to development are lifted is not known (Went, 1982; Maller and Krebs, 1980). It is, however, clearly a rare event in Drosophila melanogaster, and in Stalker's study of 1954, only two embryos hatched from more than 500,000 unfertilized eggs examined. In some way aspects of parthenogenesis are mimicked in the GNU egg, in that the block which normally inhibits DNA replication in the unfertilized egg is removed. Since it has been shown that many of the events that had been thought to be sperm-dependent are, in fact, triggered by oviposition (Mahowald et al., 1981), the unfertilized egg can be imagined to be primed, and perhaps is in a metastable state in which the only check to development is the lack of DNA synthesis. In Drosophila polymorpha Stalker (1954) found a spontaneously arising strain which caused this normally tychoparthenogenetic species to develop from unfertilized eggs at a high rate. This characteristic was heritable in a Mendelian fashion and thus was presumably due to a mutation which somehow reduced the stability of the unfertilized egg. Furthermore, Fuyama (1986) has established a line of Drosophila melanogaster that reproduces efficiently gynaegenetically (without the need for males), even though the rate of fertility is much lower than that of fertilized eggs from wild type females. However, a number of different studies have ruled out the possibility that there is a single gene that allows parthenogenesis when

mutated (Stalker, 1954; Templeton, 1983; Fuyama, 1986). Nevertheless, the fact that there are mutations that can increase the rate of spontaneous parthenogenesis by up to 1000-fold (Templeton *et al.*, 1976), suggests that the developmental block of the unfertilized egg is not insurmountable, and may not involve many gene products.

The derepression of DNA synthesis is a minimum requirement for parthenogenetic development, and this provides a link between the gnu gene and the changes that must occur to allow parthenogenesis. It is not being argued that the product of the gnu gene alone is the block to development of unfertilized eggs - the phenotype of GNU embryos demonstrates that this is not the case - but that the activation of DNA synthesis, which appears to be where the gnu gene acts, is one of the rather few necessary processes that must be involved in the parthenogenetic development of Drosophila eggs. For this reason it seems likely that the genetic and molecular analysis of the gnu mutation might provide some insights into the mechanisms that allow parthenogenetic development in Drosophila, and perhaps also in the many other species in which parthenogenesis is known to occur (Mittwoch, 1978). However, overcoming the block to DNA synthesis is not the only hurdle facing an embryo developing parthenogenetically. The experiments of Santamaria (1983) show that MH embryos die because of the failure to restore diploidy in the embryo. This result illustrates that even if the DNA synthesis block is removed, the restoration of diploidy is

another barrier that needs to be overcome during parthenogenetic development.

6.8 Secondary Effects of the gnu mutation

The hypothesis that gnu causes the derepression of DNA synthesis in the unfertilized egg explains most of the observed phenomena in GNU embryos. However, it does not provide an obvious explanation of the most striking phenotype of all, namely the development of giant nuclei. It seems likely that the growth of giant nuclei is actually a secondary effect of the gnu mutation. There are several possible explanations that stem from the inappropriate replication model that could account for the formation of these giant nuclei. For example, it is known that there are strict controls in the eukaryotic cell cycle that prevent DNA from replicating more that once in each cell cycle (Harland, 1981). It may be that such controls are overridden in GNU embryos although, since there is no information about the existence, or timing, of any master 'clock' or 'oscillator' in the mutants, it is not possible to state this fact with any confidence. However, if these re-replication controls are ignored, then it is possible that the finely-tuned regulation of mitosis is upset, and this could stop the chromosomes from being able to divide. This phenomenon would be self-reinforcing: the more rounds of re-replication that chromosomes passed through, the less able to participate

in mitosis the nucleus would be; giant nuclei would then be formed. Another possibility would be that mitosis is triggered by a signal that 'tells' the mitotic apparatus that DNA replication is complete; the signal could work by allowing an MPF activity to be induced, causing the cell to enter metaphase. Such a mechanism would ensure that division was coordinated with replication, which is crucial if the genetic material is to be passed on to daughter cells faithfully. In this interpretation, the apparently constitutive replication of DNA found in GNU embryos would not allow such a signal to be conveyed, and division would never occur. A clue as to which explanation is correct might be obtained if the stage at which replication does eventually arrest in GNU embryos (around 8 hours post-oviposition), was studied in more detail, since this is the only obvious check on DNA synthesis to which these embryos appears to be subjected.

6.9 The Molecular Analysis of gnu

It would be interesting to understand the processes that activate DNA synthesis in the developing egg at a molecular level. A molecular analysis of the gnu gene and its product is therefore a longterm aim of this project. In Chapter 5 it was concluded that the most likely approach that would be used to clone gnu would be a chromosomal walk. gnu lies in the cytological interval between 70E4,6 and 70F4,6, and it will be possible to

determine when this entire chromosomal region has been isolated, as I discussed in Chapter 5. The problem remains of identifying the gene in the cloned DNA covered by the walk, which is likely to be between 80 and 130kb. There is a variety of possible methods of identifying the gene. If an allele of the gnu mutation can be induced by radiation, the chances are reasonable that it will be associated with a visible cytological rearrangement (Sankaranarayanan and Sobel, 1976), and this will cause sequences from within, or very close to, the gene to be moved to some other cytological location. In situ hybridisation of probes from the walk to such a strain will identify the fragment of DNA that spans the breakpoint, and thus contains sequences in, or near, the gene. A more direct assay for the DNA segment that contains the gene would be to transform flies bearing the gnu chromosome with clones from the walk, using P-element mediated transformation techniques (Rubin and Spradling, 1982)), and to look for rescue of the gnu mutation by the exogenous DNA (Haenlin et al., 1985). This powerful technique allows the fragment containing the gene to be identified, and the limits of the gene to be defined. It is made easier if the genomic library in which the walk is conducted is built in a vector that already contains P-elements flanking the cloning site, so that there is no need to subclone the steps of the walk into a plasmid transformation vector. Cosmids now exist that are specifically designed for this purpose (Steller and

Pirotta, 1985; M. Speek, pers. comm.), and they could be used to facilitate the cloning of the gnu gene. As gnu is a maternal effect lethal mutation, its expression might be expected to be limited to females and, more specifically, to ovaries. This presumed specificity could also be exploited to identify the gene, by looking for clones from the walk that hybridise to RNA which has the expected pattern of expression. However, the uncertainty of whether the gene will be transcribed in this pattern, and the possibility of other ovary-specific transcripts from the same region of the chromosome, means that this technique could only be used to support other evidence of the location of gnu, and could not be trusted as the only method of identifying the gene.

Very little is known at the molecular level about the mechanisms and regulation of DNA replication in eukaryotes. It is only very recently that a suitable in vitro system in which to analyse replication has been developed (Blow and Laskey, 1986). Until now, the best system that has existed has been the injection of DNA templates into the intact Xenopus egg; these exogenous DNAs will replicate in a manner that resembles normal cellular replication (Harland and Laskey, 1980). MPF seems to play an important role in the regulation of the mitotic cell cycle, as well as being involved in the initiation of DNA synthesis in the egg, as was discussed above (Section 6.6). It is always found to be at high levels in metaphase cells and disappears rapidly just

before S-phase begins. An equivalent metaphase-specific activity has been demonstrated in a wide range of species (see, for example, Nelkin et al., 1980; Kishimoto et al., 1982). Newport and Kirschner (1984) have shown that the whole cell cycle, including DNA replication, can be driven by manipulating the levels of MPF in the egg. When the MPF level is high, metaphase is induced, and when it is removed, the egg moves into interphase, and DNA synthesis is initiated. They have also shown that when a large amount of plasmid DNA is injected into eggs that have been blocked in metaphase with CSF (cytostatic factor), there is some replication of the injected DNA even though the endogenous nuclei remain in metaphase, implying that the DNA synthesis enzymes are present during metaphase. For this reason they believe that the normal inhibition of replication during mitosis is caused by some modification of the DNA, rather than by inactivation of the replication apparatus. It appears that factors like MPF and CSF, which show cyclical activity during the cell cycle, are likely to be involved in the regulation of M-phase and S-phase events, but their molecular mechanisms are still far from clear. The study of the gnu gene will allow a molecular genetic approach to the analysis of the onset of DNA synthesis in the Drosophila egg, and this should provide new insights into these processes.

In conclusion, the product of the gnu gene appears to be a component of the maternally contributed system that ensures that DNA synthesis is repressed until the egg is

fertilized. GNU cytoplasm allows DNA to be replicated in inappropriate circumstances, and thus in some way stops the first mitotic spindle from being established; this leads to the formation of giant nuclei in the syncytial blastoderm. Since the developmental switch that occurs upon activation of Drosophila eggs is also found in a very wide range of other organisms, it is likely that the molecular characterisation of the process, which will follow the cloning of the gnu gene, will be of general interest.

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The Dissociation of Nuclear and Centrosomal Division in *gnu*, a Mutation Causing Giant Nuclei in Drosophila

Matthew Freeman,* Christiane Nüsslein-Volhard,† and David M. Glover*
* Cancer Research Campaign
Eukaryotic Molecular Genetics Research Group
Department of Biochemistry
Imperial College
London, SW7 2AZ, United Kingdom
† Max-Planck Institut für Entwicklungsbiologie
Spemannstrasse 35
7400 Tübingen
Federal Republic of Germany

Summary

We describe a recessive, maternal-effect lethal mutation of Drosophila, *gnu*. *gnu* uncouples nuclear division from many cytoplasmic events of mitosis in the Drosophila embryo. Embryos from homozygous females are defective in nuclear division, but not in DNA replication, and therefore develop a small number of giant nuclei. Centrosomes divide independently of nuclear division and migrate to the surface of the syncytial blastoderm. There they nucleate microtubules into asters, which appear normal at first but become very large. Only later, when the giant nuclei begin to break down, are spindles sometimes formed. The cortical actin of these embryos develops into a characteristic network.

Introduction

Mitotic cell division is a process that is of fundamental importance in nearly all eukaryotic cells, yet remarkably little is known about its mechanism or regulation. Since the nineteenth century there have been detailed descriptive accounts of the events of mitosis (for example Van Beneden, 1883), but molecular approaches to its study have only recently begun (for reviews see Pickett-Heaps et al., 1982; McIntosh, 1984).

One such approach that has been successful is the genetic dissection of the different events of the cell cycle in lower eukaryotes such as yeast (Hartwell et al., 1974; Beach et al., 1982). By isolating mutant strains defective for individual components, either regulatory or structural, a quite detailed pathway of the events of mitosis has been deduced. The genes responsible for these mutations are now being isolated, allowing the molecular basis of cell division in yeast to be elucidated (for example, Lorincz and Reed, 1984). However relatively little is known about the mechanisms of the cell cycle in higher eukaryotes. Such mechanisms are likely to be considerably more complex than those in unicellular organisms due to the necessity of maintaining fine spatial and temporal control over the proliferation of the different cell types within a single organism.

Drosophila is a suitable multicellular eukaryote in which to study cell division. Its embryo is a syncytium for the first 13 nuclear divisions, which occur at about 10 min intervals (Zalokar and Erk, 1976). The first nine of these divisions are within the interior of the embryo; most of the nuclei and their associated centrosomes then migrate out to the cortex, only about 10 μm below the surface membrane. These nuclei divide in parasynchronous waves of mitosis that start at the two poles of the embryo and move toward the center (Foe and Alberts, 1983). This allows several stages of mitosis to be seen in a single embryo. Cellularization occurs at about 2.5 hr of development, at which stage there are around 5000 nuclei. This characteristic mitotic activity in early Drosophila embryos is easy to observe in fixed, whole-mount preparations. Any obvious defect in mitosis at this stage is likely to be readily detected. Furthermore, the relative ease of isolating mutations and the well-developed techniques for cloning genes thus identified make Drosophila an ideal system for a genetic and molecular analysis of cell division in multicellular organisms. Indeed, there have been several reports of mutants with mitotic defects (Baker et al., 1976; Baker et al., 1978; Gatti et al., 1983; Gelbart, 1974; Ripoll et al., 1985; Smith et al., 1985).

The replication of centrosomes is a major event in the early stages of mitosis. Centrosomes are the organelles that organize the spindle poles and thus must divide once each cell cycle to maintain their ratio with nuclei. A number of groups have reported that under some conditions a limited amount of centrosomal replication can occur in the absence of nuclear division (for example, see Mazia et al., 1960; Sluder and Begg, 1985; Wolf, 1980). There has, however, been some speculation about the extent to which this occurs. It is important to know whether these major events of the cell cycle are regulated independently or whether they are part of a cascade, each event dependent on the previous one in the pathway. In the former case one would expect to be able to uncouple the events, whereas in the latter the disruption of one would necessarily lead to disruption of the other.

In this paper we describe some aspects of the phenotype of a maternal-effect lethal mutation that is defective in nuclear division but apparently not in DNA replication, thus causing the formation of giant nuclei in the syncytial blastoderm. Using immunofluorescent techniques we find that while the nuclear cycle of these embryos is disrupted, many aspects of the cytoplasmic cycle are unaffected and, specifically, that centrosomes replicate independently. We have named this mutation *gnu*, as it results in giant nuclei, and use the term "GNU embryos" to describe the embryos from homozygous females.

Results

DNA Replication without Nuclear Division in GNU Embryos

We describe aspects of the phenotype of a recessive, maternal-effect lethal mutation of a gene that we have

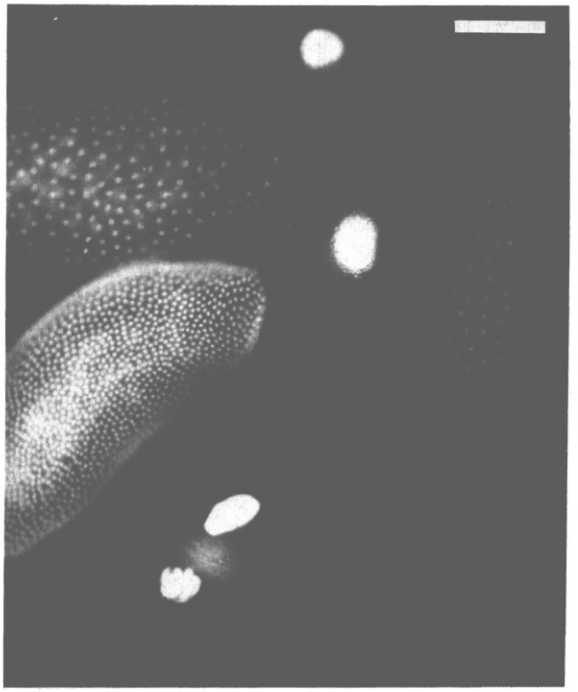


Figure 1. Nuclei of Wild-Type and GNU Embryos

Embryos were prepared as described in Experimental Procedures, and their DNA was stained with Hoechst. In this field three wild-type embryos of stages 9, 10, and 13 with approximately 500, 1000, and 3000 nuclei, respectively, are lying alongside two GNU embryos with giant nuclei. The bar represents 80 μm.

named *gnu*. The most striking characteristic of embryos from a homozygous *gnu* mother can be seen by staining their DNA with the fluorescent dye Hoechst 33258. Two such embryos are shown in Figure 1. It is clear from the size of the nuclei and the intensity of the fluorescence that DNA replication occurs within these embryos but that there is little or no nuclear division, resulting in the formation of a small number of giant nuclei. This contrasts with the appearance of the three wild-type embryos in Figure 1. These are at division cycles 9, 10, and 13 and contain progressively greater numbers of nuclei with a diploid DNA content. *gnu* maps to the left arm of the third chromosome, just to the left of *thread*, at map position 42. We have identified a deletion of a segment of chromosome 3 (*Df(3L)fz^{m21}*) that uncovers the *gnu* mutation (see Experimental Procedures). Embryos from mothers heterozygous for *gnu* and the *Df(3L)fz^{m21}* exhibit the GNU phenotype.

We have followed the development of the nuclei in GNU embryos. Ten minutes after egg deposition we can see the normal meiotic products—three polar bodies and the female pronucleus—together with the male pronucleus (data not shown). Figure 2 shows representative embryos that have been allowed to develop for increasing amounts of time before fixing and staining. At 25–40 min the embryos are already quite distinct from wild-type embryos (Figures 2A and 2B), their nuclei appearing larger and more diffuse. Most embryos have either one or two nuclei, and the number of nuclei per embryo does not appear to increase with time. Instead the number seems to be set at an early stage, and those nuclei then continue to grow without further division (Figures 2C–2F). The nuclei do not appear to undergo a significant increase in size after 8 hr (Figure 2F). In wild-type embryos, gastrulation and germ-band elongation have occurred with considerable differentiation of gut and neural tissues by this stage. By 24 hr the giant nuclei begin to break down and release pieces of chromatin throughout the embryo (Figure 2H). Examination of a similar series of GNU embryos by phase-contrast microscopy has shown that the pole cell buds never form (which characteristically occurs at cycle 10 in wild-type embryos) and that there is no cellularization of the embryo (which occurs at cycle 14 in wild-type embryos). These observations suggest that while DNA replication continues relatively unaffected, nuclear division is almost completely absent in GNU embryos.

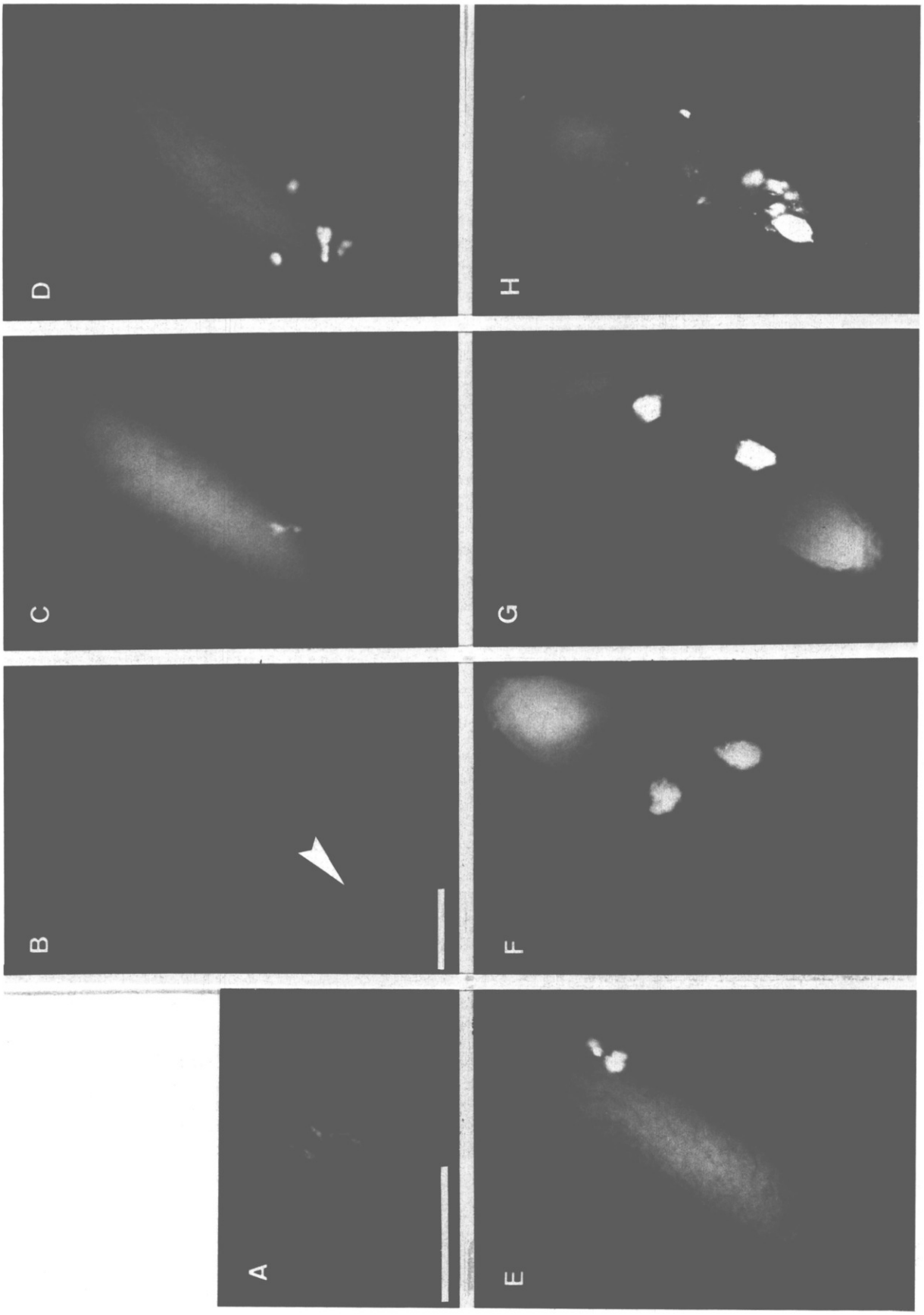
Centrosomes Continue Independent Replication and Division

In addition to the nuclear division cycle, cyclical mitotic activities occur within the cytoplasm of normal cells. One such activity is the division of the centrosome and its movement to the pole positions of what will become the mitotic spindle. In fertilized sea urchin eggs, one round of centrosomal division can be induced independently of nuclear division under certain conditions (Mazia et al., 1960). The extreme disruption of nuclear division in GNU embryos raises questions about the presence and distribution of centrosomes and related components of the normal mitotic cycle. We have used the antibody Bx63, which recognizes Drosophila centrosomes (Frasch et al., 1986), to discover whether GNU embryos have any recognizable centrosomes. A detailed description of the staining of wild-type embryos with this antibody has been given previously (Frasch et al., 1986). Briefly, there are two components to the staining pattern: strong centrosomal staining and weaker nuclear staining persisting into metaphase and anaphase in an area over the spindle. Figure 3 (WT) shows a field of nuclei in late anaphase in the cortex of a blastoderm embryo of wild-type Drosophila; centrosome staining is seen as distinct dots at the spindle poles.

As there has been no report of anything other than very limited independent centrosomal replication, we were sur-

Figure 2. The Development of GNU Embryos

Embryos were collected for a period of 15 min and allowed to develop at 25°C for a total of 25–40 min (B), 60–75 min (C), 90–105 min (D), 120–135 min (E), 8 hr (F), 16 hr (G), or 24 hr (H). They were then fixed and stained with Hoechst. A representative embryo from each age group is shown. (B–H) The same magnification; the bar represents 80 μm. (A) A high-power view of the nucleus, which is indicated by an arrow in (B); the bar represents 20 μm.


A
B
C
D
E
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G
H

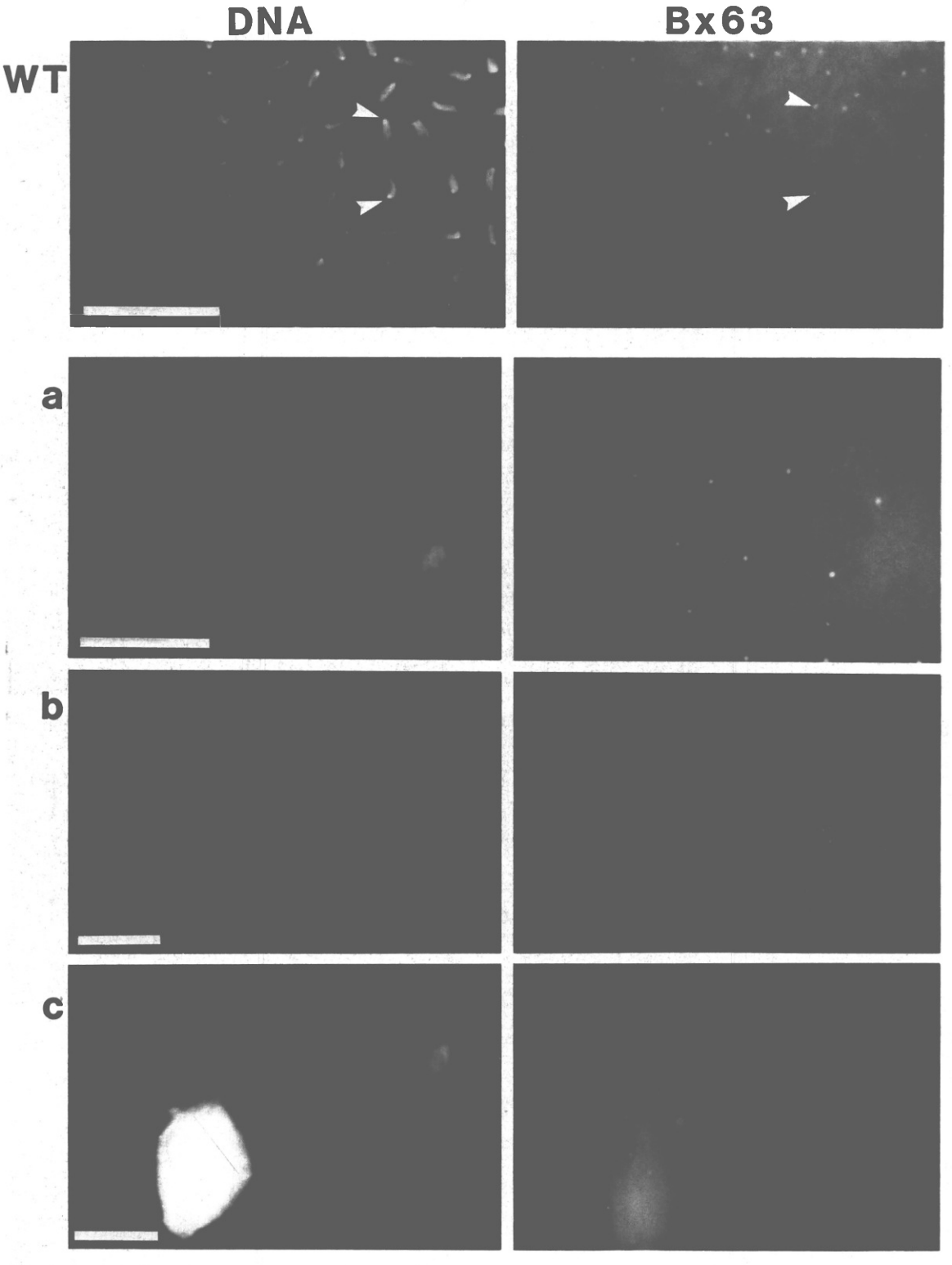


Figure 3. Centrosome Staining of Wild-Type and GNU Embryos

Wild-type (WT) and GNU (a–c) embryos were incubated with an antibody against centrosomes, Bx63 (see Experimental Procedures), then with rhodamine-conjugated anti-mouse IgG and Hoechst. (WT) shows a field of anaphase chromosomes from a wild-type embryo in which centrosomes are found at the spindle poles. The poles of one anaphase figure and their corresponding centrosomes are indicated by arrows. In GNU embryos there is no correspondence between the positions of centrosomes and nuclei. Weak nuclear staining is also sometimes apparent with the Bx63 antibody (c). The bars represent 20 μm.

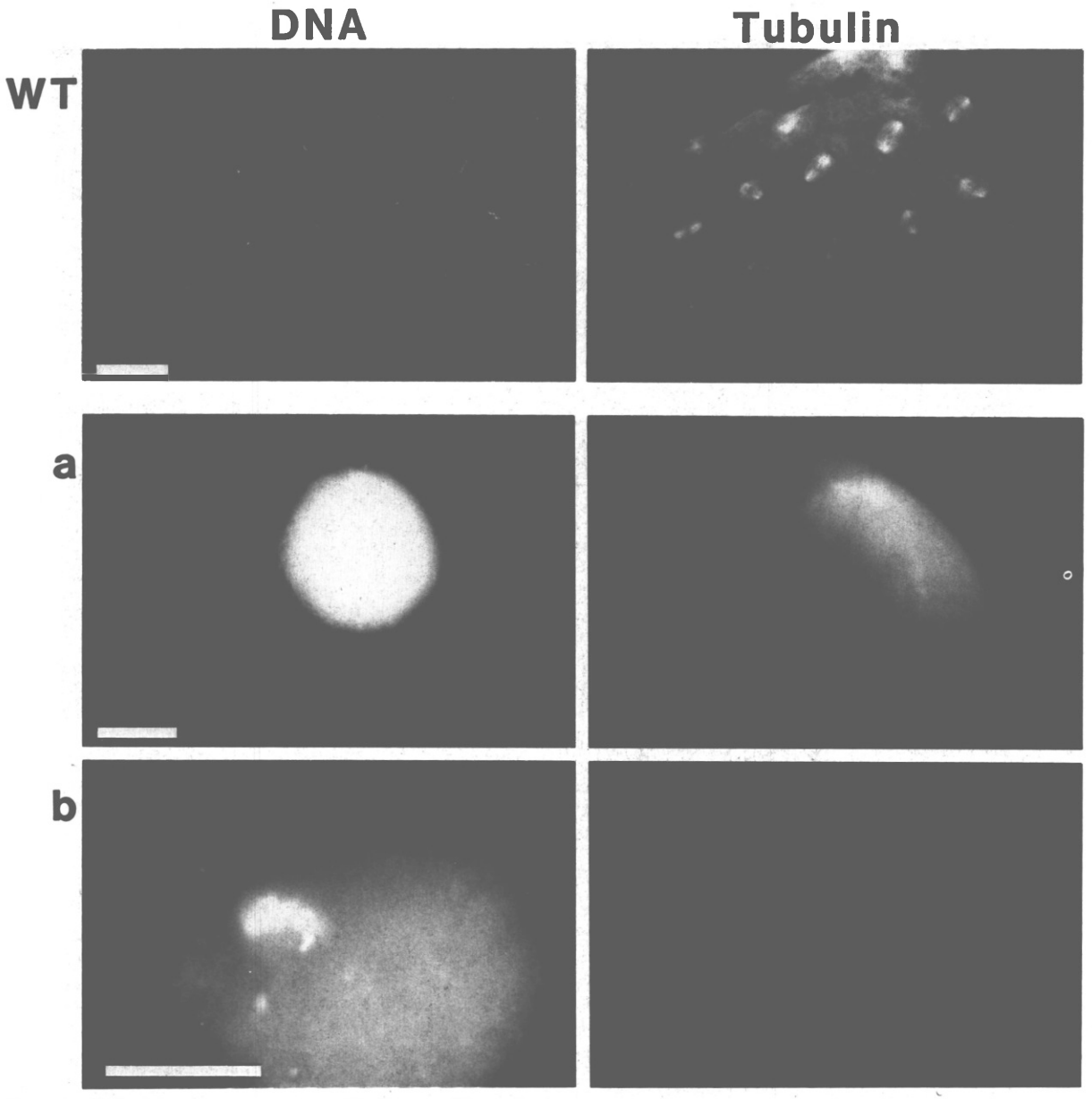


Figure 4. Tubulin Staining of Wild-Type and GNU Embryos Without Taxol

Wild-type (WT) and GNU (a and b) embryos were incubated with Bx69, an antibody against Drosophila tubulin (see Experimental Procedures), then with rhodamine-conjugated anti-mouse IgG and Hoechst. In the wild-type embryo spindle microtubules are associated with each set of metaphase chromosomes. (a) Weak anti-tubulin staining is apparent over a giant nucleus. (b) A single spindle can be seen lying over a small piece of chromatin in a late GNU embryo. The bars represent 20 μm (WT and a) and 12 μm (b).

prised to find that GNU embryos do contain centrosomes that are apparently completely dissociated from the nuclei. Since unfertilized eggs contain no visible centrosomes, their presence in GNU embryos implies that centrosomes continue to replicate and migrate to the cortex in a manner unaffected by the absence of nuclear division. Their distribution varies among embryos and among areas within the same embryo. In some regions the centrosomes form a rather regular array just below the surface of the embryo (see Figure 3a). They often form clusters, ranging in number from 3 or 4 to more than 20, which are frequently, but not exclusively, in the proximity of a giant nucleus (Figures 3b and 3c). In some embryos a weak staining over giant nuclei is also found with Bx63 (Figure 3c). Because embryos are rather large and opaque structures, the centrosomes are seen most easily when close to the surface. However, in a few cases in which the embryos have been damaged, centrosomes may be seen deeper within the cytoplasm.

The Distribution of Microtubules in GNU Embryos

Microtubules are nucleated by centrosomes into asters and, during metaphase, become attached to the kinetochores of chromosomes on the metaphase plate to form

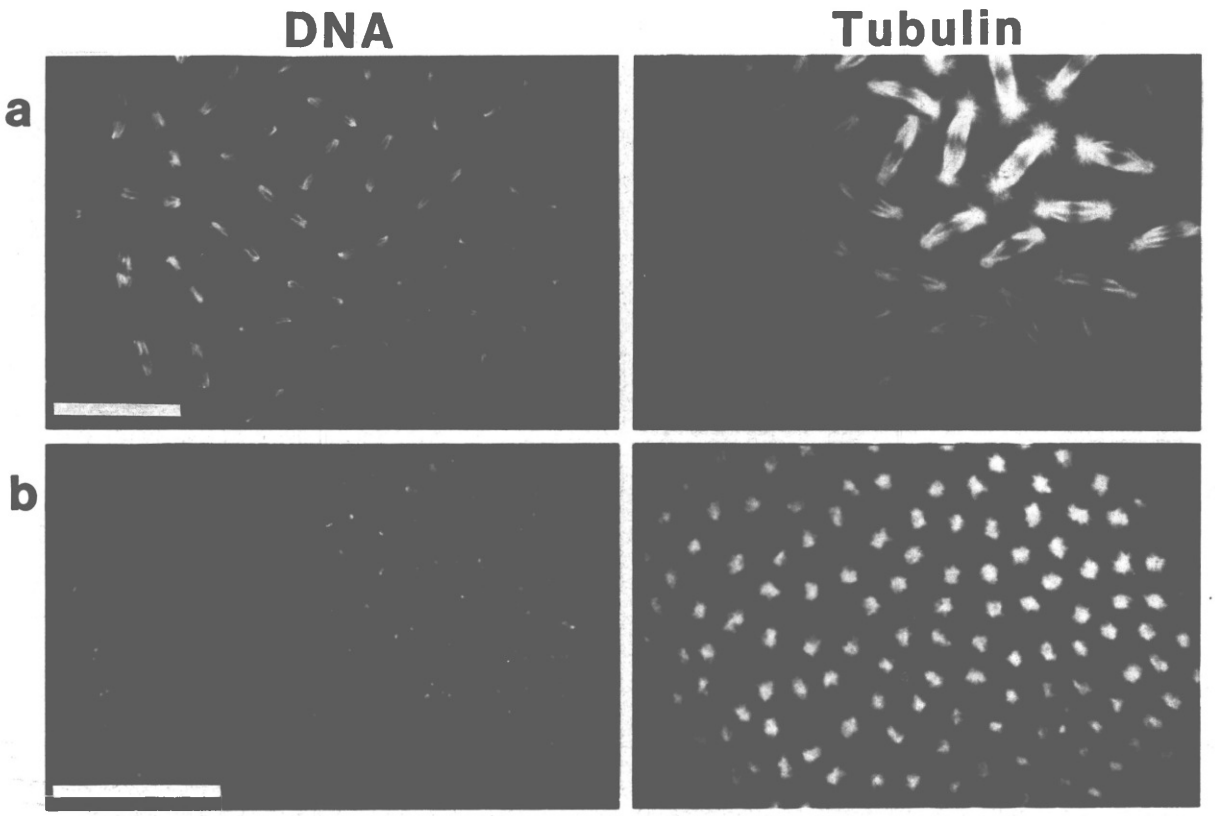


Figure 5. Tubulin Staining of Wild-Type Embryos Fixed in the Presence of Taxol

Embryos were fixed in the presence of 0.5 μM taxol and were then immunostained with Bx69 (see Experimental Procedures). Two phases of the mitotic cycle are shown. (a) Anaphase chromosomes with corresponding spindles and small asters that project from the centrosomes in the opposite direction from the spindles. (b) Interphase nuclei, each associated with a single aster. The bars represent 20 μm.

the mitotic spindle. They are thus a crucial component of the mitotic apparatus. Figure 4 shows the staining of wild-type and GNU embryos with an antibody against Drosophila tubulin, Bx69 (Frasch, 1985). Microtubules are fairly labile in the presence of formaldehyde, in which these embryos are fixed; however, the metaphase spindles are preserved and stained in the wild-type embryo (Figure 4, WT). In GNU embryos two forms of staining were observed. Figure 4a shows a rather weak staining that lies over a giant nucleus; Figure 4b shows the staining of an apparently normal spindle. Spindles like these are rather rare, but, when found, are invariably associated with small pieces of chromatin released from the breakdown of a giant nucleus, of the type shown in Figure 2H. The presence of spindles with apparently normal morphology suggests that there is no major abnormality of the spindle components caused by the *gnu* mutation.

We have also examined the distribution of microtubules by indirect immunofluorescence after a very brief exposure to taxol, a microtubule-stabilizing toxin (Wani et al., 1971). The pattern of microtubules throughout mitosis under these fixation conditions has been described in detail by Karr and Alberts (1986). We were able to repeat their observations and to show, in Figure 5, just two stages of mitosis in the cortex of the wild-type embryo. At anaphase there are spindles with small asters also growing from the centrosomes (Figure 5a), and at interphase we observe a single aster per nucleus (Figure 5b). In taxol-treated GNU embryos we see a variety of microtubule staining patterns. In early embryos (those with small nuclei) a cortical layer of tubulin with the appearance of a fine network is stained. This is similar to the cortical staining of early wild-type embryos (Karr and Alberts, 1986). Later GNU embryos show structures that are like asters except that they grow to be very large (Figure 6): Figure 6b shows one aster that is about 20 times the diameter of the wild-type interphase asters in Figure 5b. These asters invariably occur near the surface of the embryo, and their distribution appears to be independent of the location of giant nuclei. When the giant nuclei begin to break down, two new types of staining appear. Figure 6c shows the presence of spindle-like structures (marked with arrows), which are frequently found in the presence of taxol. They are formed over small pieces of chromatin and correspond to the structures we observed in embryos that had not been treated with taxol (Figure 4b). Finally, Figure 6d shows the irregular mesh of microtubules often found on the surface of old embryos whose nuclei are breaking down.

The Asters in GNU Embryos Are Nucleated by Centrosomes

To understand the unusual distribution of microtubules found in GNU embryos we asked whether centrosomes in GNU embryos are able to nucleate microtubules into

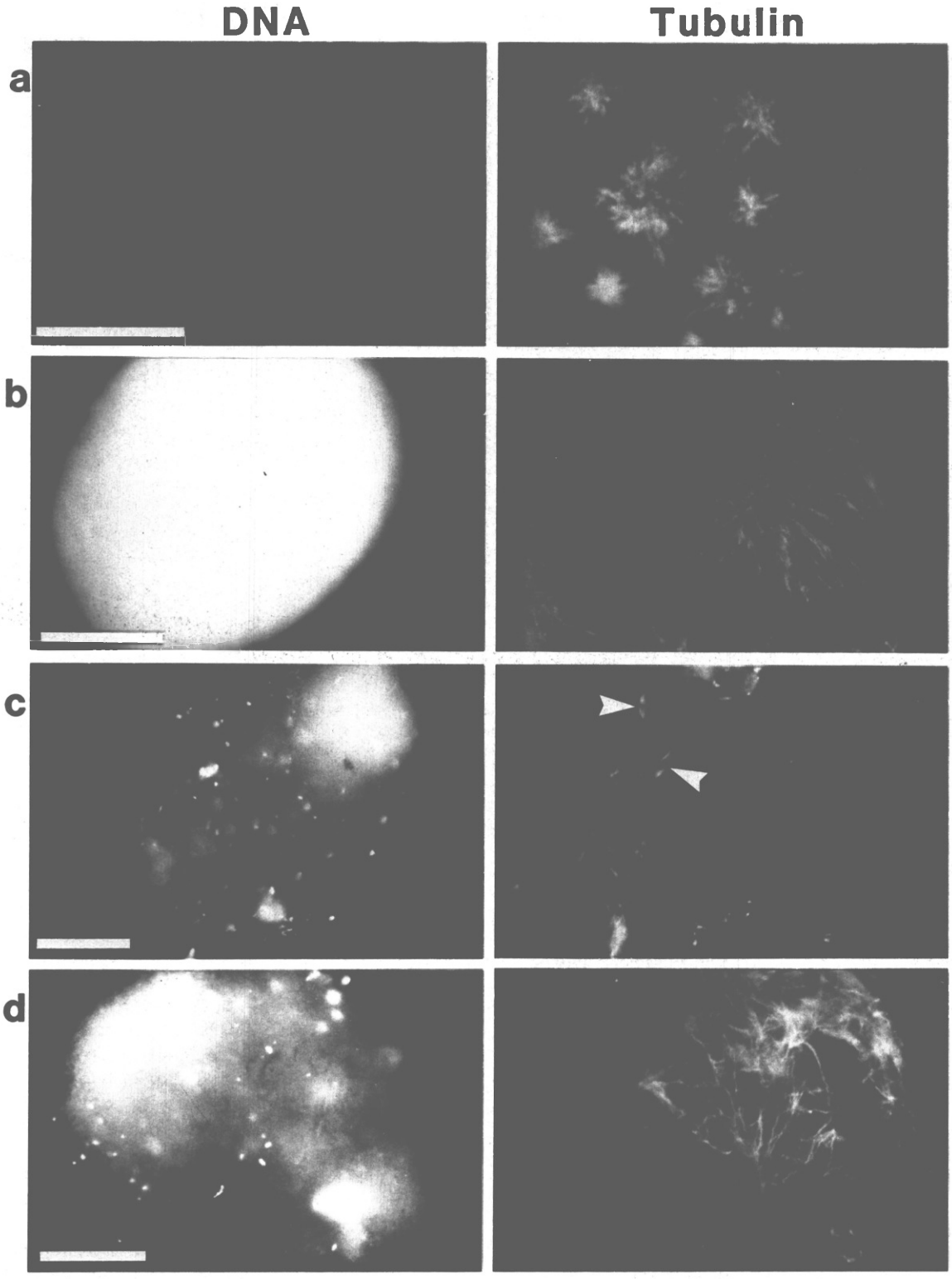


Figure 6. Tubulin Staining of GNU Embryos Fixed in the Presence of Taxol

Embryos were fixed in the presence of 0.5 μM taxol and were immunostained with Bx69 (see Experimental Procedures). The plane of focus is just below the surface of the embryo in each case. (a) Asters can be seen in a region where there is no giant nucleus. (b) A large aster with a giant nucleus lying under it. (c) The giant nucleus of an embryo is breaking down, and two spindles (indicated by arrows) can be seen associated with small discrete pieces of chromatin. (d) Another old embryo with a disintegrating nucleus, in which there is an irregular network of microtubules. Bars represent 20 μm.

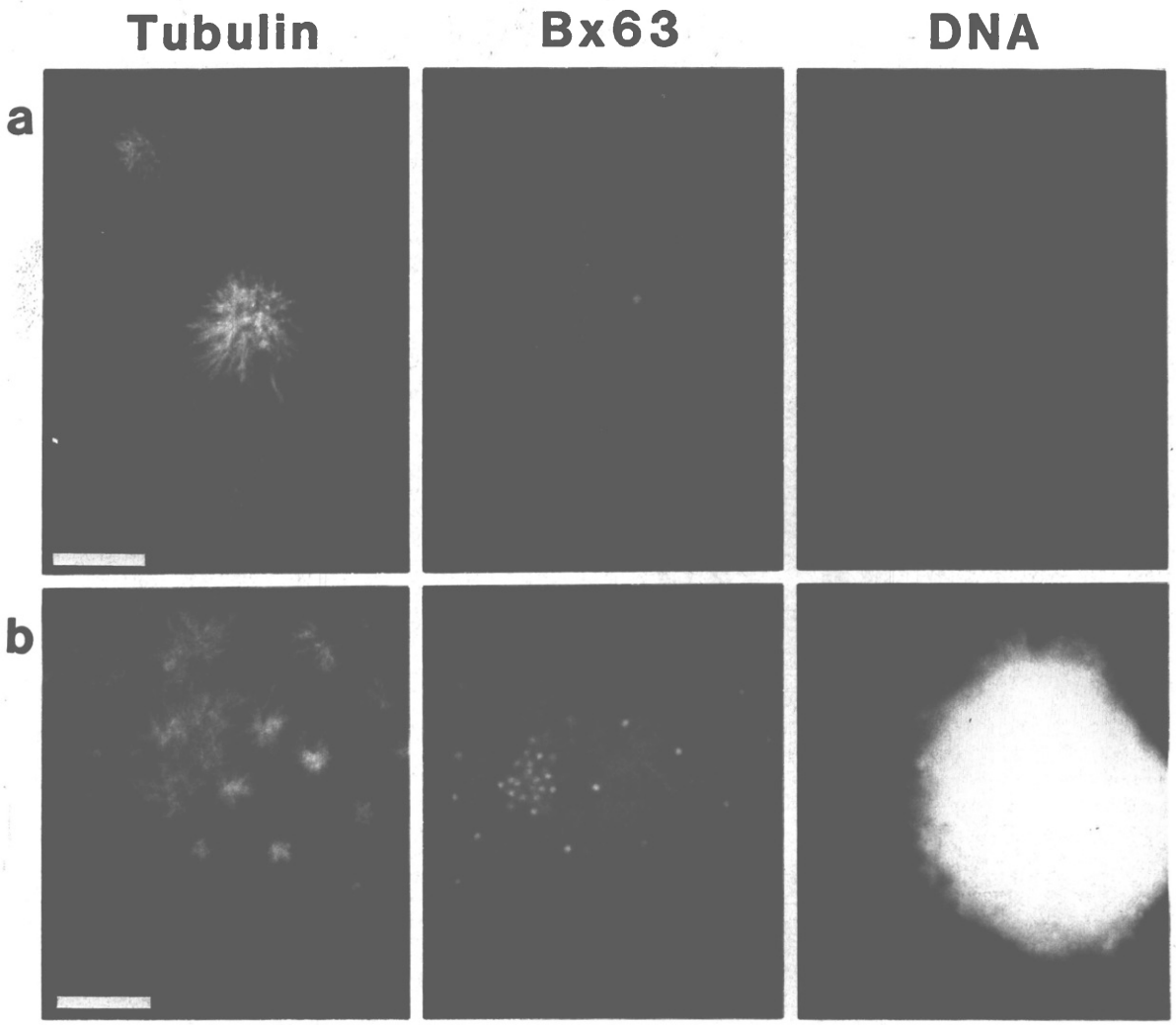


Figure 7. Double Immunostaining of GNU Embryos with Antibody against Tubulin and Antibody against Centrosomes

Embryos were incubated sequentially with Bx63 antibody (mouse anti-centrosome), rhodamine-conjugated anti-mouse IgG, YL1/2 (rat anti-tubulin), fluoroscein-conjugated anti-rat Ig, and Hoechst (see Experimental Procedures). (a) Three large asters nucleated by single centrosomes. (b) A field of centrosomes in the vicinity of a giant nucleus. Asters of microtubules radiate from the well-separated centrosomes, whereas the cluster of centrosomes appears not to nucleate microtubules. Bars represent 10 μm.

asters, as in wild-type embryos. Using well-characterized antibodies against tubulin, YL1/2 (see Experimental Procedures), and against centrosomes, Bx63 (Frasch et al., 1986), we stained both sets of structures simultaneously by indirect immunofluorescence (see Experimental Procedures). Figure 7 shows two fields: one containing three centrosomes in a field lacking nuclei, the other showing many centrosomes in the vicinity of a giant nucleus. In all cases a centrosome is seen at the center of each aster, however large. This indicates that centrosomes can nucleate microtubules in GNU embryos; thus, the structures we see are genuinely analagous to asters and not spurious microtubule arrays. Interestingly, large clusters of centrosomes appear unable to nucleate microtubules effectively (Figure 7b).

The Distribution of Cortical Actin

The surface of the early wild-type Drosophila embryo exhibits a characteristic pattern of actin-rich "caps," which divide roughly in unison with the underlying nuclei (Warn and Magrath, 1982; Warn et al., 1985; Karr and Alberts, 1986). Consequently, there is a correlation between the centrosomes associated with each nucleus and the actin cap also associated with that nucleus. During the last two or three divisions of the syncytial blastoderm, these caps of actin are closely abutted, giving the appearance of actin-rich furrows between each nucleus. Figure 8a shows such a wild-type embryo with the pole cells in the bottom right-hand corner. The unusual pattern of staining that we observe in GNU embryos is shown in Figure 8b. The surface of late embryos is covered with an irregular array of actin-sparse holes in a background of cortical actin staining. Early GNU embryos, on the other hand, show continuous cortical actin staining much like early wild-type embryos (data not shown). This network of holes in the surface actin does not correspond to any phase of actin staining in the wild-type embryo, so its cause is not obvious. However, it is clear that the surface actin distribution

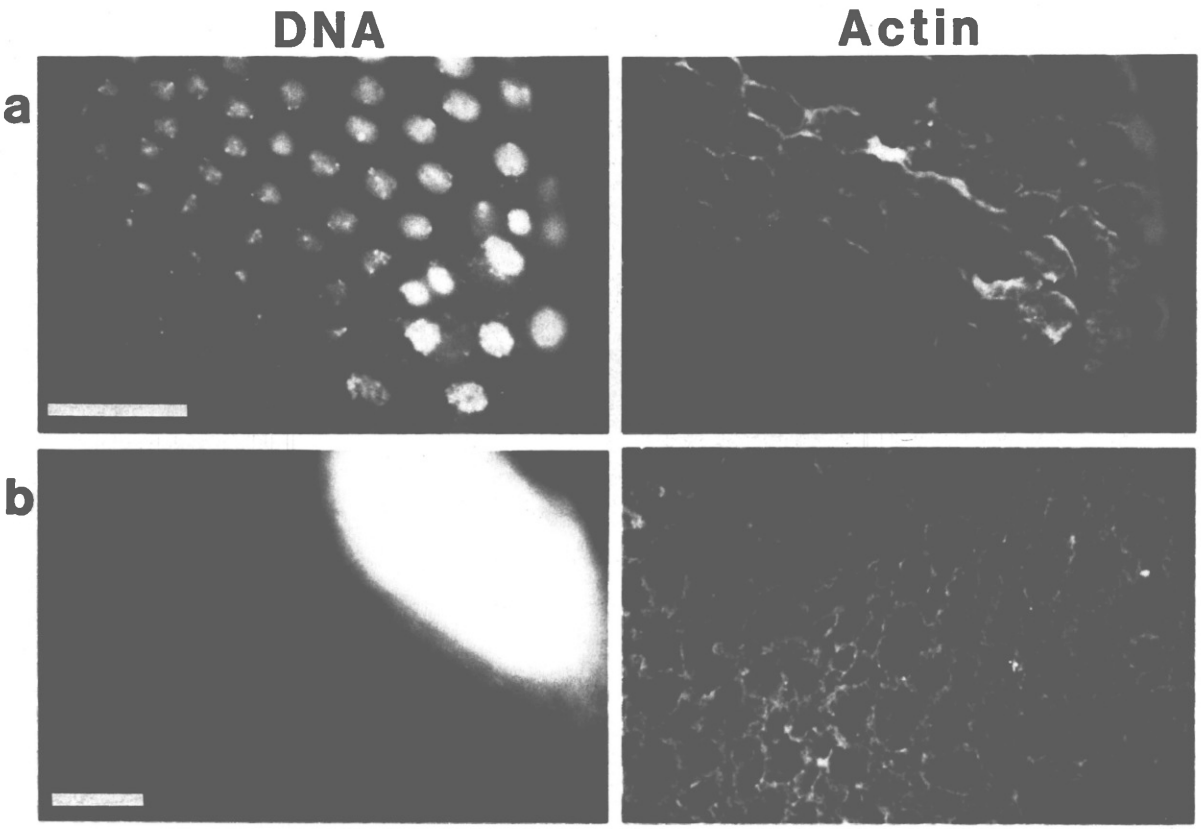


Figure 8. Actin Staining of Wild-Type and GNU Embryos

Embryos were incubated with an antibody against actin (see Experimental Procedures), then with rhodamine-conjugated anti-mouse IgG and finally with Hoechst. (a) The posterior end of a wild-type blastoderm embryo. The actin caps are closely abutted, giving actin-rich furrows between each nucleus. (b) The anti-actin staining pattern of GNU embryos. A cortical layer of actin is seen in which there is a rather irregular network of actin-sparse holes. The actin staining pattern is not altered in the vicinity of a giant nucleus. The bars represent 20 μm.

in GNU embryos does change with time in the absence of nuclear division.

We have also carried out double-label indirect immunofluorescence to monitor the distribution of both centrosomes and actin. These data (not shown) demonstrate that there is no correspondence between the distribution of actin holes and the positions of centrosomes in GNU embryos.

Discussion

The first 13 nuclear divisions in the wild-type Drosophila embryo occur within a syncytium, at approximately 10 min intervals. During cycle 14, the embryo becomes cellularized, at which stage there are about 5000 nuclei. We have described a maternal-effect lethal mutation, *gnu*, in which DNA replication continues while nuclear division is almost completely absent in embryos from homozygous females. This results in the formation of embryos with a small number of giant nuclei, which we refer to as GNU embryos. The envelopes of these nuclei stain to a variable degree with an antibody against lamin (data not shown). This suggests that the giant nuclei may go through phases in which the nuclear membrane undergoes considerable structural change, as occurs during prometaphase in wild-type embryos. Our observations demonstrate that although nuclear division is disrupted, the centrosomes continue to divide in the syncytial blastoderm. Furthermore, the centrosomes still migrate from the interior to the cortex of the syncytium, where they nucleate the formation of giant asters. Later, the giant nuclei appear to break down, and we then see some spindle-like structures formed about small fragments of chromatin. Finally, we have also shown that the cortical actin in GNU embryos forms an unusual network of holes that is unlike any wild-type phase of actin staining.

In some way the *gnu* mutation uncouples nuclear division from cytoplasmic events of the mitotic cycle in Drosophila embryos. The autonomy of cytoplasmic events has been demonstrated by the elegant experiments of Hara et al. (1980), who showed that the enucleated egg of X. laevis continues to undergo periodic surface contractions. These cortical waves have exactly the same frequency as those occurring normally at the metaphase–anaphase transition in control fertilized eggs. This led to the suggestion that there is an autonomous cytoplasmic oscillator responsible for timing the mitotic events of each cell cycle. Our observations suggest that centrosome replication continues independently of nuclear divisions in GNU embryos, which is consistent with there being

continued cytoplasmic cycles. We have not, however, determined whether the timing of such cycles is affected in GNU embryos.

We are intrigued by the observation that the division of centrosomes is not dependent on the presence of nuclear division or the formation of spindles since these processes are normally so tightly coupled. Rattner and Phillips (1973) showed that in mouse L cells treated with arabinosyl cytosine to inhibit DNA synthesis, centrioles (the microtubular structures within centrosomes) divide independently of DNA replication. In this case the centriole cycle subsequently arrested in a configuration normally found at prophase and the centrioles did not undergo further division. Sluder and Begg (1985) have analyzed in detail the phenomenon first described by Mazia et al. (1960) in which centrosome division is induced in sea urchin eggs by mercaptoethanol. In this system, centrosomes split rapidly upon mercaptoethanol treatment but the newly formed centrosomes quickly associate with chromosomes via spindle fibers. Wolf (1980) has studied the distribution of asters (which appear to be nucleated by centrosomes) during development of cleavage embryos of the gall midge, Wachtliella persicariae. He describes a process of "pseudocleavage" of unfertilized eggs in which the asters sometimes undergo up to four divisions in the absence of nuclear division. Our observations in GNU embryos are, to our knowledge, the first report of multiple rounds of independent centrosome division.

The observations that we have presented here suggest a possible course of events for the abnormal development of GNU embryos. The centrosomes divide in the absence of nuclear division and at some stage migrate from the interior of the embryo to the cortical region. On reaching the cortex, the centrosomes recruit the layer of surface microtubules into forming asters (as has been proposed in wild-type embryos by Karr and Alberts, 1986). The asters are originally small but they continue to grow, perhaps indefinitely, to sizes 20 or more times larger than wild-type asters. When the giant nuclei begin to disintegrate (at about 24 hr), small pieces of chromatin are released and these provide functional kinetochores that capture free microtubule ends. The microtubules are stabilized and this leads to the formation of some spindles as in wild-type embryos. Later still, the embryo begins to decay and the microtubules start to form an irregular network. This interpretation is consistent with the model proposed by Mitchison and Kirschner (1985) from their studies of microtubule nucleation in vitro. They suggest that microtubules grow in random directions from the centrosome. Those growing toward the chromosome are captured by kinetochores and thus stabilized against the eventual depolymerization that happens to uncaptured microtubules on the loss of their GTP caps. The feature of this model is that it requires no directionality of fiber growth: microtubules do not "know" in which direction the chromosome lies. This would certainly appear to be the case in GNU embryos, in which the huge, round asters show no sign of growth directed toward the giant nuclei. On the other hand, there appears to be a requirement for the nuclei of GNU embryos to be fragmented before the chromatin will participate in forming spindle-like structures. This suggests that the microtubule organizing centers of the chromosomes within the giant nuclei are not available, either because the nuclear membrane acts as a barrier or because the chromatin is decondensed or in an equally inappropriate state. Karsenti et al. (1984) have shown that preparations of nuclei devoid of centrosomes can mediate the formation of anastral microtubule arrays when microinjected into Xenopus eggs. It would be interesting to follow the behavior of such preparations in the GNU embryo. Karsenti et al. (1984) have also concluded that in the egg of X. laevis, it is the association with the nucleus that renders an otherwise inactive centrosome capable of nucleating microtubules during mitosis. We observe that centrosomes are capable of nucleating microtubules independently of nuclei in the GNU embryo.

The network of holes that we observe in the cortical actin of GNU embryos is difficult to explain. The pattern is unlike any that occurs in the wild-type syncitial blastoderm. However, it is apparent that with time the distribution does change from the smooth actin staining of early embryos to the network that we see later (Figure 8b). In the wild-type embryo, caps of actin divide in unison with the nuclei close under the surface. The absence of nuclei in GNU embryos may be instrumental in giving rise to the abnormal actin staining pattern. It is interesting to note that Euteneuer and Schliwa (1985) have shown that in a neuroblastoma cell line, the movement of centrosomes is dependent on actin microfilaments. We do not know whether the centrosome movement in GNU embryos is due to diffusion or to some kind of directed movement, but the rather regular array of centrosomes that we often see (for example, Figure 3a) suggests that at least there may be some mechanism for positioning the centrosomes at the cortex. It is possible that actin filaments play a role in this process. However, the fact that there is not a simple correspondence between holes in the actin network and the position of centrosomes implies that if such a mechanism does occur, it is not simple.

It is difficult to assign even a putative function to the wild-type GNU product. Our observations suggest that many of the cytoplasmic components of the mitotic apparatus are functionally adequate. Thus, centrosomes can divide, nucleate microtubules, and, when the giant nuclei break down, form spindle-like structures. This last observation implies that there are at least some functional kinetochores associated with the chromatin that are presumably inaccessible to the spindle microtubules when in giant nuclei. The defect therefore appears to be associated specifically with nuclear division. Many processes could be affected including, for example, topology of DNA replication, chromosome condensation, structure of the nuclear envelope, or even a direct signal coupling nuclear division to cytoplasmic oscillations. We are in the process of isolating other *gnu* alleles to determine whether these have similar phenotypes affecting nuclear division. A detailed localization of *gnu* will allow us to clone the *gnu* gene and thereby enable its full molecular and developmental analysis.

Experimental Procedures

Origin of the *gnu* Mutation

The *gnu* mutation was recovered in a systematic screen for maternal-effect lethals in EMS-treated *ru th st ri roe p^p e^s ca* chromosomes (C. Nüsslein-Volhard, G. Jurgens, K. Anderson, and R. Lehmann, unpublished data). To map *gnu*, we scored recombination between *th* (43.2 map units), *st* (44.0 map units), and *ri* (47.0 map units) (all markers as listed in Lindsley and Grell, 1968), and *gnu*. We find that *gnu* lies to the left of all three markers, and it is separated from *th* in 4/563 chromosomes. On the basis of these figures, we place *gnu* about 0.7 map units to the left of *th*, at approximately 42.5 map units. Scoring 2267 flies for recombination between *th*, *st*, and *ri* demonstrates that there is no major rearrangement associated with the *gnu* mutation. The deletion *Df(3L)fz^{m21}* (70D2-3;71E4-5) deletes the region containing the wild-type gene but the deletion *Df(3L)fz^{d21}* (70D3;70E3-8) does not.

Fixation and Devitellinization of Embryos

Embryos were fixed and prepared for staining by a slightly modified version of the method of Dequin et al. (1984), which itself is based on the technique of Mitchison and Sedat (1983). Embryos were dechorionated in 60% hypochlorite bleach in 0.7% NaCl, 0.1% Triton X-100 and then rinsed extensively in NaCl, Triton. Following removal of excess liquid by blotting on tissue paper, the embryos were transferred to a 7 ml glass vial containing 25 μl 37% formaldehyde (Fisons Scientific, UK), 100 μl water, 25 μl 10× Buffer B (100 mM potassium phosphate buffer [pH 6.8], 450 mM KCl, 150 mM NaCl, 20 mM $MgCl_2$), and 2.25 ml heptane, and the vials were shaken vigorously for 10 min. In some experiments 0.5 μM taxol (1.25 μl of a 1 mM solution in DMSO) was included in the mixture to which the embryos were added; in these cases the vials containing the embryos were shaken for 15–30 sec before addition of formaldehyde.

To ensure the validity of the results using taxol, it is important that the taxol only stabilizes existing structures and does not cause a change in the microtubule distribution, as it has been shown to do under certain conditions (Horwitz et al., 1981; De Brabander et al., 1981). De Brabander et al. (1981) have shown that in mammalian cells two kinds of microtubule artifact are found upon treating them with doses of taxol ranging from 2.5 to 100 μM for periods greater than 30 min: they see bundles of parallel microtubule fibers forming where none usually exist and asters that are not nucleated by centrosomes. Under the extremely mild conditions of taxol treatment in the present study (0.5 μM for 15–30 sec before fixing), we see no obvious artifacts in wild-type embryos (Figure 5). This agrees with the results of Karr and Alberts (1986), who found no microtubule artifacts under identical conditions or when using 5 times as much taxol. Spurious microtubule arrays were seen when they used 10 μM taxol for 5 min prior to fixation. Furthermore, we have shown that all the asters in GNU embryos are nucleated by centrosomes (Figure 7). Taken together these arguments convince us that taxol is not forming spurious structures under our conditions and that the microtubule distribution that we see is a real phenomenon which occurs in vivo.

After shaking for 10 min, 2.5 ml of methanol was added and the mixture was shaken for an additional 30 sec to 2 min. 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 floating embryos were then aspirated away, and the divitellinized embryos were rinsed twice in methanol for a total of 10 min. They were then rinsed at least 3 times over an hour in Buffer A (15 mM Tris-HCl [pH 7.4], 60 mM KCl, 15 mM NaCl, 1.5 mM spermine, 0.5 mM spermidine) plus 0.1% Triton X-100 and left in fresh Buffer A and Triton X-100 for at least 2 hr before staining.

Antibody Staining of Embryos

Between 50 and 200 embryos were transferred into staining nets that were made by cutting off the bottom 3 cm of a 1.5 ml plastic microfuge tube and replacing it with a stainless steel mesh of a gauge fine enough to retain embryos. All subsequent stages were carried out in these nets, placed in wells of a 24-well tissue culture plate. The embryos were incubated in Buffer A containing 10% fetal calf serum for 1 hr at room temperature, blotted dry, and incubated in first antibody for 12–16 hr at 4°C. After this period, excess liquid was removed by blotting and the embryos were washed in Buffer A for 1–2 hr with three changes and incubated in second antibody for 12–16 hr at 4°C. At the end of this incubation they were washed in Buffer A for at least 2 hr with four changes, washed in Buffer A containing 1 μg/ml of Hoechst 33258 (Riedel De Haen AG, Hannover, FDR) for 10 min, and rinsed twice in Buffer A. The stained embryos were mounted in 85% glycerol containing 2.5% n-propyl gallate (Giloh and Sedat, 1982). Slides were viewed with a Zeiss Standard microscope fitted with epifluorescent optics with appropriate filters for Hoechst, fluoroscein, and rhodamine. Embryos were photographed on Kodak 2415 Technical film and developed with Kodak D-19 developer.

The level of nonspecific staining was determined by incubating embryos with second antibody alone. There was found to be a low level of uniform background staining using both rhodamine- and fluoroscein-conjugated antibodies, but no specific structures were detected.

Antibodies

We have used the following antibodies in this study: Bx63, against centrosomes (Frasch et al., 1986) and Bx69, against tubulin (Frasch, 1985). In the double-labeling experiment a well characterized rat monoclonal antibody against tubulin (from Sera-lab, UK), YL1/2, was used (Kilmartin et al., 1982); this has been shown to recognize specifically α-tubulin in Drosophila (Warn and Warn, 1985). Anti-actin was a mouse monoclonal from Amersham International (UK). We used dilutions of between 1 in 5 and 1 in 15 for hybridoma culture supernatant and dilutions of 1 in 500 for ascites fluid, all in Buffer A (see Antibody Staining of Embryos). Rhodamine- and fluoroscein-conjugated second antibodies were bought from Jackson Immunoresearch Laboratories Inc. (USA) and used at dilutions of 1 in 500 and 1 in 100, respectively.

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