Genetic and molecular analysis of mitotic chromosome condensation in *Drosophila*

John Myles Axton
Department of Biochemistry
Imperial College of Science, Technology and Medicine
London SW7 2AZ

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Abstract

The advanced cytology, genetics and molecular biology the fruit fly *Drosophila melanogaster* make it suitable for the analysis of genes encoding functions required for mitotic and meiotic cell division. My approach has been to identify such essential genes by mutation. Using the techniques of molecular biology I have attempted to characterise several of the genes whose products are needed for the correct condensation or packaging of chromatin in mitotic cell division.

l(3)snap mutants fail to condense fully their mitotic chromatin resulting in condensed blocks linked by extended chromatin threads. Breakage of tangled chromosomes at anaphase causes aneuploidy and cell death. The X-linked locus *mutagen-sensitive(1)101* encodes a function required for the condensation of mitotic heterochromatin in larval neuroblasts. Gross defects in DNA replication have not been reported for *mus(1)101* mutants and there may be no specific requirement for the gene in S phase. However, such mutants are hypersensitive to killing by mutagens and defective in post-replication DNA repair and chorion gene amplification. I have identified a female-sterile *mus(1)101* allele which shows a temperature-sensitive maternal effect on the mitotic chromosomes of the embryo. *l(1)d.deg4* produces a colchicine-like block to mitosis in larval neuroblasts, arresting cells with condensed chromosomes. This mutation is not allelic to *mus(1)101* but by cytological criteria the genes are very closely linked at 12B. In order to characterise these genes at the molecular level, I have isolated DNA from 12B1-C1 by chromosome walking.

Much of the macromolecular reorganisation during mitosis is regulated by protein phosphorylation resulting from the opposing activities of mitotic protein kinases and protein phosphatases. I have identified null mutations in the major type 1 protein serine-threonine phosphatase gene *PP1 87B* causing defects in mitotic spindle organisation, chromatid separation and chromatin decondensation. This work indicates a role for this phosphatase in coordinating mitotic progression.

For Eva J. Neer, who started the ball rolling.

"Il faut imaginer Sisyphe heureux." (Camus 1942).

Originality

I wrote this thesis myself. The work it describes is entirely my own work with the exception of experiments clearly indicated in the text as having been performed by co-workers.

Support

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Acknowledgements

I am grateful to my thesis supervisor, David Glover, for his well-timed advice and incisive editing. Many colleagues, including Mike Bate, Alan Cheshire, Patricia Cohen, Viktor Dombrádi, Erich Frei, Mike Goldberg, Cayetano González, Roger Karess, Santosh Krishnan, Peter Little, Alfonso Martinez-Arias, Kevin O'Hare, Robert Saunders, Abe Schalet, Debbie Smith and Will Whitfield provided essential advice, materials and enthusiasm.

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Chapter 1: Introduction.

Great progress has been made over the last few years in our understanding of the intiation and co-ordinate regulation of the cellular decision to enter mitosis. In contrast, much remains to be discovered of the ways in which the cell responds to this decision. Of particular interest is the rapid and astonishingly well-ordered reorganisation of the interphase nucleus to form discrete, packaged mitotic chromosomes. This process of chromosome condensation, and its systematic reversal (decondensation) after nuclear division needs to be addressed on at least three levels: how does chromosome organisation change at different stages of the cell cycle, what are the integral components of the chromosomes, and which enzymes and other control systems act upon these molecules? These requirements strongly favour *Drosophila melanogaster* as an experimental organism of choice. Firstly, the structure of the mitotic chromosome may be examined cytologically. Individual genes encoding proteins required for chromosome packaging may be identified by analysis of mutations and by molecular analysis of the genes themselves. Finally, it may soon be possible to analyse the interactions of some of these products in the process of chromosome packaging using a combination of genetics and cell biology.

1.1 Nuclear cycles and cell division in the Drosophila life cycle

The analysis of genes encoding essential cell cycle functions in *Drosophila* has made use of the developmental biology of this multicellular organism (reviewed by Ripoll et al. 1988, Glover 1989, O'Farrell et al. 1989). The first 13 nuclear divisions in the *Drosophila* embryo take place within a large syncytium filled with sufficient maternally-provided RNA and protein to enable these divisions to be completed in the absence of zygotic transcription (Edgar et al. 1986). The first mitosis is gonomeric, with haploid male and female pronuclei dividing on a common mitotic spindle prior to the fusion of nuclei (Sonnenblick 1950). Nuclei then divide almost synchronously and move outward to the surface of the embryo during the telophases of cycles 7-10. After this, in cycles 10-13, nuclei enter and leave mitosis in rapid metasynchronous waves which sweep the embryo from the anterior and posterior poles and move medially. These nuclei may differ in phase by up to 2 minutes so that nearly all stages of the cycle are simultaneously present within an embryo (Foe and Alberts 1983).

Although the mean length of the first 8 cycles is about 8 minutes at 25°C (Rabinowitz 1941, Zalokar and Erk 1976), the mitotic cycle time slows progressively with increasing nuclear density to 21 min. by cycle 13 (Foe and Alberts 1983). As in other large yolky eggs such as those of *Xenopus* and starfish, the cytoplasmic volume remains constant during these "cleavage" divisions. The ratio of nuclei to cytoplasm is thus able to act as an important developmental signal (Newport and Kirschner 1982, Mita 1983.). The interphases of nuclear cycle 14 are at least 70min. long in diploid embryos at 25°C. During this phase, nuclei elongate, replicate and many genes become transcriptionally active. Cell membranes form around the nuclei, and soon after, discrete stereotyped domains of cells enter mitosis asynchronously but in a fixed temporal order over a period of about an hour (Foe 1989). Haploid embryos, however, complete a 14th syncytial mitosis before the onset of cellularisation and nuclear cycle lengthening. This means that it is the ratio of the number of chromosomes (or amount of chromatin) to amount of cytoplasm rather than the number of nuclei that determines cell cycle timing (Edgar et al. 1986).

Although zygotic transcription is not required to activate entry into mitosis until interphase 14, the translation of maternally-provided messages is. This may reflect a requirement for periodic synthesis of the periodically degraded mitotic cyclins as in the embryos of other species with large eggs (Evans et al. 1983, Whitfield et al. 1989, Lehner and O'Farrell 1989). In *Xenopus*, cyclin B is the sole message whose translation is required for cleavage mitoses (Murray and Kirschner 1989, Murray et al. 1989). However, there is as yet no evidence for cycling of cyclin protein levels in syncytial *Drosophila* embryos. The level of accumulation of cyclin A or B protein does not appear to control entry into mitosis in the cellular embryo (Lehner and O'Farrell 1989, O'Farrell et al. 1989). Indeed, the "mitotic domains" of the 14th mitosis are foreshadowed exactly by zones of expression of another, zygotic RNA transcribed from

the *string* (*stg*) gene (Edgar and O'Farrell 1989). Maternal *stg* message has been degraded by the start of cycle 14, and in mutants lacking zygotic *stg* expression, the first cellular (14th) mitosis does not occur. For this reason, the stg^+ product is considered to be an activator of mitotic initiation. This view is further supported by the findings that *stg* message is expressed 20 min before mitosis, and that the *stg* protein shares sequence homology with a mitotic activator in *Schizosaccharomyces pombe*, the product of the $cdc25^+$ gene (Section 1.3).

In contrast to these regulators whose pattern of synthesis and degradation is important in control of mitosis, many of the maternally-encoded functions required in the basic cell cycle are expected to be stable until relatively late in development. This perdurance has been reported for the maternal asp^+ gene product (Carmena et al. 1990) and histones 2A and 2B (Minden et al. 1989). The embryo does not change in volume until after hatching so dilution of maternal products by cell growth will be minimal until after this stage, by which time most larval tissues are formed. Except for part of the CNS, most larval cells do not divide but become polyploid or polytene and grow by cell enlargement. In the ganglia of the CNS, neuroblasts differentiate during the first instar and begin asymmetric stem cell divisions yielding smaller ganglion mother cells which themselves divide symmetrically to form non-dividing ganglion cells (Poulson 1950, White and Kankel 1978, Truman and Bate 1988). The stages of mitosis readily studied in these cells are shown in Fig. 1.

According to one model of the requirements for cell division in the *Drosophila* life cycle, it should therefore be possible to obtain mutations in genes encoding essential cell-cycle functions, which nevertheless exert their lethal effects late in ontogeny. Progeny homozygous for a mutation in such a gene may thus be expected to survive embryogenesis using wild type products provided by their heterozygous mothers. After embryogenesis, diploid cell division is restricted to the gonad, imaginal discs and the developing thoracic and ventral ganglia. Late in the third larval instar these tissues show most mitotic activity. Hence it is at this stage that stable products required for cell division will be exhausted leading to late-larval or early pupal lethality with failure of proliferation of the imaginal discs (Shearn et al. 1971, Baker et al. 1982, Szabad and Bryant 1982). Examining mutants fitting this description, Gatti and Baker (1989) found nearly all (19 of 20) were abnormal in some aspect of neuroblast mitosis. Half of all larval and early pupal lethals examined (30 of 59) displayed a high frequency of abnormal neuroblast chromosomes, regardless of imaginal disc status. The classes of mutation discovered in this way can be divided into four broad categories: Mutants with few or no cells in division might identify genes controlling mitotic initiation but, unfortunately, this screen would be unable to distinguish these from a large class of mutations deficient in metabolic processes required in interphase. Mutants arresting the cycle at metaphase might be defective in components of the mitotic apparatus, or functions required in chromatid disjunction.

Mutants with highly polyploid cells may have a defect in chromatid segregation or cytokinesis. Finally, a large class of mutations were found to cause abnormal chromosome condensation.

This last category described a variety of mutant effects on chromosome morphology. Mutants displayed highly pycnotic chromosomes, with or without chromatid disjunction. Chromosomes could be evenly decondensed, fuzzy or swollen, or coiled and banded in appearance. In other mutants, region-specific decondensation was common. Many mutations produced irregular, regionally non-specific condensation into blocks and threads, or a high frequency of chromosome breakage (Fig. 2). Mutations causing chromosome breakage without gross effects on condensation have been previously described by the same authors (Gatti 1979, Baker et al. 1982).

Because each mutation can cause the simultaneous appearance of more than one of each of these defects within a population of cells, further analysis using more alleles would be of use in assigning specific mutant effects to particular loci. Even within an individual homozygous for a zygotic null mutation, some heterogeneity of terminal cellular phenotype is expected to result from variation between cells in the residual level of maternal product. To overcome this uncertainty, conditional (e.g. temperature-sensitive) mutations may also be employed with some success in cytological analysis (Gatti et al. 1983, Smith et al. 1985).

Because the nuclear divisions of the syncytial embryo are dependent upon maternal products, another source of mutants defective in cell cycle functions is the class of female sterile mutations which have a lethal maternal effect before cellularisation of the embryo. Maternal effect lethal mutations which cause uneven distribution of nuclei and cytoskeletal components appear relatively common. However, mutations at several loci affect both embryonic nuclear division and neuroblast mitosis in third instar larvae. The normal role of these genes in the cell cycle is clearly worthy of investigation (Sunkel and Glover 1988, Glover et al. 1989). Other mutations such as *gnu* (Freeman et al. 1986, Freeman and Glover, 1987) and *plutonium* (T. Orr-Weaver, pers. comm.) are of interest because they disrupt the temporal coupling of DNA synthesis to mitosis in the pre-blastoderm embryo.

Fig. 1

Chromosomes and mitosis in wild type giant neuroblasts.

Squashes were prepared from ganglia of third instar larvae without colchicine or hypotonic treatment. The stain is acetic orcein.

a) XY interphase showing heteropycnotic elements (arrowed). The presence of such structures is dependent on tissue and cell cycle stage (Section 1.4.1).

b)XY prophase, the pericentric heterochromatin is condensed and chromatids are closely paired in heterochromatic regions. Euchromatic arms are still thin and threadlike. The Y and heterochromatic X (Xh), and pericentric heterochromatin of one pair of autosomes are arrowed. Note the somatic pairing of homologous autosomes.

c)XY, late prophase.

d)XX, pro-metaphase. Somatic pairing of homologues is maintained.

e)XY, pro-metaphase, the metacentric second chromosomes can be distinguished by a conspicuous secondary constriction in the left arm. The third chromosomes are also metacentric. The fourth chromosomes are small and dot-like.

f-i) XY, anaphases. Sister chromatids are separated to the poles of the mitotic spindle.

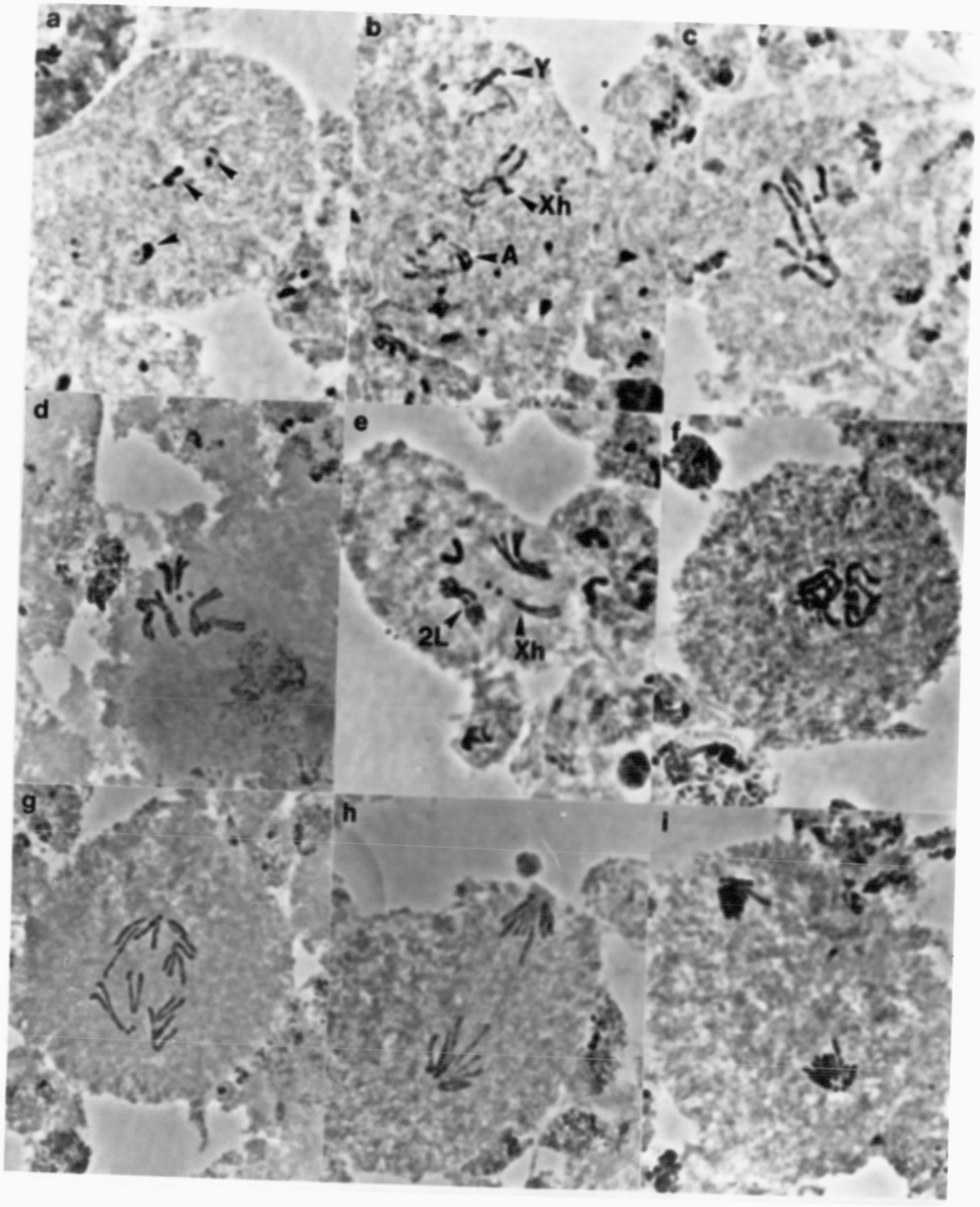

a
b
Y
Xh
A
c
d
e
2L
Xh
f
g
h
i

Fig. 2

Examples of mutations causing abnormal mitotic chromosome condensation and chromosome breakage in larval neuroblasts

Several different mutations produce similar effects in mitotic cells of *Drosophila* third instar larval ganglia. Irregular chromosome condensation and chromosome breakage are often found in cells of all of these mutants. These squashes were stained with acetic orcein after treatment with colchicine to accumulate cells in metaphase, and a brief osmotic shock in hypotonic saline, which normally improves the morphology of wild type mitotic chromosomes. The abnormally condensed chromosomes of these mutants are similar in appearance if prepared with or without colchicine and hypotonic treatment.

a) Wild type XX female cell. The colchicine and hypotonic treatment has resulted in wide separation of the euchromatic portions of the chromatids (the chromosome arms). Somatic pairing has also been disrupted (compare with Fig. 1e).

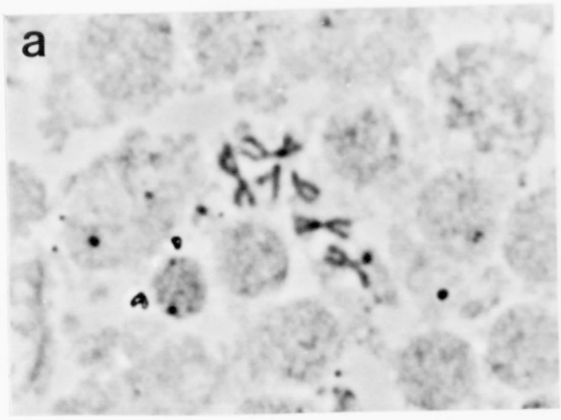
b)*l(3)K43* homozygote. The few metaphases present in ganglia from this mutant characteristically show a coiled or beaded appearance.

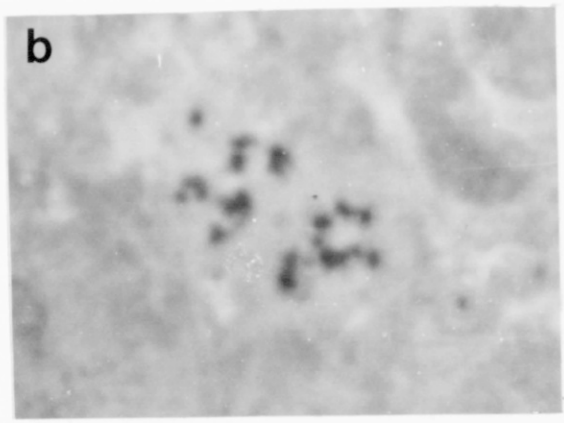
c)*l(3)snap* homozygote (see Chapter 3). The abnormally condensed chromosomes in this mutant frequently consist of regularly-sized chromatin blocks linked by threads of chromatin. Chromosome fragments are frequently seen.

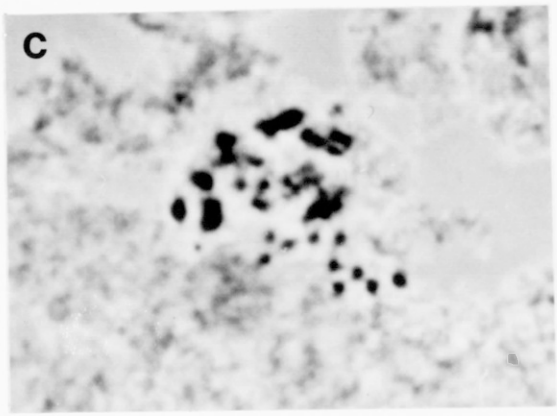
d)*l(3)g60A* homozygote. The chromosomes are extremely unevenly condensed. It is not possible to distinguish this kind of irregular condensation from extensive chromosome breakage.

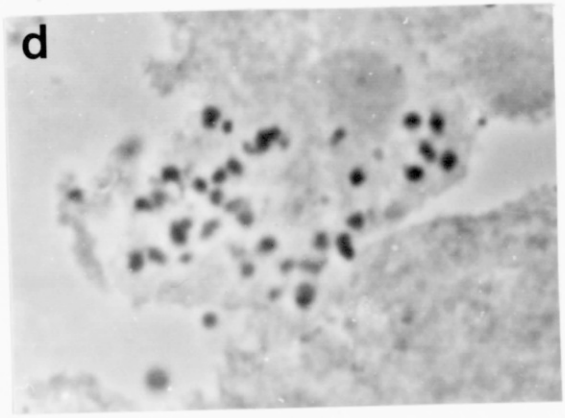
e)*mus(1)101*^{lcd}/Y hemizygote (Chapter 4). This mutant shows cells with different degrees of aberrant chromatin organisation within the same ganglion. In the least affected cells only the pericentric heterochromatin is undercondensed (Fig. 5b). The blocks of chromatin shown here are probably whole chromatid arms no longer held together by the decondensed heterochromatin.

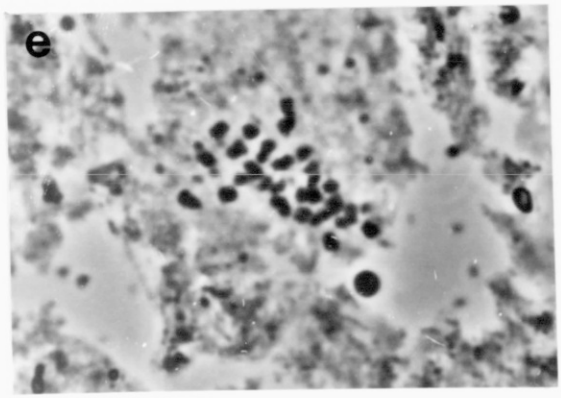
f)*l(1)d.deg10* hemizygote without colchicine or hypotonic treatment. This metaphase is hyperploid with strongly overcondensed chromosomes. Some breakage is also apparent. (This photograph (f) was kindly supplied by Dr. M. Gatti)

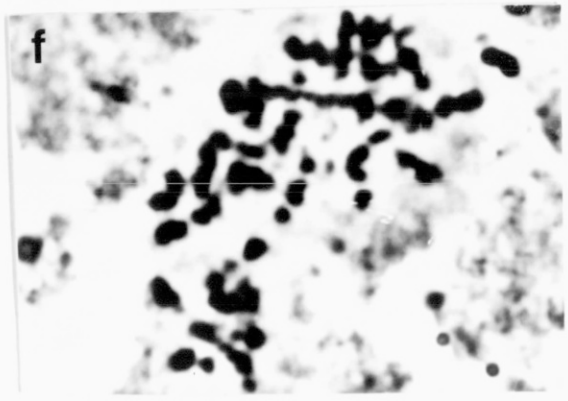

a


b


c


d


e


f

1.2 Chromosome structure

Rabl concluded in 1885 that "there must be order" within the nucleus. More recent descriptions of nuclear structure support the idea that this is so (reviewed by Newport and Forbes 1987). A non-random distribution of chromosomes within the nucleus has profound implications for those who study the organisation of replication, transcription, macromolecular transport and the spatial relationship of chromosomal loci to sub-compartments of the cytoplasm. However, I shall concentrate on aspects of nuclear structure which are relevant to problems of mitotic chromatin condensation and decondensation.

Four major questions stand out: How is the interphase nucleus transformed into discrete chromosomes and chromatids without confusion and tangling? What is the physical organisation of a metaphase chromosome in terms of coiling or gathering of DNA strands and their associated chromatin proteins? To what extent does this process require *de novo* addition (or removal) of specialised packaging proteins and to what extent does the process depend on modification and reorganisation of existing macromolecules? Finally, how are chromosome condensation and decondensation coordinated with the mitotic reorganisation of the cytoskeleton?

It is generally accepted that the chromatids of diploid eukaryotic cells typically comprise single linear DNA molecules non-covalently bound to nucleosomal histones and other chromatin proteins (reviewed by Taylor 1990). Comparison of prophase and telophase nuclei in a number of different eukaryotic organisms has led to the conclusion that chromosomes retain their anaphase (or Rabl) orientation throughout interphase (reviewed by Fussell 1987). Fluorescence microscopy of embryonic and polytene interphase nuclei in *Drosophila* confirms that centromeres are attached to the nuclear envelope at one side of the nucleus, the telomeres at the other (Agard and Sedat 1983, Foe and Alberts 1985, Hochstrasser and Sedat 1987a,b). In the polytene nucleus chromosomes coil in predominantly right-handed turns but do not wind about one another, although they may have different neighbours in different nuclei (Hochstrasser et al. 1986). Specific chromosomal regions associate with the nuclear envelope. These loci correspond well with the known distribution of intercalary heterochromatin (see below) and may be responsible for maintaining the relative positions of the interphase chromosomes. If diploid nuclei are organised in the same way, for which there is some evidence (Avivi et al. 1982a,b, Skaer et al. 1976, Moroi et al. 198[illegible], Lewis and Laemmli 1982) then the spatial restriction of chromosomes to particular regions of the nucleus by envelope attachment could prevent tangling upon chromosome condensation.

The packaging ratio of DNA in a mitotic chromosome is about 10^4. Typically a 5μm human chromosome 1.3×10^8bp long contains about 5cm of DNA which contributes about a third of the mass of the chromosome. The remainder is made up of histones and non-histone proteins in approximately equal masses. The organisation of

DNA and histones into chromatin accounts for a 40-fold reduction in length (reviewed by Felsenfeld and McGhee 1986). This leaves a factor of some $2.5x10^2$ which needs to be accounted for in models of mitotic chromosome condensation.

Although models have been proposed in which the chromatin of a mitotic chromosome is packaged by several sequential levels of helical supercoiling (Sedat and Manuelidis 1977, Bak et al. 1977), there is abundant evidence for chromatin gathering or loop formation and most current models incorporate this feature (reviewed by Earnshaw 1988). Interphase nuclei contain loops or topologically isolated domains (Cook and Brazell 1976a,b) analogous to the radial lampbrush loops of meiotic prophase chromosomes (Callan 1982). Preparations for the electron microscope (EM) of metaphase chromosomes from which histones have been extracted by a variety of treatments reveal loops of DNA attached by their bases to residual protein. Loops of 30nm chromatin fibre are also seen in whole mount EM preparations (Miller spreads) of mitotic metaphase chromosome but the bases of the loops are not usually resolved (Rattner and Lin 1985).

Based on the data from the histone-extracted chromosomes, models were proposed in which radial loops of 30nm fibre are arranged about an axial protein "scaffold" (Paulson and Laemmli 1977, Adolph et al. 1977, Marsden and Laemmli 1979, Earnshaw and Laemmli 1983). The helical structure of the mitotic chromatid axis in EM whole mount preparations of chromosomes from detergent-lysed cells led to a compromise model in which the scaffold itself coiled helically, permitting a further level of condensation of a 200-300nm radial loop structure (Rattner and Lin 1985).

These models are susceptible to criticism on technical grounds because of the extreme variability of morphology and protein composition of structures variously termed scaffold, nuclear matrix, or karyoskeletal elements which remain after different biochemical procedures employed to extract histones and DNA. For a discussion of the extraction techniques used and their implications for structural models see Fisher (1989). The structure of mitotic chromosomes *in vitro* is very sensitive to environmental conditions presumably reflecting the structural plasticity of chromosomes in the living cell. But this has meant that chromosomes prepared in different ways exhibited different features of organisation. Few EM studies of native mitotic chromosomes in three dimensions have been attempted because the fully-condensed structures appear too dense to yield useful information.

However, Belmont et al. (1987) have used high voltage transmission electron microscopy, axial tomography and electronic image processing to reconstruct images of *Drosophila* chromosomes from embryos and Kc cells, in various states of condensation. Distinct homogeneous classes of structural element were uncovered, the smallest of which was 12nm in diameter, corresponding in size to extended polynucleosomal chromatin. In the least condensed chromosome examined, the 200nm diameter embryonic anaphase chromatid, a 24nm loop element predominated. However, no

evidence for radial organisation of loops was found. The 24nm element is less frequently found in more condensed chromosomes which are made up of 40-50nm and 80-100nm elements. At the highest level of compaction, in metaphase-arrested Kc cell chromosomes, large 130-160 nm elements occasionally appeared as transverse bands. The authors stressed that the larger elements found in the more condensed chromosomes need not be composed of previously-formed stable elements of smaller diameter. Because of the possibility that the hierarchy of structures was not constructed in a strictly sequential way, they recommended that chromosomes should be examined at different points during the condensation and decondensation cycle rather than relying on biochemical techniques of expansion and extraction.

The main impetus to the effort to understand how loops of chromatin could be brought together to form an axial element of mitotic chromosomes was the discovery that a large proportion of the protein remaining bound to the bases of DNA loops after extraction of histones proved to be topoisomerase II.

1.2.1 DNA topoisomerases

DNA topoisomerases are classified by the mechanism of DNA strand breakage and rejoining they catalyse (reviewed by Wang 1985). Type I enzymes break and rejoin one strand at a time and may mediate the degree of supercoiling of DNA. In contrast, type II topoisomerases catalyse double strand breakage and rejoining, thus they additionally capable of decatenation, passing one entire DNA molecule through another. This property is likely to be required at the time of mitosis both in chromatin packaging and in sister chromatid separation (Holm et al. 1985). *Drosophila* topoisomerase II is distributed throughout polytene chromosomes, in contrast with topoisomerase I which appears specifically associated with transcriptionally active genes (Berrios et al. 1985).

Non-histone proteins of the "metaphase chromosome scaffold" remaining bound to DNA after extraction with 2M NaCl constitute only 3-4% of the total chromosomal mass. Two proteins are enriched in this fraction, Sc1 and Sc2 (Lewis and Laemmli 1982). The most abundant protein, Sc1, was shown immunologically and functionally to be topoisomerase II (Earnshaw and Heck 1985, Earnshaw et al. 1985). Immunofluorescence and immuno-EM techniques localised this protein to an axial position within hypotonically swollen, unextracted mitotic chromosomes. Its distribution was not uniform but in the form of separate 120-200nm "islets" which might represent "clusters or rosettes" of chromatin loops bound by the topoisomerase. Scaffold-associated DNA sequences have been characterised and proposed to contain topoisomerase II cleavage sequences (reviewed by Mirkovitch et al. 1987). Although it is not known how such sequences aggregate upon chromosome condensation, genetic data strongly implicate topoisomerase II in the processes of condensation and decondensation where it could play both a structural and enzymic role.

Cold-sensitive double *top2-nda3* mutations of *Schizosaccharomyces pombe* arrest earlier in the cell cycle than do mutations in the *nda3* (β-tubulin) gene alone. Chromosomes of the double mutant are extended, in contrast with the highly condensed chromosomes of the *nda3*-arrested mutant cells (Uemura et al. 1987). If double mutant cells were permitted to recover their topoisomerase II activity while prevented from forming mitotic spindles in the presence of the inhibitor of microtubule assembly, nocodazole, the chromosomes rapidly converted to the condensed form.

In *in vitro* extracts of activated *Xenopus* eggs, the specific inhibitor of topoisomerase II, VM-26, prevents the appearance of highly ordered "metaphase-like" organisation of exogenous DNA but does not affect nucleosomal formation (Newport 1987). Decondensation of metaphase chromosomes in these extracts cannot proceed in the absence of topoisomerase II activity. It must be noted, however, that DNA breakage resulting from inactivation of topoisomerase II in mid-reaction might prevent transmission of packaging forces along chromatin fibres, thus interfering with both processes of condensation and decondensation.

Many other factors are likely to be needed to achieve the final packaging of a metaphase chromosome. Some are expected to be stoichiometrically added structural proteins. Evidence for this is provided by the observation of Newport and Spann (1987) of the way in which naked DNA acts to inhibit chromosome condensation in *Xenopus* extracts. It appears to be necessary for other proteins to leave the DNA for chromatin condensation to occur. The RCC1 protein is bound to chromatin in interphase human cells but dispersed into the cytoplasm at mitosis (Ohtsubo et al. 1989). This protein has a role in regulating the onset of mitotic chromosome condensation. Cells bearing conditional mutations in the hamster homologue of this gene (tsBN2) undergo premature chromosome condensation when shifted to the non-permissive temperature (Yamashita et al. 1984a, reviewed by Nishimoto 1988). There is also a multitude of rather poorly-characterised factors involved in chromosome condensation and decondensation which have been summarised by Adlakha and Rao (1986).

The hyperphosphorylation of histone H1 on particular serine and threonine residues, observed in many organisms, is synchronous with chromatin condensation (reviewed by Hohmann 1983, Matthews and Huebner 1984, Wu et. al. 1986). The ability of histone H1 to aggregate DNA is greatly increased by these modifications (Matthews and Bradbury 1978). Condensation can be induced in late S or G2 phase *Physarum* plasmodia exposed to purified M-phase-specific H1 kinase (Bradbury et. al. 1974a,b). What also seems clear is that phosphorylation of H1 is not in itself sufficient to cause chromosome condensation in most systems. Phosphorylation of histone H3 and nonhistone proteins is also observed. These modifications may also be necessary in the correct regulation of the condensation process (Yamashita et al. 1984b).

1.3 Coordinate regulation of mitotic processes

It is now thought that the concerted phosphorylation of a particular set of proteins is required not only to initiate chromosome condensation, but to coordinate this with the radical reorganisation of the nuclear envelope and cytoskeleton that occur on entry into mitosis. A crucial regulator linking these processes is the protein serine-threonine kinase encoded by the essential cell division cycle gene *cdc2*$^+$ of *Schizosaccharomyces pombe*. This gene is functionally conserved between evolutionarily distant organisms such as *Saccharomyces cerevisiae*, *Drosophila melanogaster* and humans (for review, see Norbury and Nurse 1989). Evolutionary conservation of function has also been noted for the dosage dependent activator of p34^{cdc2}: the product of the *cdc25*$^+$ gene (Russell and Nurse 1986, Edgar et al. 1989).

The complex regulation of the systems of protein phosphorylation governing entry into mitosis has been genetically dissected in several fungi. In *Schizosaccharomyces pombe*, *cdc2*$^+$ is negatively regulated by *wee1*$^+$, which is itself negatively regulated by *nim1*$^+$ (Russell and Nurse 1987a,b). Both *wee1*$^+$ and *nim1*$^+$ are protein kinase cognates, as is *nim A*$^+$ of *Aspergillus nidulans*, a G2-specific activator of mitotic initiation (Osmani et. al. 1987, 1988).

Although specific proteins have been described that are phosphorylated on entry into mitosis (Davis et al. 1983) the identity of most of the substrates of p34^{cdc2} *in vivo* remain to be determined. Many workers have assayed p34^{cdc2} kinase by its ability to phosphorylate histone H1 *in vitro* (e.g. Labbé et. al. 1988) and *in vivo* (e.g Arion et. al. 1988). The process of nuclear envelope breakdown (NEBD) is facilitated by the depolymerisation of the underlying nuclear lamina, synchronous with the phosphorylation of nuclear lamins (Gerace and Blobel 1980). However, Newport and Spann (1987) found that lamin depolymerisation could occur without NEBD in *Xenopus* extracts. Indeed, in many species, such as *Drosophila*, mitosis takes place within a lamin-depleted nuclear membrane (Fuchs et al. 1983). p34^{cdc2} from starfish will cause solublisation of lamins B from chicken nuclei *in vitro*, phosphorylating the same amino acids that are modified in mitosis *in vivo* (Peter et al. 1990a). This p34^{cdc2} will also phosphorylate nucleolar proteins on sites that become phosphorylated in M phase, and these authors have suggested that the mitotic reorganisation of the nucleolus results directly from the action of p34^{cdc2} kinase (Peter et al. 1990b).

The p34^{cdc2} kinase may also bring about the reorganisation of the cytoskeleton as cells enter mitosis. Its association with centrosomes at the G2/M transition suggests it may influence the behaviour of microtubules (Booher et al. 1989, Bailly et al. 1989). Verde et al. (1990) added starfish p34^{cdc2} to interphase extracts of activated *Xenopus* eggs and observed that the dynamics and steady state length of centrosome-nucleated microtubules switch from an interphase state to those characteristic of a mitotic cell.

The phosphorylation state of proteins is dynamic, dependent on the relative activities of protein kinases and protein phosphatases. It is not surprising, therefore to find that protein phosphatases, too, contribute to mitotic regulation. $p34^{cdc2}$ is phosphorylated on serine, threonine and tyrosine residues in exponentially growing *S. pombe* cells (Gould and Nurse 1989). Although dephosphorylation of tyrosine may be a critical step in $p34^{cdc2}$ activation in mammalian cells (e.g. Morla et. al. 1989) and *S. pombe*, threonine dephosphorylation is also noted (Gould and Nurse 1989). Felix et al. (1990)ㅋ have demonstrated that the protein phosphatase(s) responsible for final activation of $p34^{cdc2}$ in extracts of activated *Xenopus* eggs is insensitive to okadaic acid, a potent inhibitor of protein serine/threonine phosphatases 1 and 2A (reviewed by Cohen et al. 1990), and thus distinct from these type 1 and 2A enzymes. A protein tyrosine phosphatase (reviewed by Hunter 1989) capable of activating $p34^{cdc2}$ remains to be identified.

In contrast, type 1 and/or 2A enzymes are required in regulating the extent of $p34^{cdc2}$ kinase activity. Microinjection of okadaic acid or anti-PP1 antibodies into starfish oocytes maintains a high level of $p34^{cdc2}$ protein kinase activity (Picard et al. 1989). Inhibition of PP2A by okadaic acid promotes the activation, and decreases the rate and extent of inactivation of $p34^{cdc2}$ kinase in "cycling" extracts of *Xenopus* eggs whereas inhibition of PP1 with I-2 does not (Felix et al. 1990). Protein phosphatases are also required to return mitotic protein kinase substrates to their interphase levels of phosphorylation, events which must play an important part in mitotic steps after metaphase. These phosphatases could be among the chromosome decondensation factors specifically active in telophase and G1 cells (Adlakha and Rao 1986).

1.4 Heterochromatin

"From the time of the nineteenth century cytologists, odd assortments of densely staining flecks, blobs, rods and agglomerations have been seen in the cell nucleus of various species of plants and animals." (Brown, 1966).

Most genes, that is, chromosomal DNA sequences with defined cellular products, are located in euchromatin which characteristically occupies a medial position in disparate chromosome arms. Despite being distributed in this way, euchromatin behaves as a relatively homogeneous unit during the mitotic cell cycle, replicating, condensing and separating quite synchronously. It must be noted, however, that some regions of euchromatin may exhibit somewhat asynchronous replication, and that this may correlate with the tissue-specific expression of these regions (Stubblefield 1975, Goldman et al. 1984). Heitz (1928) introduced the term "heterochromatin" to describe regions or chromosomes which failed to decondense at telophase with the rest of the chromatin. Subsequently the term has been applied to almost any interphase chromatin which is visibly pycnotic and thus differs from the rest of the genome in the timing of

its condensation. The timing of replication, and of anaphase sister chromatid disjunction of morphologically heterochromatic regions may also exhibit such "allocycly". Heterochromatin ought therefore to be of particular interest to cell cycle researchers.

Regions of constitutive heterochromatin comprise much repetitive DNA, very little of which appears ever to be transcribed or to encode cellular products. Constitutive heterochromatin is thus distinct in primary sequence and origin from that euchromatin which has been facultatively inactivated in a sex- or tissue-specific manner. Such inactive chromatin, which has also been described as being "heterochromatinised" or even "facultative heterochromatin" has been reviewed by Gartler and Riggs (1983). I shall use the term "heterochromatin" to denote constitutive heterochromatin, including that which is formed both by the repetitive satellite sequences and by the less redundant genetic elements interspersed among them.

Much of heterochromatin may well be without consequence, with few effects and no functions. This view is most persuasively put in an excellent review by John (1988). However, at very least, there exist structural differences between eu- and heterochromatin in DNA sequence and protein composition that determine their response to the oscillating signals of the mitotic cell cycle. Investigation of these structural differences throughout the cycle will undoubtedly improve our understanding of the ways in which cells control the timing of DNA replication and mitotic chromosome condensation.

In the terminology of current models it is obvious that the heterochromatic portions of the genome differ in their response to a cytoplasmic cell cycle oscillator. However, constitutive heterochromatin (forming heteropycnotic chromocentres in interphase) has been most extensively reported as characteristic of complex somatic cell cycles where it is clear that transcriptional activity of the nucleus, cell size and integrity of replicated DNA all influence the ability of the cell to enter mitosis. In such cells, the nucleus itself is also part of the oscillator. Thus it is hard to be sure that heterochromatin, replicating and condensing out of phase with the euchromatin, is without effect on the timing of the cycle itself.

The location of heterochromatin at centromeres and telomeres induces inquiry into its structural role in cell division. In addition, heterochromatin might actually perform some function in mitotic cells, though there is little direct evidence for this. Finally there is also the possibility that the heterochromatic environment could permit expression of genetic functions during periods when euchromatic genes are not transcriptionally active (Pimpinelli et al. 1985).

1.4.1 *Drosophila* heterochromatin

About one third of the genome of *Drosophila melanogaster* is heterochromatin, as has been pointed out in several useful reviews (Hilliker et al. 1980, Pimpinelli et al. 1986). The Y chromosome is entirely heterochromatic. Heterochromatin contributes the proximal one third to half of the prometaphase length of the mitotic X and the proximal fourth of each arm of autosomes 2 and 3 (Heitz 1933, Kaufmann 1934). These conclusions have been confirmed by the C-banding technique (Pimpinelli et al. 1976). Of a haploid genome of 0.18pg or 170Mb (Rasch et al. 1971) approximately 20% consists of satellite DNA, simple 5, 7, 10bp repeats or more complex 359bp sequences in tandem array (Hsieh and Brutlag 1979, reviewed by Lohe and Roberts 1988). *In situ* hybridisation with bulk satellite DNA (Peacock et al. 1977) or cloned satellite DNA probes (Lohe and Roberts 1988) reveals that simple satellite repeats are restricted to the proximal ends of the mitotic chromosome arms of neuroblast cells, and to the heterochromatic aggregate (chromocentre) of polytene nuclei where they form large blocks (Lohe and Brutlag 1987). Although small arrays of simple and complex satellite DNA have been found by sequencing in cloned euchromatic loci (Tartof et al. 1984, Spradling et al. 1987), evidence from P element mediated transformation with tandem arrays of satellite sequences suggests that such sequences may be largely restricted to the heterochromatin because they are unstable when inserted in euchromatic positions even in the absence of P transposase (unpublished work described by Lohe and Roberts (1988)).

Intercalary sites of different sequence composition have also been found within the euchromatin (Zhimulev et al. 1982, Bolshakov et al. 1985, Kholodilov et al. 1988). These have been classified as being heterochromatic because many contain repeated DNA, are late replicating, under-replicated in polytene chromosomes and display affinity for each other or for the chromocentral heterochromatin (ectopic pairing).

Embedded at sparse intervals in centric heterochromatin are also found a number of genetic loci. Few such loci have been detected by mutational analysis on the sex chromosomes. The X has a nucleolar organiser: the bb^+ or rDNA locus, and a site (collochore) responsible for male meiotic pairing of the sex bivalent (Cooper 1964). The Y bears six male fertility factors of exceptional size (Gatti and Pimpinelli 1983, reviewed by Pimpinelli et al. 1986), bb^+, collochore, and a locus *Su(Ste)* which interacts with, and shares homology with a tandemly repeated euchromatic locus *Stellate* on the X (Livak, 1984, 1990). 21 lethal complementation groups have been localised to the centric heterochromatin of autosomes 2 and 3 (Hilliker et al. 1980). The best characterised of these genes is *light* (*lt*) (Devlin et al. 1990) required for both viability and pigmentation which spans at least 17kb of single copy sequence interspersed with a heterogeneous array of middle repetitive DNA (25-60 copies per

nucleus). The expression of the *lt* gene does depend on its proximal location (see Section 1.5 for discussion of position effect).

Late replication of heterochromatin is a common finding in a number of different cell types in different organisms (reviewed by Lima de Faria and Jaworska 1968). Barigozzi et al. (1966) have shown by labelling *Drosophila* primary embryonic cells in culture with tritiated thymidine that in these cells the heterochromatin replicates later than euchromatin. However, as suggested by Masubuchi (1976) replication of heterochromatin may better be described as occupying a period shorter than that of the euchromatin as there are organisms in which heterochromatin is first to replicate (reviewed by John 1988). Heterochromatin in diploid tissues is not sparsely supplied with replication origins as was suggested by Blumenthal et al. (1974). In the larval brain of *Drosophila virilis*, satellite DNA replicates in short (<30kb) tracts, but the mean replicon size for the rest of the genome is about 100kb (Steinemann 1981a,b).

The constitutive heterochromatin of the Y chromosome and pericentric regions of the other chromosomes is not replicated in polytene nuclei. This means that there must be a intermediate zone of complex structure linking the unreplicated heterochromatin with the fully polytenised euchromatin. It is possible that this zone, sometimes called "β-heterochromatin", comprises a stepped series of branched DNA junctions resembling stabilised replication forks (Plaut 1963, Laird 1973, Spradling and Orr-Weaver 1987). The control of replicative behaviour of regions within heterochromatin remains to be elucidated. The DNA sequence itself cannot be the sole determinant, for example Endow and Glover (1979) have shown that the rDNA of only one of the nucleolar organisers undergoes polytenisation.

During the brief interphases of the cleavage stages in the syncytial embryo, no heteropycnotic structures are visible. During cellularisation of the embryo, in the interphase of nuclear cycle 14, individual heterochromatic structures (chromocentres) appear, associated with nucleoli. Huettner (1933) shows superb examples, also Sonnenblick (1950). Blastoderm nuclei transplanted into unfertilised eggs lose their chromocentres and stain homogeneously in E.M. preparations (Illmensee 1972). Despite the absence of heteropycnotic interphase chromatin in syncytial embryonic nuclei, late decondensing chromosomal sites associated with the nuclear envelope have been reported by Hiraoka et al. (1989) during nuclear cycles 12 and 13 in observations made with fluorescent histones in real time. These sites also stain brightly with the fluorescent stain DAPI in fixed preparations as do the centric regions. However, the position of these late-decondensing/ early-condensing sites at the nuclear mid-line, is inconsistent with their being the centromeric heterochromatin shown by Foe and Alberts (1985) to be apically located. The late sites disappear fully in interphase but this does not mean that they are not "classically" heterochromatic despite this assertion by Hiraoka et al. (1989).

It is clear that interphase heterochromatin is pycnotic in some but not all interphase giant neuroblasts in iron-haematoxylin, aceto-orcein or aceto-carmine stained squash preparations (Cooper 1959). This may reflect differences in the cell cycle timing or differential regulation of interphase condensation in various cell subtypes. Kaufmann (1934) has reported that telophase heterochromatin of giant neuroblast chromosomes cannot be cytologically distinguished from condensed euchromatin but is clearly distinct in smaller (ganglion mother) cells. This difference may merely be attributable to the relatively greater degree of condensation of the mitotic ganglion mother cell chromosomes. These two cell types also have different cell cycle lengths, 55min. for giant neuroblasts and 6.5h from birth to mitosis for a ganglion mother cell (Truman and Bate 1988). The relative condensation of the heterochromatin of these cells during G1, S and G2 phases remains to be described.

Not only does the condensation state of interphase heterochromatin depend upon the tissue and developmental stage, but so, apparently, does the ability of heterochromatin to form a collective chromocentre. Lifschytz and Hareven (1982b) hybridised heterochromatic probes with squashed interphase cells of ganglia heterozygous for an inversion of the X heterochromatin, In(1)sc^8. They concluded that while centromeres certainly aggregate in telophase or Rabl orientation (Rabl 1885, reviewed by Fussell 1987), inverted heterochromatin could be resolved at a distinct site. Their conclusion that heterochromatin plays no active role in determining interphase structure certainly deserves investigation now that less disruptive techniques are available (Sedat 1990 in press).

Polytene nuclei display a strong chromocentral aggregation of heterochromatin regardless of chromosomal position. The subject is well described by Lefevre (1976). However, this aggregation may be an unusual tissue-specific adaptation which controls the position within the polytene nucleus of under-replicated DNA. Alternatively the association of heterochromatic sequences in polytene nuclei may be sufficiently strong to withstand the relatively gentle squashing pressure required to separate chromosome arms. The endoreduplication of chromatids, even to the limited degree seen in the proximal polytene divisions may also reinforce the chromocentral association.

Most of the pericentric heterochromatin in a chromosome, as distinct from the centromere proper, is unlikely to be important for nuclear division, although specific requirements have not been intensively investigated. Yamamoto and Miklos (1977) found that deficiencies of X heterochromatin did not affect the processes of meiotic pairing and segregation in female meiosis. However, several authors have speculated on the roles of specific sites located in heterochromatin which could potentially be important for mitotic segregation. Discrete sites within pericentric heterochromatin of the X chromosome have been found, each capable of interacting with specific maternal effect lethal mutations in the autosomal euchromatin. One such maternal effect mutation, *dal*, causes irregular nuclear divisions in the pre-blastoderm embryo (Sandler

1977, Pimpinelli et al. 1985, 1986, Sullivan, W. GSA *Drosophila* Conference 1989). Baker (1975), investigating the somatic loss of paternal chromosomes in embryos fathered by males homozygous for the meiotically abnormal *pal* mutation, mapped a site in the "basal region of the X chromosome" responsible for a 6-fold difference in chromosome loss between two different *pal*-bearing strains. He proposed that the normal *pal* product might bind near the centromere during male meiosis, affecting centromeric function during these divisions and the first two embryonic mitoses. González et al. (submitted) observed anaphase disjunction of neuroblast sister chromatids containing rearrangements which placed blocks of heterochromatin at some distance from the centromere. They found that these heterochromatic blocks, but not the euchromatin, maintained the pairing of chromatids after the centromeres had disjoined. Finally, these blocks separated as the chromatids were pulled apart by the spindle forces acting at the kinetochores. These authors concluded that it would be advisable to study the distribution of "centromere-associated" proteins not only at the centromere (Crooke et al. 1987, Rattner et al. 1988, Earnshaw et al. 1990) but within all heterochromatic regions if we are to understand the roles of these molecules in regulating chromatid disjunction.

1.5 Position-effect variegation

Heterochromatic position-effect variegation is the cell-autonomous inactivation of the expression of a gene brought into the proximity of a region of heterochromatin by a chromosomal rearrangement or transposition. The "variegating" gene is transcriptionally silenced in a proportion of the cells in a tissue, leading to a mosaic or variegated pattern of expression (reviewed by Spofford 1976, Eissenberg 1989). This inactivation of euchromatic genes is in some ways reminiscent of the facultative inactivation of one X chromosome in female mammals to form the Barr body (reviewed by Gartler and Riggs 1983). Although position-effect variegation in *Drosophila* reflects the consequences of abnormal chromosome structure on gene expression, it is thought that investigation of this phenomenon is likely to illuminate the role of interphase chromatin structure in determining patterns of gene expression at the chromosomal level.

The affected genes themselves are not altered in sequence so they do not become (constitutive) heterochromatin as I have defined it above. Although to my knowledge there is as yet no evidence for interphase heteropycnosis of a variegating euchromatic locus in *Drosophila* diploid cells, variegating loci in polytene chromosomes display several features usually attributed to heterochromatic regions. Here, inactivated euchromatin appears compact and unbanded. Variegation may result in under-polytenisation, for example *Sgs4* was replicated to only 66% of the wild type level under strongly variegating conditions (17°C in T(1,4)$w^{m258-21}$: Kornher and Kauffmann 1986). However, normal polytenisation of the variegating gene was found in nuclei of

T(Y;3)A78 larvae in which the proximity of the heterochromatin of the Y chromosome prevented puffing and RNA accumulation from the 87C heat shock locus (Henikoff 1981). Normal levels of polytene replication were also detected in malpighian tubules variegating for *ry* (Rushlow et al. 1984). Under-replication cannot be the cause of variegation in diploid tissues.

The frequency of inactivation of genes on a variegating chromosome decreases with distance from the breakpoint. This polar "spreading effect" is probably initiated at sites at some distance inside the heterochromatin and may extend up to 2.4 Mb (80 chromosome bands). The ability of heterochromatic sequences to initiate formation of an inactive chromatin structure was investigated in revertants generated by re-inverting the In(1)w^{m4} chromosome with X-rays. Tartof et al. (1984) concluded that these revertants could retain some DNA sequences derived from heterochromatin in a position adjacent to the *w* gene without exhibiting variegation. Reuter et al. (1985) demonstrated that there was probably a quantitative dependence on the residual heterochromatin-forming sequences adjacent to the gene of rather than a requirement for specific heterochromatic sites by showing that 48 of 51 such revertants of w^{m4} still variegated in the presence of a dominant extragenic enhancer of position-effect variegation. A component of heterochromatin, possibly satellite DNA, may preferentially nucleate a heterochromatin complex of protein and DNA which would then extend by cooperative binding of protein subunits to the preformed complex until assembly was terminated. Assembly might stop at particular sites, or because the concentration of available subunits had become limiting. We should be able to identify genes encoding such proteins by their effect on the expression of variegating genes. Indeed many dominant suppressors (*Su(Var)*) and enhancers (*E(Var)*) of the variegation of w^{m4} have been mapped. One such modifier, *Su(Var)*205, has been mapped to the *C1A9* locus, which encodes a nonhistone protein component of heterochromatin (James and Elgin 1986, Eissenberg 1989).

Locke et al. (1988) have split into two classes those *Su(Var)* and *E(Var)* mutations whose dominant effect is dependent on the dose of the $Su(Var)^+$ or $E(Var)^+$ gene. Class I genes enhance variegation when duplicated and suppress when deficient. Class II modifiers suppress variegation when duplicated and enhance when deficient. The class I modifiers are likely to be stoichiometrically required structural proteins which bind combinatorially, inactivating stretches of DNA. These authors proposed an intriguing model based on mass action to explain the sensitive dependence of modulation of variegation upon the dosage of these genes. According to this model, the amount of inactivated chromatin formed is proportional to the *n*th power of the concentration of a class I modifier protein, where the inactive complex comprised *n* types of protein monomer.

The different regions of heterochromatin are not equally effective in inducing variegation (Spofford 1976). A set of class I duplications can all enhance variegation of

several different rearranged genes but with different relative efficiencies of enhancement depending on the rearrangement. For these reasons, Locke et al. have put forward two ideas. Firstly that each region of heterochromatin may be composed of a subset of these proteins, some of which may be common to all regions. Secondly, inactivated euchromatin is likely to be a continuation of this structural association of DNA with these proteins established within constitutive heterochromatin. According to this model, class II molecules may be normal components of euchromatin or components limiting the assembly of type I molecules. Formally, these might also be transcription factors in competition with the spreading inactivation mechanism. With probes for such proteins it would be possible to determine whether inactivated euchromatin is, as many authors have implied, organised in the same way as constitutive heterochromatin; or alternatively, whether the proximity of heterochromatin causes the reorganisation of proteins normally bound to euchromatin.

Although inactivation of a variegating gene is normally dependent on the presence of the rearrangement breakpoint *in cis*, Henikoff and Dreesen (1989) have demonstrated inactivation of the bw^+ gene *in trans* with a breakpoint. This *trans*-dominant variegation depends on the synapsis of homologues, an example of transvection (Lewis 1954). The propagation of altered chromatin structure from one homologue to the other has been suggested by Ashburner (1977) to account for synapsis-dependence of polytene chromosome puffing.

Variegation of rearranged genes normally located within heterochromatin may result from the failure to establish normal heterochromatin structure at these loci when these genes are displaced from the heterochromatic environment. In many ways, variegation of heterochromatic genes, such as the eye-colour gene *lt*, resembles the converse of the classic (w^{m4}-type) position-effect variegation. *lt* variegation is enhanced by *Su(Var)* mutations, relatively high temperatures, and the presence of extra heterochromatin (Y-chromosomes), all of which act to suppress variegation of w^{m4}. Two models were considered by Wakimoto and Hearn (1990) to explain why the lt^+ gene in heterochromatin of 2L only variegates when displaced to a position in the 75% of the euchromatic arms most distal to the heterochromatin (Hessler 1958). In their favoured model, the normal heterochromatic structure required for lt^+ expression is formed by propagation of structure between regions of heterochromatin located together in a chromosomal compartment. In terms of the model of Locke et al. (1988), the diffusion of heterochromatin-forming proteins, including those required for transcription of the lt^+ gene might be limited, a chromocentral aggregate would then represent a local intranuclear region with a high steady-state concentration of these factors. The alternative model proposed by Wakimoto and Hearn (1990) would require longitudinal differentiation of the euchromatic arms into a gradient or domains of differing chromatin structure providing environments compatible with the expression of different classes of gene.

The molecular analysis of suppressors and enhancers of position-effect variegation, and structural analysis of proteins required in the assembly of large chromatin domains could potentially illuminate mechanisms controlling programs of gene expression at the level of the whole nucleus. It is clear from clonal inheritance of gene inactivation by position-effect variegation that some structural conformations of chromatin may be stable throughout the cell cycle. It will be especially interesting to determine to what extent the mechanisms causing the structural reorganisation and transcriptional silencing of the genome on entry into mitosis overlap with those determining the patterns of gene expression in interphase nuclei.

This thesis describes the use of direct cytological screening to identify mutations in essential genes required for the structural integrity of mitotic chromosomes. I shall discuss the possible roles of the products of these genes in the processes of chromosome condensation and decondensation which are central to an understanding of the dynamic organisation of the nucleus through the cell cycle.

Chapter 2: Experimental procedures

2.1 Abbreviations

1xSSC: 0.15M Nacl, 0.015M tri-sodium citrate pH7.5.

SDS sodium dodecyl sulphate.

PP1 protein phosphatase type 1

PEG poly-ethylene glycol mwt. 8000

EDTA ethylene diamine tetra-acetate, di-sodium salt.

TE 10mM Tris pH7.5, 1mM EDTA

HGT high gelling temperature

LGT low gelling temperature

TBE Tris borate EDTA electrophoresis buffer

TAE Tris acetate EDTA electrophoresis buffer

PBS phosphate-buffered saline (0.16M NaCl, 50mM sodium phosphate, pH 6.9)

100x Denhardt's solution 2% polyvinylpyrrolidone, 2% bovine serum albumin (Sigma fraction V), 2% ficoll 400, 20mM EDTA.

BSA bovine serum albumin

FCS fetal calf serum

2.2 *Drosophila* culture

Flies were raised on standard *Drosophila* medium in plastic vials (Regina) or 1/3 pint glass milk bottles stoppered with non-absorbent cotton wool to permit ventilation but prevent access by mites to the cultures. No extra humidification was used but trays of vials were covered with Saran wrap. A few grains of "active" dried yeast (BeRo) were added to nourish the parents. Adults were transferred to new media every 12-20 days at 25°C or every 35 days at 18°C.

We made fly food as follows:

200g glucose

80g dried live yeast

4l water 37°C

These ingredients were left at 37°C for 2h while the remaining ingredients boiled:

900g glucose

150g agar (BDH)

1kg ground maize meal

620g killed brewers yeast

10l hot water

The boiled ingredients must be stirred and heated while the agar melts. After 2h boiling the two mixtures were combined and heated to 90°C to kill the live yeast. 38g of methyl-4-hydroxybenzoate was added as an anti-fungal agent. This was dissolved in a minimal volume of ethanol and added to the food in 2l of water. 45ml of propionic acid, another anti-fungal agent, was then added. After mixing, the medium was dispensed hot with a peristaltic pump into vials and allowed to cool. The food keeps well for up to 2 weeks at 18°C wrapped in plastic to prevent dehydration.

2.2.1 Virgin collection

Cultures from which virgins were to be collected were left at 18°C overnight and cleared of flies in the morning. During the day the cultures were incubated at 25°C and cleared of flies after 2h and again after a further 6-8h. Virgin females were recognised by the presence of the meconium, unexpanded wings or soft pale cuticle. Virgins were never scored by time alone, all virgin females used showed physical signs of recent eclosion.

2.2.2 Embryo collection

Embryos were collected at 25°C on plates of grape juice agar daubed with actively growing live yeast to feed the laying females. Grape juice agar was made as follows:

12l water
1173g sugar
105g dry yeast
450ml grape juice
297g agar

After boiling to dissolve the agar, 225ml of a 10% solution of methyl-4-hydroxybenzoate in ethanol and 87.5ml of propionic acid were added and stirred in. This medium can still be used after one year of storage at 4°C. Females were allowed 1h on fresh medium to deposit eggs retained overnight before collections were made. Large collections were made from population cages of wild type flies. Collections on a smaller scale were made using a 1/3 pint milk bottle from which the bottom had been cut and replaced with a removable petri dish of grape juice agar.

2.3 Cytology

2.3.1 Neuroblast squashes

Third instar larvae grown at 25°c on standard *Drosophila* medium were separated from food by floatation on 1.5M NaCl, washed in distilled water and placed in new food for at least 6h at 18°c. Mutants were selected using the larval markers *y, cho, red*, Tb^{+}. All cytological preparation and solutions were at 18°c. Squashes were prepared by

the method of González et al. (1988). Larvae were dissected in 0.7% NaCl with Dumont Inox #5 forceps. Ganglia were dissected away from adhering eye-antenna discs, fixed for 30sec. in 45% glacial acetic acid, stained in 45% acetic acid, 3.5% Gurr synthetic orcein, destained briefly in 45% acetic acid and stained in 60% acetic acid, 3.5% orcein on a coverslip for 30s before squashing. Siliconised coverslips (Chance Propper) and untreated slides were cleaned with a Kimwipe paper towel dipped in ethanol, rinsed in ethanol and blown dry with compressed air (Kenro Photographic). Greater air pressure was obtained by immersing the air can in water at 50°C. Clay Adams Gold Seal slides were found to give the best squashes. Squashes were made by picking up the coverslip with the slide, wrapping both in a fold of soft bibulous paper (Gold Medal Blotting Paper) and pressing firmly for 30s with full body weight on one thumb atop the other. A library embossing press bearing down on a hemispheric centrifuge cushion is a useful substitute if uncial trauma is to be avoided. Squashes were examined within 12h using a Nikon Microphot FX microscope equipped with a Zeiss 63x phase contrast objective. A "field" of nuclei was defined as the area covered by the photographic viewfinder at 63x10 magnification. Metaphases and anaphases per microscope field were recorded, as were the total nuclei per field (mean=146 for wild type). Tests of statistical significance were performed on tables of cell numbers (Chi squared) and on mean figures per microscope field (Student's t). Incubation in 10^{-5}M colchicine in 0.7% NaCl at 18°C was sometimes used to accumulate cells in a metaphase-like state (c-metaphase). In this case, ganglia were incubated in hypotonic 0.5% sodium citrate pH7.0 for 10min to swell the cells before fixation and squashing.

2.3.2 Polytene chromosomes

Propionic orcein was made by refluxing 1% synthetic orcein (Gurr) for 30min in 45% propionic acid. This was mixed 1:1 with lactic acid to make propionic lactic orcein which keeps indefinitely at 4°C. Salivary glands from crawling third instar larvae were dissected in 0.7% NaCl, fixed for 30sec. in 45% glacial acetic acid, squashed in propionic lactic orcein under an unsiliconised 18mm coverslip by tapping with a pencil eraser through bibulous paper. The breakpoints of all deficiency stocks used were confirmed using Df/+ heterozygous chromosomes.

2.3.3 *In situ* hybridisation to polytene chromosomes

This procedure is an amalgam of several standard protocols, the technique has been reviewed by Pardue (1986). Salivary glands were dissected in 0.7% NaCl, stripped of any adherent fatbody, fixed for 30s in 45% glacial acetic acid and squashed in 1:2:3 lactic acid: water: acetic acid (Ashburner 1989b) on slides coated with Denhardt's solution. Glands were squashed first by outward spiral tapping over the coverslip with a mounted needle, then by wrapping the slide in bibulous paper and pounding over the chromosomes with the blunt end of a Bic ballpoint pen encased in a rubber pipette

teat. Squashes were often left overnight at 4°C to flatten by evaporation. They were then frozen in liquid nitrogen, the coverslip was lifted with a scalpel blade and the chromosomes allowed to thaw and desiccate for 10min. in absolute ethanol with one change of ethanol. Slides were heated to 65°C in 2xSSC for 30min. to preserve chromosome morphology (probably by inactivating residual proteases and nucleases), and allowed to cool before denaturation of the DNA in 70mM NaOH for 3min. Slides were washed in 2x5min. changes of 70% ethanol and dehydrated in 2x5min. changes of absolute ethanol. Slides were frequently stored dry for up to a year at 4°C before denaturation.

In situ hybridisation with tritiated probes.

Probe DNA was prepared by TAE agarose gel electrophoresis of restriction fragments. The gel was dissolved in saturated NaI. The DNA was purified by adsorbtion to glass (Vogelstein and Gillespie 1979). We use the commercial preparation Gene Clean (Bio 101). DNA was labelled to $2x10^7$ dpm/μg using 5μCi each of [^{3}H]dCTP and [^{3}H]TTP (ICN), and random oligomer-primed synthesis (Feinberg and Vogelstein 1983). Probe DNA was purified on a Sephadex G50 column and denatured by boiling for 10min. Hybridisation took place under a 24x24mm coverslip during 16h at 58°C in 25μl of a solution of 50ng/ml probe DNA, 0.2mg/ml salmon sperm DNA, 4xSSC, 1x Denhardt's solution, 10% dextran sulphate. Slides were washed at 53°C in 2xSSC and desiccated in ethanol before being coated in 50% Ilford L4 emulsion. After exposure for 2-3 days at 4°C in a box of dry silica gel the slides were developed for 3min in Kodak D19 developer, and stopped in 1% acetic acid. Emulsion was hardened for 1min in 60mM chromium potassium sulphate, 0.42M sodium sulphate and fixed for 5min in a 1/4 dilution of Ilford Ilfospeed fix. After 10min washing, the slides were counterstained with 5% Giemsa's stain (Gurr) in 10mM sodium phosphate pH6.8. Slides were photographed on a Nikon Microphot FX microscope with a Zeiss 63x phase contrast objective and a green filter, Kodak 2415 Technical Pan film and HC110 developer.

In situ hybridisation with biotin-labelled probes.

This procedure was found to be simpler and faster than the tritium method. Probes were prepared by random oligomer-primed labelling with 20μM biotin-16-dUTP (Boehringer) in place of TTP (Ashburner (1989 and pers. comm.)). Probe DNA was purified and hybridised as detailed above. After 3 20min. washes in 2xSSC at 53°C, slides were washed twice for 5min. in PBS, once for 2min. in PBS with 0.1% Triton X-100, and then rinsed in PBS. To detect the probe, 100μl/slide of a 1/250 dilution of streptavidin-horseradish peroxidase (Enzo) in PBS was applied for 30min. at 37°C in a humid chamber. During this time, the chromosomes were covered with 22x40mm coverslips. Coverslips were removed in a beaker of PBS. Slides were washed again in PBS and PBS-Triton as detailed above. The chromogenic peroxidase substrate 3,3-diaminobenzidine (DAB) was then applied at 0.5mg/ml in PBS with 0.01% H_2O_2

and left, covered with a 22x40mm coverslip for 10-30min. Slides were then rinsed in water and counterstained with Giemsa's stain for 2min. as detailed above. When dry, the chromosomes were mounted in DPX mountant and sealed with a siliconised 24mm coverslip. The residual, carcinogenic DAB solution was routinely mixed with Chloros bleach for 1h before disposal.

2.3.4 Indirect immunofluorescence of whole embryos

This protocol is derived from that of Frasch et al. (1986). Embryos were collected and washed by gently brushing them off the yeasted grape agar plate in water with 0.03% Triton X-100 into a suction funnel equipped with a Nitex nylon gauze filter. The embryos were dechorionated in 3:1 bleach:water for 3min. with occasional stirring, then washed well in water with Triton. Embryos were then transferred to 0.9ml of Buffer B (10mM K phosphate buffer (1M K_2HPO_4, 1M KH_2PO_4) 45mM KCl, 15mM NaCl, 2mM $MgCl_2$) within 9ml of n-heptane in a glass screw-top vial. 5μM taxol was added at this time to preserve microtubular structure. 30sec. after addition of taxol, 0.1ml of 37% formaldehyde and 10μl of methanol were added and the embryos fixed for 5-10 min. with continuous shaking. The vitelline membranes were removed by the repeated movement of the embryos through the solvent interfaces, without them, the embryos rapidly sink. The solvents were removed by aspiration and replaced with 10ml of methanol. After 2x5min. washes in Buffer A (15mM Tris pH 7.5, 60mM KCl, 15mM NaCl, 1.5mM spermine, 0.5mM spermidine) with 0.1% Triton, the embryos were incubated for 30min. in the same buffer supplemented with 10% fetal calf serum (FCS). To reveal microtubules and centrosomes, embryos were incubated for 12-16h at 4°c in a 1/5 dilution, in Buffer A with Triton and FCS, of rat anti-tubulin antibody YL1/2 (Sera-Lab) and a 1/500 dilution of the antibody Rb188, directed against a centrosomally-located antigen (Whitfield et al. 1988). Embryos were given two 15 min. washes in Buffer A with Triton. They were then incubated in a 1/200 dilution of fluorescein-conjugated goat anti-rat and a 1/500 dilution of rhodamine-conjugated goat anti-rabbit antibodies (Jackson) for 2h at 25°C or 12-16h at 4°C. Embryos were washed once for 15min. in Buffer A with Triton and then for 15min. in Buffer A alone. It is important to remove the Triton because it precipitates in the mounting medium. To visualise chromatin, the DNA-binding fluorochrome Hoechst 33258 was added at 0.5μg/ml and allowed to stain for 5min. before the embryos were mounted in 85% glycerol containing 2.5% n-propylgallate (to retard photobleaching) beneath a siliconised coverslip. Slides were viewed on a Nikon Microphot FX microscope using epifluorescence illumination and filters specific for the emissions of the three fluorochromes used. Micrographs were recorded on Kodak 2415 technical Pan film developed in D-19 high contrast developer.

2.3.5 Indirect immunofluorescence of whole ganglia

Ganglia were dissected in 0.7% NaCl, fixed in 3.7% formaldehyde in PBS (0.16M NaCl, 50mM sodium phosphate, pH 6.9) for 1h in the presence of either 5μM taxol or 1mM GTP (González et al. in prep.). After blocking for 1h in 10% fetal calf serum, 0.3% Triton X100 in PBS, ganglia were incubated for 12-16h at 4°c in a 1/5 dilution of rat anti-tubulin antibody YL1/2 (Sera-Lab), and given two 15 min. washes in PBS, 0.3% Triton X100. They were then incubated in a 10^{-2} dilution of fluorescein-conjugated goat anti-rat antibody (Jackson) for 2h at 25°C or 12-16h at 4°C.

After 2x15min. washes in PBS containing 0.3% Triton, ganglia were stained for 5min. in 1μg/ml propidium iodide in PBS and mounted in 85% glycerol, with 2.5% n-propylgallate to retard photobleaching, on slides coated with 1μg/ml poly L-lysine (to promote adhesion of the specimen). A siliconised coverslip was sealed in place with nail varnish. The inclusion of 2.5μg/ml boiled RNase A in the first antibody incubation was found to reduce the cytoplasmic staining by propidium iodide and improve visualisation of the chromatin. No differences in spindle morphology were noted between neuroblasts fixed in the presence of taxol or GTP or if 0.7% NaCl was substituted for PBS in the fixation step. Spindles were only rarely preserved in ganglia fixed in the absence of taxol or GTP. Tubulin and chromatin were visualised simultaneously using the split video display of an MRC 500 confocal microscope. 3-5 serial optical sections were recorded differing in focal depth by 1μm. Color images were permanently recorded using a Sony video printer.

2.4 P element mediated germline transformation of flies

w^{1118} embryos were collected for 40min, then manually dechorionated and placed on a line of Sellotape^{TM} glue applied in n-heptane to a coverslip. Embryos were desiccated for 7min. over dry silica gel and then covered in Voltalef 10S halocarbon oil. Transformation was accomplished as described by Karess (1985). The P*w*8 constructs were injected at an initial concentration of 200μg/ml just inside the posterior pole of the embryo. Transposase was provided *in trans* by defective helper element phsΔ2-3 (kind gift of Dr Andrew Tomlinson, Dept. of Zoology, University of Cambridge) at 400μg/ml. Coverslips were placed on grape agar plates for 48h and hatching larvae removed to food vials in batches of 20. Approximately 25% of embryos survived injection, 30% of these emerged as adults, 75% were fertile of which 5% (0.3% of injected embryos) gave transformed progeny bearing the w^{+} eye color marker. 9 independent transformed limes were isolated containing the wild type *PP1 87B* construct p*w*813, and 6 lines with the disrupted construct pAM12

G_0 (injected) flies and their transformed G_1 progeny were crossed to w^{1118} G_2 and G_3 flies were crossed *inter se* to generate lines homozygous for the insertions. *In*

situ hybridisation with vector probe was used to determine insertion sites. Lines with a single euchromatic insertion site were chosen for use in rescue experiments.

2.5 DNA preparation from flies

DNA was prepared by a modification of the protocol of McGinnis and Beckendorf (1983). 0.5-1 Eppendorf microcentrifuge tubes full (about 0.5-1g) of flies were homogenised in 0.5ml of 100mM NaCl, 30mM Tris pH8, 10mM EDTA, 0.5% Triton X-100, 8mM 2-mercaptoethanol in a 5ml Dounce homogeniser with a motor-driven teflon pestle. Fresh flies or recently-thawed flies stored at -70°C may be used. The homogenate was transferred to a 1.5ml Eppendorf microcentrifuge tube and the homogeniser was cleaned with a further 0.5-1ml of homogenisation buffer which was then used to fill the Eppendorf tube. Nuclei were pelleted in the microfuge for 1min and the pellet resuspended in 0.5μm of 100mM Tris pH 8.4, 100mM NaCl, 20mM EDTA, respun and resuspended in 0.3ml of the same buffer. 30μl of 10% sodium lauryl sarcosine and 30μl 1mg/ml proteinase K was added and the solution incubated at 45°C for 1h. Proteins were twice extracted with 600μl of 1:1 phenol:$CHCl_3$ and once with 600μl of chloroform. The aqueous layer was washed twice with 1ml of diethyl ether and DNA precipitated with 0.3M sodium acetate and 2 volumes of ethanol followed by centrifugation. The pellet was resuspended in 200μl TE, and an equal volume of 7.5% ammonium acetate. DNA was then re-precipitated with 1ml of ethanol, washed in 70% ethanol and dried by vacuum desiccation. DNA dissolved well in TE if heated to 60°C for 1h and then left overnight with occasional agitation. 200μl was a suitable quantity of buffer for the DNA extracted from half an eppendorf tube of flies. 50μl was suitable for the DNA from 25 flies. I have always measured the OD_{260} of a 1/100 dilution of the DNA and have found this to be a consistent measure of DNA quantity. Little RNA is evident on agarose gels of DNA prepared in this way. 2μg of genomic DNA was usually used in a 20μl restriction enzyme digest to be run on one lane of an agarose gel.

2.5.1 Preparation of neutral phenol

Phenol used in purification of DNA from contaminating protein was purchased in a redistilled water-saturated liquid form (Rathburn). To neutralise this with minimal loss 75ml of water-saturated phenol was mixed with an equal volume of TE in a tap funnel. Approximately 300μl of 10M NaOH was added until the aqueous phase was pH7.5. The phenol phase was run off and stored for up to 6 months under a layer of fresh TE. The total wastage of phenol by this method is about 5% which compares favourably with the 50% or so lost by neutralising phenol with an equal volume of 1M Tris base (Maniatis et al. 1982). An additional disadvantage of using 1M Tris base is that the aqueous and phenolic phases separate more slowly.

2.5.2 Genomic DNA from fly stocks

In order to confirm the P or M status of stocks when working with mutations induced by hybrid dysgenesis I prepared genomic DNA from several stocks. (M) denotes that the stock was free from P elements as judged by DNA blotting. (P) means that P elements were detected in the stock but does not necessarily imply the presence of active elements. Genomic DNA from recombinant chromosomes was also used in Southern blotting experiments in an attempt to correlate the presence or absence of particular P elements with presence or absence of the mutation under investigation. Genomic DNA from *ck19* mutations was used to detect the mutations in *PP1 87B* described in Chapter 6. Genomic DNA from *l(1)d.deg4*, *g* and *mus101* stocks was investigated with probes from the 12BC walks for the presence of polymorphisms specific for these mutations.

DNA from fly strains	mg/ml
Oregon R (Oxford)(M)	1.1
Canton S (M)	1
$\pi 2$ (P)	1
Basc; bw^D/SM1; *Sb*/TM2*Ubx* (H41 (RM))	1
X chromosome	
P[ry^+]SRS3.9-2(12E)	0.593
P[ry^+]R301.2(12C8-D1)	0.638
Df(1)HA92 *y w*/FM7c (M)	0.800
Df(1)g^1 *f B*/Dp(1;f)LJ9	0.325
Df(1)w^{LCD}/FM7c	1.1
T(1;2)g^{R1}/FM7c	0.495
In(1)g^w	0.525
g^{S2}	1.3
g^{S3}	0.800
T(1;Y)B166/Y	0.750
DfBasc/FM6*ml(1)*/sc^8Y (M)	0.190
FM7c (M)	0.850
FM7	0.500
FM6/In(1)N^{262-84} (M)	1
C(1)DX *y f* (M)	0.220
C(1)DX *y f*/sc^8Y/Dp(1;f)LJ9	0.600
C(1)DX *y f*; ry^{506} (M)	1
y w ct^6 *m f* (M)	0.240
y sn^3 *v* (M)	0.365
In(1)z^+64b9	0.950
mus(1)101 stocks	

mus101^{A1}/*sc*^{8}Y	0.525
mus101^{D2}/*sc*^{8}Y	0.193
fs(1)K451/Y	0.278
mus101^{SM}/*y*$^{+}$ g^{+} *na*$^{+}$ Y	0.850
mus(1)101^{lcd} stocks (P)	
ychomus101^{lcd} *f*/DFB	0.950
y cho mus101^{lcd} *f*/*sc*^{8}Y/Dp(1;f)LJ9	0.280
y w ct^{6} *mus101*^{lcd} *f*(55AB)/FM6	0.100
y w ct^{6} *mus101*^{lcd} *f*(55AE)/FM6	0.100
y w ct^{6} *mus101*^{lcd} *f*(55AF)/FM6	0.100
y w ct^{6} *mus101*^{lcd}(55ABD)/FM6	0.700
y w ct^{6} *mus101*^{lcd}(55ABF)/FM6	0.325
y w ct^{6} *mus101*^{lcd}(55ABJ)/FM6	0.640
y w ct^{6} *mus101*^{lcd}(55ABL)/FM6	0.210
y w ct^{6} *mus101*^{lcd}(55ABT)/FM6	0.335
m mus101^{+} *f*/FM6(55rec1)	0.100
y w ct^{6} *mus101*$^{+}$ *f*/FM6(55recõ)	0.100
l(1)d.deg4 stocks	
l(1)d.deg4/FM7	0.950
l(1)d.deg4^{0820}/*y*$^{+}$ g^{+} *na*$^{+}$ Y	0.500
Chromosome 2	
C(2)EN	0.170
Sco/CyO; *ry*^{506}	0.445
thr$^{BH9.8}$/CyO	1
thr$^{BH9.9}$/CyO	0.290
Chromosome 3	
TM1/TM3 (M)	1
TM3/TM6B (P)	0.585
TM3/TM6B (M)	1
ru h th st cu sr e ca/TM6B (M)	0.160
ru h th st cu sr e^{+} *ca* (M)	0.300
ru h th st cu sr Pri e ca/TM6*Ubx* (M)	0. 480
mwh red e/TM3 *ry*^{RK} *Sb e* (M)	0.350
ry^{506} *e*/TM3 *ry*^{REK} *Ser ri e* (M)	0.370
red e (M)	0.200
P[*ry*$^{+}$]Δ2-3 99B (P)	1
l(3)snap stocks (P)	
revertant r40	0.400

r40/TM6B	1
R7.1/TM3*Ser*	1
R10.1/TM3*Ser*	1
R7.4A/TM3*Ser* P-clean	1
R7.4A P-clean recombinants (P)	
ru h th st cu l(3)snap^{+}	
4.5v/TM1	1
4.1v/TM1	1
4.6v/TM1	0.515
4.7v/TM3	1
ru h th st l(3)snap	
5.11/TM3	1
l(3)snap sr ca	
12.21/TM3	1
12.61/TM3	1
12.71/TM3	1
Stocks used to study *PP1 87B*	
e280/TM3*Sb Ser*	0.370
e180/TM3*Sb Ser*	0.545
ck19^{e078}/TM3*Sb Ser*	0.435
ck19^{e211}/TM3*Sb Ser*	0.635
ck19^{e211}/TM6B	0.400
ck19^{hs46}/TM6B	0.620
ck18^{e312}/TM3*Sb Ser*	0.535
ck18^{e290}/TM3*Sb Ser*	0.290
Df(3R)E-079/TM3*Sb Ser*	0.400
Df(3R)Kar^{3Q}/TM6B	0.210
Df(3R)Kar^{3Q}/TM3*Sb Ser*	0.185
Df(3R)Kar^{Sze-12}/TM3*Sb Ser*	0.650
Df(3R)E-079/*ck19*^{hs46} (from larvae)	0.400
Df(3R)E-079/*ck19*^{e211} (from larvae)	1
Df(3R)E-079/*ck19*^{e078} (from larvae)	0.615

2.6 DNA electrophoresis and DNA (Southern) Blotting

2.6.1 Restriction enzyme digestion and gel electophoresis

Restriction enzymes were usually incubated for 2h at 37°C with 0.1-10μg of DNA in 20-100μl volumes of 50mM Tris-HCl pH 7.5, 100mM NaCl, 10mM $MgCl_2$ 1mM dithioerythritol, 0.1mg/ml BSA (high salt buffer). Many enzymes required the use of 10mM Tris-HCl pH 7.5, 50mM NaCl, 10mM $MgCl_2$ 1mM dithioerythritol, 0.1mg/ml BSA (medium salt buffer) or 10mM Tris-HCl pH 7.5, 10mM $MgCl_2$ 1mM

dithioerythritol, 0.1mg/ml BSA (low salt buffer). Digestion with more than one enzyme was achieved by sequential incubation, starting with the one requiring the lowest salt concentration and subsequently adjusting the NaCl concentration to suit the next enzyme. 1-5U of enzyme per μg of DNA was usually sufficient to achieve complete cleavage. Because restriction enzymes were supplied in 50% glycerol to permit storage at -20°C, care was taken to limit the volume of enzyme added to 10% of the reaction volume because concentrations of glycerol greater than 5% often inhibit digestion.

Digestion was arrested by adding 1/5 volume of 6x concentrated loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 40% sucrose, 5mM EDTA). Samples were then loaded and run on a 20x25x0.5cm horizontal, submerged gel of 0.3-2% HGT agarose (Miles SeaKem) with 0.5μg/ml ethidium bromide buffered with TBE (45mM Tris-borate pH8, 1mM EDTA) at about 1-5V/cm for 4-20h. DNA size standards run on each gel were obtained by digestion of lambda cI^{857} DNA with *Hind*III. The 1-12kb molecular size markers obtained from BRL were also found to be useful. DNA was visualised by the fluorescence of the ethidium bromide-DNA complex lit by a 365nm UV transilluminator. Gels were recorded on Polaroid 665 film using an orange filter. Negatives were printed to life size, this was made easier by including a clear plastic ruler alongside the gel. A backing of autoclave tape on this ruler provided sufficient fluorescence under UV light for photography.

2.6.2 Labelling probe DNA by randomly primed synthesis

Probe DNA was prepared by TAE agarose gel electrophoresis of restriction fragments. TAE buffer was 40mM Tris-acetate pH8, 1mM EDTA. 0.5μg/ml ethidium bromide was used to visualise the DNA. The gel was dissolved in saturated NaI. The DNA was purified by adsorbtion to ground glass and elution into TE (Vogelstein and Gillespie 1979). We use the commercial preparation Gene Clean (Bio 101). DNA was labelled with [α-^{32}P]dCTP to about $9x10^8$ dpm/μg using random oligomer-primed synthesis (Feinberg and Vogelstein 1983). A 50μl reaction volume comprised 50mM Tris pH8, 5mM $MgCl_2$, 400μM each of (dATP, TTP, dGTP), 50μCi of [α-^{32}P]dCTP (>3000Ci/mmol Amersham), 1mM 2-mercaptoethanol, 0.2M HEPES (initially pH6.6 with 4M NaOH), 0.2mg/ml random hexanucleotides (Pharmacia)) 100ng denatured template DNA, 1mg/ml BSA, 5U Klenow fragment of DNA polymerase I from *E. coli*. 80% incorporation of [α-^{32}P]dCTP into DNA was routinely obtained after incubation for 2h at 37°C.

2.6.3 Measuring incorporation by CTAB precipitation

To measure the incorporation of radiolabelled nucleotides into DNA, I used the cationic detergent cetyl trimethyl ammonium bromide (CTAB) to precipitate DNA from the labelling reaction. 1μl of labelling reaction was mixed with 500μl 2xSSC, 250μl of 1M sodium acetate pH5, 0.5 mg yeast RNA, 250μl of 4% CTAB. The mixture

was chilled on ice for 5min. and the precipitate collected on a Whatman GF/C glass fibre filter, washed with water to remove unincorporated nucleotides, dried and counted in a scintillation counter. A further 1μl of the labelling reaction was spotted onto a dry filter to count the total amount of radioactivity present. ^{32}P incorporation could be measured without scintillation fluid, by counting Cerenkov radiation.

2.6.4 Separation of unincorporated nucleotides

DNA probes were purified from unincorporated nucleotides by gel filtration on a Sephadex G50 column poured in a 1ml syringe. The column was first spun at 1500rpm within a disposable 50ml Falcon tube in the Damon Centra 3 benchtop centrifuge for 5min. A fresh microcentrifuge tube was placed below the column to collect the flow-through volume. The reaction was loaded onto the dry column and a further 50μl of TE added. The column was then spun for a further 5min at 1500rpm. Unincorporated nucleotides remain bound in the gel matrix, higher molecular weight DNA is excluded and flows through. With ^{32}P probes, the approximate incorporation may be estimated by comparing the relative radiation emitted by column and effluent with a Geiger-Muller monitor.

2.6.5 Hybridisation

Denaturation buffer pH10-13

1.5M NaCl	1577.7g
0.5M NaOH	360g
	18l

Neutralisation buffer pH 7-8

1M NH_4Acetate	1386g
20mM NaOH	14.4g
	18l

To blot restriction enzyme digests of cloned DNA, the agarose gel was left for 30min. in denaturation buffer to ensure separation of complementary DNA strands, then neutralised for 2x30min. The gel was then inverted onto a glass plate and nylon-reinforced nitrocellulose membrane (e.g. Hybond-C Extra, Amersham International) was placed onto the gel followed by 3 sheets of Whatman 3MM chromatographic paper soaked in neutralisation buffer and rolled with a pipette to remove air bubbles. A 2-4cm stack of absorbent paper (postslip paper, Gold Medal blotting paper or Kimwipe) was then laid on top of this topped with a glass plate and 4 lead pigs (too much weight leads to smeared or double bands). Transfer takes at least 1h, longer if the gel is >0.5% agarose. To blot digests of genomic DNA with maximal efficiency, it was additionally necessary to depurinate the DNA in the gel in 0.25M HCl (1/50 neat) for 15min. prior

to denaturation. Transfer of these smaller quantities of DNA, was achieved by capillary (Southern) blotting in 20x SSC for 4-16h using a 5-10cm stack of absorbent paper.

The position of the sample wells was marked with ballpoint pen (which fades during hybridisation) or pencil (which doesn't fade but does absorb probe). The filter was then rinsed in 2xSSC and baked for 1-2h at 80°C in a drying oven. The filter may be stored indefinitely at room temperature after this treatment.

Hybridisation

The filter was then rehydrated in 2xSSC and incubated for 15min-1h at 65°C in a 25-40ml volume of 0.5M $NaHPO_4$ pH 7.2, 7% SDS (Sigma MB grade), 1mM EDTA. 1M $NaHPO_4$ pH 7.2 was made by dissolving 134g $Na_2HPO_4.7H2O$, 4ml 85% H_3PO_4 in a final volume of 1l (Church and Gilbert 1984). This is 0.99M Na^+ or equivalent to 5x SSC for purposes of calculating stringency of hybridisation.

Radiolabelled ^{32}P DNA probes were denatured by boiling for 10min. and then placed on ice. When cool, the probe was added to the hybridisation fluid. Hybridisation took place over 8-16h at 65°C in a shaking water bath. Probes in 0.25M $NaHPO_4$ pH 6.5, 7% SDS, 50% deionised formamide (eg. RNA probes or DNA probes previously used for RNA blots) were used on DNA blots at 42°C after treatment ("pre-hybridisation") of the blot with fresh 50% formamide mix. Blots were washed after hybridisation in 0.2x SSC, 0.5% SDS (cheap grade) for 2x15min. at 60°C, wrapped in Saran wrap and exposed to Fuji RX X-ray film at -70°C with DuPont Cronex Quanta 3 intensifying screens. Filters were sometimes re-used as many as four times as the probe could be stripped off by boiling the filter for 2x10min. in wash buffer.

2.6.6 Synthesis of end-specific RNA probes from cloned DNA.

DNA inserted into the lambda Dash vector is flanked by the promoters specific for the RNA polymerases from T3 and T7 bacteriophages permitting synthesis of end-specific radiolabelled RNA probes. These are of use in orienting clones in a chromosome walk without the need for restriction mapping or subcloning. When a library is probed with transcripts from both ends of a clone, new clones may be isolated which only overlap the original clone at one end. The standard transcription reaction was incubated for 30min. at 37°C and contained 25μl of 40mM Tris-HCl pH7.5, 6mM $MgCl_2$, 2mM spermidine HCl, 5mM NaCl, 10mM dithiothreitol, 100μg/ml BSA, 1μg lambda Dash DNA, 500μM each of rATP, rCTP, rGTP, 20μCi of [α^{32}P]UTP (>3000Ci/mmol; 10μCi/μl), 1U/μl placental RNAase inhibitor (RNAse Block, Stratagene), 10U T3 or T7 polymerase. No unlabelled UTP was added, in order to limit the length of transcripts. Transcription resulted in 25-50% incorporation but unincorporated nucleotides were not found to create significant background problems with the hybridisation conditions used. Consequently, probes were only rarely purified on Sephadex G50 spun columns before use. Probes were hybridised to Southern blots and library filters in the same buffer used for hybridisation of RNA blots with DNA

probes and washed using similar conditions (see below). A filter with a sample of the clone used as a template for transcription was included in each hybridisation as a positive control for hybridisation.

2.7 RNA preparation from flies and RNA blotting

1-5g of *Drosophila* frozen at -70°C were thawed at room temperature in 5ml REB lysis buffer (8M urea, 0.35M NaCl, 10mM Tris pH 8.3, 10mM EDTA). An equal volume of 90% neutral phenol, 10% chloroform, previously equilibrated with REB was then added. The tissue was disrupted using a Polytron homogeniser. The homogenate was vortexed with 1% sarkosyl (sodium dodecyl sarcosinate) and the phases were separated by centrifugation. Extraction was repeated twice more with phenol/chloroform then thrice with chloroform alone. RNA was precipitated from the aqueous phase with 0.3M sodium acetate pH5.5 and 3 volumes of ethanol, then resuspended in distilled water.

RNA was briefly heated to 65°C then bound to a 1ml oligo-dT cellulose (BRL) column (Aviv and Leder, 1972) in a 5ml syringe in 10mM Tris pH 7.5, 0.4M Nacl, 1mM EDTA, 0.5% SDS at 22°C (SDS precipitates at lower ambient temperatures). The column was washed with an excess of this buffer, the RNA eluted in 2ml 10mM Tris pH 7.5, 1mM EDTA, 0.05% SDS. Polyadenylated RNA was precipitated with sodium acetate and ethanol and redissolved in distilled water. Aliquots of such RNA were routinely stored frozen in water for up to 2 weeks or up to a 2 years as ethanol precipitates at -20°C. 5g of tissue yielded approximately 15mg total RNA which yields about 500μg poly A^+ RNA after one cycle of enrichment on the oligo-dT column.

5μg of poly A^+ RNA (or 0.5μg of 1-12kb double stranded DNA molecular size markers (BRL)) was denatured for 1h at 50°C in 7.5% deionised glyoxal, 50% DMSO, 10mM $NaHPO_4$ pH 6.5 and loaded in the presence of 10% sucrose, 0.01% bromophenol blue, 0.01% xylene cyanol FF onto a 1% agarose gel in 10mM $NaHPO_4$ pH6.5 (McMaster and Carmichael 1977, Thomas 1980). The gel was run at 5V/cm with vigorous continuous buffer recirculation. RNA was transferred to Hybond N (Amersham) by capillary blotting for 10-20h in 20x SSC. The membrane was irradiated 5min at 1200$\mu W/cm^2$ with UV light at 254nm to fix the nucleic acid, baked 1h at 80°C, then boiled 10min in 0.2x SSC, 0.5% SDS to ensure reversal of the glyoxalation.

Hybridisation occurred in 0.25M $NaHPO_4$ pH 6.5, 7% SDS, 50% deionised formamide at 48°C for 12-16h. The Na^+ concentration of this modified buffer is approximately equivalent to 3x SSC. Filters were incubated in this solution for 1h prior to addition of the denatured, [α-^{32}P]-labelled probe (see above) Filters were washed in 0.2x SSC, 0.5% SDS, 15min at room temperature, 2x15min at 60°C, wrapped wet in Saran Wrap for autoradiography. Filters may be reused as many as ten times, we strip off the probe by boiling for 2x10min in wash buffer.

2.8 *E. coli* as a host for cloning *Drosophila* DNA

Strains

The following genotypes and the original references are all listed by Sambrook et al. (1989) with the exception of NM514 which is from Murray (1983).

BHB2688: N205 *rec*A$^-$ (lambda *imm*34 cI^{ts} *b*2 *red*3 *E*am4 *S*am7)

BHB2690: N205 *rec*A$^-$ (lambda *imm*34 cI^{ts} *b*2 *red*3 *D*am15 *S*am7)

C600: *sup*E44 *hsd*R *thi*-1 *thr*-1 *leu*B6 *lac*Y1 *ton*A21

DH5: *sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi-1 rel*A1

NM514: *hsd*R *lyc*7 (rk^- mk^+)

Q358: *sup*E *hsd*R $\phi 80^r$

Q359: *sup*E *hsd*R $\phi 80^r$ P2

TG2: *sup*E *hsd*Δ5 *thi* Δ(*lac-pro*AB) delta(*srl-rec*A)306::Tn10(tet^R)

XL1-Blue: *end*A1 *hsd*R17 (rk^- mk^+) *sup*E44 *thi*-1 lambda$^-$*rec*A1 *gyr*A96 *rel*A1 (*lac*$^-$) [F'*pro*AB *lac*^{q}ZΔM15 Tn10 (tet^R)]

2.8.1 Preparation of microbiological media

SM phage buffer is 100mM NaCl, 50mM Tris pH 7.5, 10mM $MgSO_4$,0.01% gelatin. LB broth is 5% NaCl, 5% yeast extract (Difco), 10% tryptone (Difco), for work involving phage lambda, this medium was supplemented with 10mM $MgSO_4$ and is referred to here as LBM. Plates were made with LB or LBM and 1.25% Difco agar, top agar was LBM with 0.7% HGT agarose. To make media for antibiotic selection, ampicillin was used at 100μg/ml in LB plates and broth, tetracycline at 20μg/ml.

2.8.2 Preparation of phage by caesium chloride density centrifugation

Plaque-purified phage were grown to confluent lysis ($2x10^4$ p.f.u./plate), the plates overlaid with 5ml of SM and agitated for 1h. The supernatant contained 10^8-10^{10} p.f.u./ml. This was used to inoculate bulk cultures. We have found the most reliable way to achieve lysis of the culture and a high yield of phage was to mix phage and cells at a measure of infection of 0.01-0.05 p.f.u./cell before co-inoculation. In practice 100μl of plate lysate of a poorly-growing phage such as EMBL4 or lambda-DASH, or 1μl of vigorous phage such as gt10 or NM1149 was added to 2ml of an overnight culture or 200μl of plating cells. After 20min. adsorbtion at 37°C, the inoculum was added to 250ml of pre-warmed LBM broth in a 500ml conical flask and grown with powerful agitation to lysis, 5-16h. 0.5ml of $CHCl_3$ and 15g of NaCl were added for an additional 5min. with gentle agitation. Crude RNAase and DNAase were also added, to a final concentration of 10μg/ml. When the salt had dissolved, the cell debris was pelleted at 8krpm for 20min in the Sorvall GS3 rotor and the flasks rinsed with distilled water. Supernatants were mixed with 40ml of 50% PEG (7-8% final concentration) and left in the flasks for 1-16h at 4°C to precipitate the phage particles.

The precipitate was harvested at 8krpm for 30min. in the GS3 rotor, the supernatant discarded and pellet resuspended in 10ml SM using an air-driven pipetting aid and 10ml glass pipette. I found that larger yields of phage were obtained with careful resuspension and it may be advisable to leave the resuspended phage for an hour or so before proceeding if the yield is critical. The PEG was removed by extraction with an equal volume of $CHCl_3$ and centrifugation. 7.6g of caesium chloride was dissolved in 10ml of the aqueous supernatant. The phage were then banded by equilibrium density gradient centrifugation in the Beckman 50Ti rotor at 45krpm for 16h. Greyish bands of phage could be seen in bright light and were extracted in a volume of 1ml through a 19G hypodermic needle into a 2ml syringe. 0.5ml was reserved and 0.5ml used to make phage DNA. To do this, phage particles in CsCl were dialysed against 4l of 10mM NaCl, 50mM Tris pH7.5, 10mM $MgSo_4$ for 1h. 10μl of 1mg/ml proteinase K, 40μl 0.25M EDTA and 25μl 10% SDS were added (final concentrations 20μg/ml proteinase K, 20mM EDTA and 0.5% SDS) and the solution left at 60°C for 30min. to lyse the phage particles. Protein was extracted twice with 1:1 phenol:$CHCl_3$, the aqueous layer was washed twice with diethyl ether, and the DNA precipitated with 0.3M sodium acetate and 2 volumes of ethanol. After a wash in 70% ethanol, the DNA pellet was dried and redissolved overnight in 200-500μl of TE.

2.8.3 Small scale preparation of phage DNA from confluently-lysed plates

This method, which has its origins in the Amersham International company, was used to prepare DNA from large numbers of lambda clones picked from libraries of microdissected DNA. The principle is to use the affinity of DEAE52 cellulose for RNA and DNA to deplete a plate lysate of contaminating *E. coli* nucleic acids. It was first necessary to prepare a slurry by mixing 100g of DEAE52 cellulose (Whatman) with several volumes of 50mM HCl. When the pH was below 4.5, the suspension was neutralised to pH7.5 with 10M NaOH. The solid was washed twice with changes of L-broth with 10mM $MgSO_4$ and resuspended in L-broth, 10mM $MgSO_4$, 0.1% sodium azide. 5ml of SM phage buffer was left on each confluent plate of phage for 1h at room temperature. 4ml of supernatant was recovered and a drop of chloroform added. It was found to be important at this stage to clarify the suspension at 3krpm in a bench-top centrifuge (Centra 3). 4ml of 20% PEG, 2.5M NaCl was then added to the cleared supernatant and incubated on ice for 1h. Phage aggregated by the PEG were pelleted at 3krpm for 20min. The pellet was re-suspended in 0.5ml L-broth with 10mM $MgSO_4$ and 0.75ml of a slurry of approximately 75% (v/v) DEAE52 cellulose in the same broth was added and mixed by vortex. The DEAE52 was pelleted after 5min. and the supernatant, which contained phage particles but little *E. coli* RNA or genomic DNA, was removed to a fresh tube. In order to lyse the phage capsids 32μl of 10% SDS, 30μl 0.5M EDTA, 13μl 0.1mgml^{-1} proteinase K were added to 0.75ml of supernatant, mixed and incubated for 10min. at room temperature. 130μl of potassium

acetate was added to precipitate SDS-protein complexes; residual DNAases were inactivated by heating the mixture to 85-90°C for 20min. followed by 10min. on ice. The supernatant was separated by centrifugation and removed to a fresh tube, then precipitated with an equal volume of isopropanol and 10min. incubation at -20°C. After washing in absolute ethanol, the pellet was dried and resuspended in 20μl of TE.

2.8.4 Ligation and transformation

Ligations into plasmid vectors

5μg of the plasmid vector pBs^+ (Bluescribe $M13^+$, Stratagene) was cleaved with the appropriate restriction enzyme. DNA was extracted with phenol and precipitated with ethanol after restriction enzyme digestion, resuspended in sterile distilled water and dephosphorylated with 1U calf intestinal alkaline phosphatase (CIP) in 10mM Tris pH8.3, 1mM $ZnCl_2$, 1mM $MgCl_2$. The extraction, precipitation and resuspension steps were then repeated. To prepare vector for subcloning single restriction fragments purified by agarose gel electrophoresis and adsorbtion to glass (see above), it was sufficient to include the CIP in the restriction enzyme reaction, and then to run the digested and dephosphorylated vector DNA on the same preparative gel as the insert fragment, processing the vector in parallel with the insert DNA. Ligations were performed in a final volume of 10μl of 50mM Tris pH8, 7mM $MgCl_2$, 1mM dithiothreitol, 1mM rATP, 1-5 Weiss units of T4 DNA ligase. DNA was left at 22°C for 2h to ligate and then used to transform competent *E. coli* cells.

Preparation of competent *E. coli* cells

A culture in LB broth was established from a single colony of XL1-Blue which had been grown on a tetracycline LB plate to select for the F'. This culture was grown to OD_{600}=0.3 and used to inoculate fresh broth. The new culture was grown to OD_{600}=0.5. The cells from 50ml of this were centrifuged for 10min. at 3krpm in the Damon Centra 3 benchtop centrifuge and resuspended in 25ml of ice cold 50mM $MgCl_2$ and allowed to sit for 30min. on ice. Cells were then pelleted again and gently resuspended in 2.5ml of 50mM $CaCl_2$. and left on ice for 1-24h. Competent cells could be frozen at this stage if glycerol was added to 15%.

0.2-0.4μg of DNA in 2-4μl was added to 100-200μl of competent cells and left on ice for 30min.-1h, heat-shocked at 42°C for 2min., returned to ice for 5min. and added to 1ml of LB at 37°C for 30min. Cells were pelleted in the microcentrifuge and resuspended in 50μl of LB with 100μg/ml ampicillin. Recombinant colonies were detected by their lac^- phenotype on indicator medium. LB plates with ampicillin were spread with 100μl of 5mg/ml each of isopropylthio-β-D-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) and allowed to dry at 37°C before spreading the transformed cells on top. Each experiment consisted of transformation with an experimental ligation, and control transformations with cleaved vector and insert without ligase treatment, vector ligated in the absence of insert DNA, uncut

vector DNA and ligation buffer without DNA. Competent cells prepared in this way gave about 10^6 colonies per microgram of uncut vector DNA.

Ligation into lambda replacement vectors EMBL4 and lambda DASH

Ligations were performed as above. A molar ratio of insert DNA (genomic DNA cleaved with restriction enzymes) to arms of approximately 1 was employed, in practice this meant that 1.5μg of vector and 0.5μg of insert were used in each ligation. DNA was left at 22°C for 2h or 16°C for 16h to ligate, then packaged *in vitro* as detailed below.

2.8.5 Preparation of *in vitro* phage packaging extracts

Extracts were prepared by the procedure of Hohn and Murray (1977). Two lambda lysogens, BHB2688: N205 recA$^-$ (lambda *imm*34 *c*I^{ts} *b*2 *red*3 *E*am4 *S*am7) and BHB2690: N205 recA$^-$ (lambda *imm*34 *c*I^{ts} *b*2 *red*3 *D*am15 *S*am7) were streaked onto plates incubated at 30°C and 42°C to confirm temperature sensitivity. Single colonies from the 30°C plates were used to inoculate cultures grown overnight at 30°C. 3 2-litre flasks each containing 500ml of LB broth at 30°C in were inoculated with 6ml of BHB2688. one flask of 500ml was inoculated with 10ml of BHB2690 from the overnight cultures. Cultures were grown for about 3h to OD_{600}=0.5-0.6 and then phage were induced by temperature shift to 45°C for 15min. After a further 1h of incubation at 37°C an aliquot of culture was tested for lysis with $CHCl_3$. If this resulted in clearing, the flasks were chilled on ice and the cells spun down at 8krpm in the Sorvall GS3 rotor at 4°C for 10min. All successive steps were performed at 4°C, on ice where possible. The supernatants were removed and the centrifuge bottles drained.

Freeze-thaw lysate of BHB2688

The cell pellets were gently resuspended in 0.5ml ice cold 50mM Tris pH7.4, 10% sucrose. The resuspended material was transferred to two 10ml screw-cap ultracentrifuge tubes (Oakridge) and 75μl of fresh 2mg/ml lysozyme in 0.25M Tris ph7.5 was added. Tubes were snap-frozen in liquid nitrogen, thawed and mixed with 75μl of Buffer M1 (6mM Tris pH7.5, 25mM spermidine, 50mm putrescine, 10mM $MgCl_2$, 15mM ATP, 30mM 2-mercaptoethanol). Extracts were spun at 35krpm for 40min at 4°C in a chilled Beckman 50Ti rotor without braking. The supernatant was transferred to 50μl aliquots and frozen at -70°C.

Sonicated extract of BHB2690

The cell pellet was gently resuspended in 0.5ml of Buffer A (20mM Tris pH7.5, 3mM $MgCl_2$, 0.05% 2-mercaptoethanol, 1mM EDTA.), transferred to a 15ml polypropylene tube with a further 2.6ml of Buffer A. The suspension was then sonicated for four 10 second bursts interspersed with 1min chilling periods. The viscosity rose during the first two sonication bursts but later declined as genomic DNA was sheared to shorter lengths. The lysate was spun in 15ml Corex tubes at 10krpm at

4°C in a chilled Sorvall SS34 rotor. The supernatant was frozen to -70°C in 20μl aliquots.

Packaging ligated vector DNA

The following were combined in the order given: 7μl of Buffer A, 0.2-0.4μg DNA, 1μl Buffer M1, 2μl sonicated extract and 6μl freeze-thaw lysate. These were gently mixed and incubated at 20°C for 1-2h. After this time, the resultant phage were diluted in 0.5ml SM buffer and 10μl $CHCl_3$ added as a preservative. Plating cells were prepared from overnight cultures of C600, NM514, Q358 or Q359 strains grown in LBM. Cells were pelleted and resuspended in 10mM $MgSO_4$. These cells were useful for 3-4 days if stored at 4°C.

2.8.6 Hybridisation-selection of phage and cosmid clones

Bacteriophage lambda

Libraries in bacteriophage lambda vectors were plated at a density of $2x10^4$ pfu/plate in 9ml of LBM top agarose on carefully-dried 15cm LB petri plates. After growth overnight at 37°C, plates were chilled at 4°C for 1h to harden the top agarose and then overlaid with Schleicher and Schull BA85 nitrocellulose discs. Orientation marks were made in the plate, through the membrane with a hypodermic needle dipped in India ink. Filters were peeled off when thoroughly wet, denatured for 5min in 1.5M NaCl, 0.5M NaOH, neutralised for 5min in 1M ammonium acetate, 20mM NaOH and rinsed in 2xSSC. Filters were dried and baked for 1h at 80°C and then processed exactly like a DNA blot (see above). Autoradiographs of probed filters were aligned with the plates and positive areas picked with a pasteur pipette into 0.5ml of SM with 10μl of $CHCl_3$. Two further rounds of hybridisation-selection were usually employed to ensure that a single positive plaque could be picked. Densities of about 10^3 and 10^2 plaques per 9cm plate respectively were ideal for this purpose.

Plasmids and cosmids

Cells containing recombinant plasmids or cosmids were spread on membrane filters laid onto plates of antibiotic LB medium and grown until colonies were 2-3mm in diameter. Pall Biodyne nylon membranes were used with 15cm plates of cosmid and cDNA libraries which were multiply reprobed. 20x20cm GeneScreen Plus filters were also used for cosmid libraries on 22x22cm square plates. Replica filters were made by sandwiching the filter with the colonies with a fresh filter, moistened on a fresh plate. The two filters were encased in sterile blotting paper and rolled with a 25ml glass pipette. Orientation marks were made with a large hypodermic needle. The two filters were peeled apart and placed, colonies uppermost on fresh plates and the colonies were re-grown for 2-3h before further replicas were made. The master filter was re-grown and then placed on a plate of LB agar with antibiotic and 15% glycerol for 30min before sandwiching with a fresh filter moistened on a similar plate. The master sandwich was frozen at -70° C between sheets of 3MM paper soaked in LB with

antibiotic and 15% glycerol. Temporary master plates were made by freezing a glycerol plate with a filter of colonies at -20°C. Colonies could be picked from this without thawing the plate. Filters to be probed were processed exactly like filter replicas of phage plates but occasionally needed boiling in 0.2xSSC, 0.5% SDS to remove debris after baking and immediately before the first hybridisation. Selected colonies were resuspended in 0.5ml of LB with antibiotic and re-plated at low density on BA85 nitrocellulose discs over 9cm plates. These were only probed once or twice owing to their relative fragility. As with hybridisation-selection of phage, a total of three rounds of plating, hybridisation and selection were needed to obtain a pure colony.

2.8.7 Preparation of cosmid and plasmid DNA from bacterial strains

Small Scale preparation

Plasmid DNA was prepared on a small scale (1-5μg) from 5ml cultures grown overnight in LB broth containing appropriate antibiotics. An aliquot was prepared for long-term frozen storage at -70°C by the addition of 15% sterile glycerol. Cells from 1.5ml of culture was pelleted in the microcentrifuge and resuspended in 100μl of 8% sucrose, 50mM Tris pH8, 50mM EDTA, 5% Triton X-100. Lysozyme was added to 1mg/ml, and the tubes were left on ice for 10min Each tube was punctured, and the cells were lysed by a 2min immersion of the tubes in boiling water. Cell debris and genomic DNA were pelleted for 15min in the microcentrifuge. Supernatants were removed and mixed with ground glass (Gene Clean, Bio101). Plasmid DNA and glass were pelleted and washed in Tris-buffered 70% ethanol ("NEW" Wash Bio101 - a classic "chicken soup" cocktail). The pellet was dried and the DNA resuspended in 10-20μl of TE.

Preparation by centrifugation in caesium chloride/ethidium bromide gradients.

250ml cultures in LB broth with antibiotic were inoculated from single colonies and grown in 500ml conical flasks for 16-20h. Cells were pelleted 10min at 8krpm in the Sorvall GS3 rotor and resuspended in 10ml of 50mM glucose, 25mM Tris-HCl pH8, 10mM EDTA. Lysozyme was added to 0.5mg/ml to weaken cell walls. The solutions were left on ice for 5min and then 20ml of 0.2M NaOH, 1%SDS was added and swirled until clear. Solutions were left to stand at room temperature for 10min, then 10ml of ice cold 3M potassium acetate (5M acetate) was added and bottles were shaken gently until the resulting flocculent aggregates of protein-SDS complex resembled dried milk powder. After 10min on ice, the precipitates were separated by centrifugation for 15min at 8krpm, 4°C. The supernatants were strained through cheesecloth into fresh GS3 pots and 0.6 volumes of isopropanol was added to precipitate DNA (10min at room temperature). The precipitate was collected by centrifugation at 8krpm for 15min and washed gently with 70% ethanol. The pellet was resuspended in 3.5ml of 20mM Tris base, 10mM EDTA (the pH should be 7-8 at this stage) and 1.2g of CsCl added for each initial ml of solution. 0.75ml of 10mg/ml ethidium bromide was added and

the purple furry precipitate of protein and ethidium bromide removed by centrifugation for 10min at 3-10krpm in the Sorvall SS34 or Damon Centra 3 bench-top rotor. The refractive index of the solution should be 1.395, corresponding to a density of 1.59g/ml. The preparation was sealed into high density polyethene tubes and spun for 16h at 50krpm in the Beckman VTi65 rotor. The radial density gradient established in this rotor was permitted to reorient itself smoothly as a vertical gradient by removing the centrifuge brake for the deceleration from 2krpm to the end of the run. Bands of supercoiled plasmid were harvested through the wall of the tube with a 19G hypodermic needle. Ethidium bromide was extracted 2-4 times with water-saturated butan-1-ol until the organic phase was clear. DNA was diluted 3-fold with water and precipitated with 2 volumes of ethanol at room temperature (CsCl precipitates at 4°C), washed in 70% ethanol, dried under vacuum and resuspended in 1-2ml of TE. The concentration of plasmid DNA was ascertained by measuring the OD_{260} of a 1/100 dilution, and subsequently adjusted to 0.5-1mg/ml.

Chapter 3: Identification of *Drosophila* mutants with abnormal mitotic chromosome condensation

3.1 Cytological screen for mitotic mutants induced on chromosome 3 by hybrid dysgenesis.

Mutations causing late larval or early pupal lethality have been found to be a plentiful source of those affecting mitotic processes including chromosome condensation and mitotic chromosome integrity (Gatti and Baker 1989). The possibility of isolating the mutated genes by transposon tagging (Searles et al. 1982) led us to use P-M hybrid dysgenesis as a mutagen. Mutations were induced by Roger Karess and Alan Cheshire on a lethal-free *red e* chromosome using $\pi 2$ or Harwich strains as a source of wild type P elements. 727 lethal mutations were isolated and maintained in stocks heterozygous for the TM3 balancer (*l(3) red e* / TM3 *ri* p^p *Ser e*). Homozygous larvae could be selected using the *red* malpighian tubule phenotype. 227 of the stocks exhibited early lethality (fewer than 1 homozygote per 100 heterozygous third instar larvae). 51 of the stocks were not investigated further because homozygotes isolated to fresh media as third instar larvae developed into fertile adults. These may be stocks containing revertants of originally lethal mutations or leaky mutations which were judged too difficult to map. 363 lethal stocks which yielded late larvae were examined cytologically in acetic orcein squash preparations of colchicine-treated third instar larval ganglia by Roger Karess, Alan Cheshire, Robert Saunders and me. In 14 of these mutants, no neuroblasts in mitosis were seen. 3 stocks displayed a variable but weak mutant effect on mitosis: G1.1 was an early pupal lethal with a low frequency of isochromatid breaks, H3.25 larvae had few neuroblasts in mitosis, J2.1 displayed a low frequency of polyploid cells. 12 stocks exhibited a mutant mitotic phenotype that was both characteristic and consistently expressed in all mutant larvae examined. The E2.1 mutation (now lost) produced a high frequency of polyploid cells. 7 mutations were alleles of *rough deal* (*rod*) which aberrantly segregates chromosomes at anaphase (Karess and Glover 1989). In addition I identified three mutant alleles R7.1, R7.4 and R10.1 at a new locus *l(3)snap*, which have a defect in mitotic chromosome integrity. R7.1 and R7.4 are probably separate isolates of the same mutagenised chromosome because they both came from the same bottle in the mutagenesis experiment. R10.1 should be an independent mutation because it was isolated from a different bottle.

Mitotic mutations residing on chromosomes bearing other, embryonic lethal mutations may be missed in such a screen of larval ganglia but may be detected by screening for failure to complement an existing mutation. K3.1 carries an zygotic larval lethal allele of *polo*, a mutation which can produce polyploid cells. The larval lethal allele was detected by its failure to complement a female sterile allele which has a mutant maternal effect on embryonic mitosis (Sunkel and Glover 1988).

3.2 *l(3)snap* mutants have abnormal mitotic chromatin condensation and exhibit anaphase tangling.

Metaphase chromosomes from ganglia of *l(3)snap* homozygotes of both sexes characteristically show irregular condensation (Fig. 3). Blocks of condensed chromatid arm are often found to be linked by thin threads of undercondensed chromatin. Because these threads are thin and may break during squashing, it was not possible to estimate the relative frequencies of irregular condensation and chromosome breakage. Anaphases were also found, but were often abnormal, with tangles of chromatin spread irregularly over the mitotic spindle (Fig. 3). This phenotype is somewhat reminiscent of the abnormal anaphases found in $mus101^{D2}$ embryos (Section 4.1.2). The mitotic spindle probably attempts to separate irregularly condensed chromatids, resulting in tangling and chromosome breakage. Such a defect has been seen in chromosome condensation mutants of other species. In *S. pombe*, mutations in topoisomerase II produce "streaked" anaphase chromosomes indicating a packaging defect in the presence of a functional mitotic spindle.

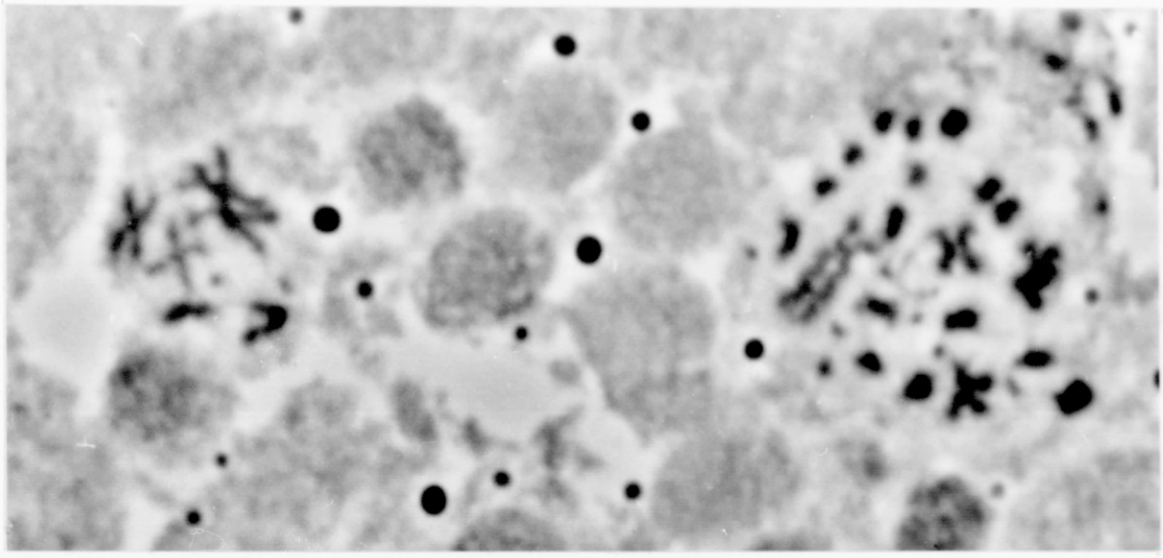
l(3)snap mutant third instar larvae have normally-sized imaginal discs and undergo metamorphosis to produce pharate adults which appear normal but die before eclosion. This late lethality indicates that most of the diploid cell proliferation necessary for development of adult occurs normally in these mutants. Eyes, legs and cuticle are all well-formed in these flies. Given the gross abnormality of neuroblast mitosis in these mutants it would be interesting to examine the nervous systems of mutant pharate adults for developmental abnormalities.

Fig 3

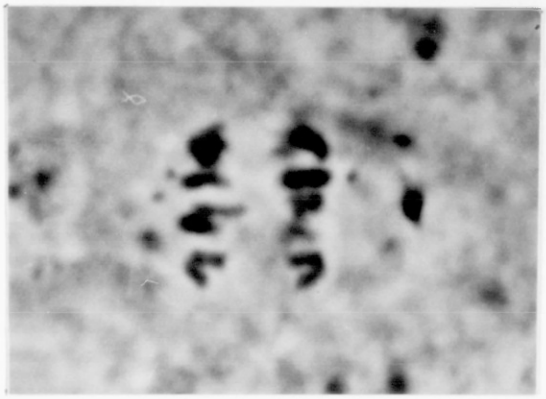
Acetic orcein squash preparations of mitotic cells from ganglia of larvae homozygous for *l(3)snap* allele R7.1

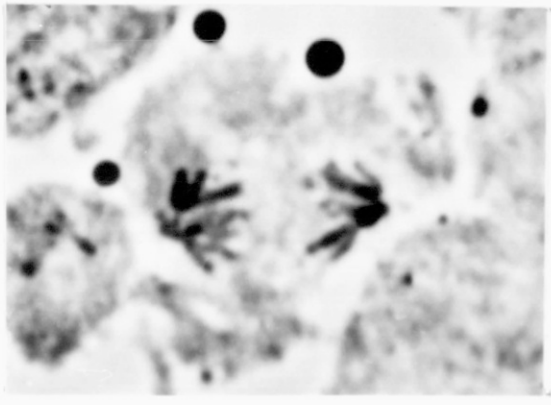
The upper panel shows a typical metaphase in a cell squashed after colchicine and hypotonic treatment. The mutation causes irregular chromosome condensation, producing blocks and threads as well as broken fragments. Aberrant anaphases from untreated mutant cells (below right) indicate one way in which breakage may occur, due to tangling of irregularly condensed chromatids. Normal anaphases from untreated ganglia of the Canton S wild type strain are shown below, at left.

I(3)R7.1 colchicine

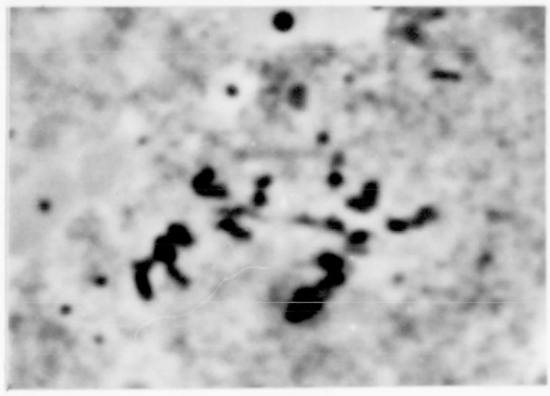


Wild type





I(3)R7.1



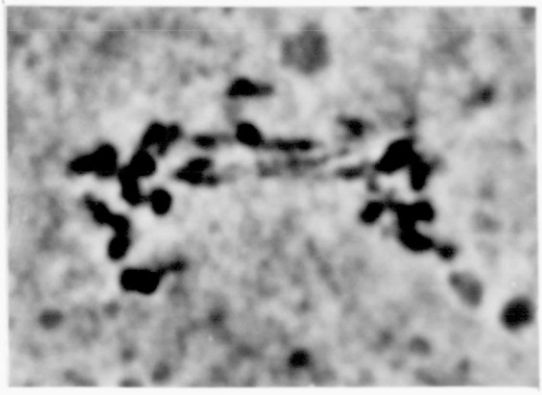


Table 1

Frequency of abnormal mitosis in *l(3)snap* ganglia

This table records the frequencies of mitotic figures (metaphases plus anaphases) exhibiting abnormally condensed chromosomes in ganglia of *l(3)snap* third instar larvae. Examples of such aberrant figures may be seen in Fig. 3. Three alleles of *l(3)snap* (R7.4A, R7.1, R10.1) were used. Canton S is a wild type strain. Results obtained from neuroblast squashes prepared after treatment with colchicine and hypotonic sodium citrate are marked "+", results from untreated ganglia are marked "-". Only clearly interpretable mitotic figures were recorded. A greater proportion of mitoses with abnormal chromosomes were present when no colchicine was used. This is probably because cells containing chromosome fragments can be delayed in progress through mitosis (Gatti and Baker 1989). Colchicine arrests cycling cells at metaphase, causing accumulation in mitosis of cells from the sub-population still unaffected by the mutation.

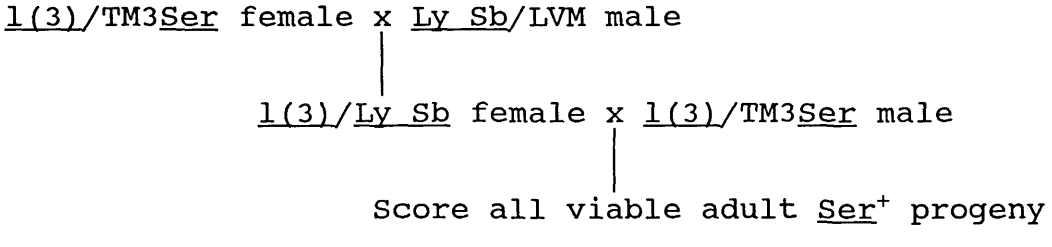
Stock	Aberrant(%)	Wild type	Ganglia	Colchicine
R7.1red e	61(33)	123	6	+
R7.1red e	152(74)	53	6	-
R7.4Ared e	110(35)	204	8	+
R7.4Ared e	69(62)	42	5	-
R10.1red e	119(70)	51	5	-
R7.4Ared e/ Df(3R)T61	0	128	5	+
Canton S	0	371	8	+
Canton S	0	229	10	-

3.2.1 *l(3)snap* is a novel mitotic mutation.

I tested *l(3)snap* (R7.4) for complementation with a number of mitotically abnormal mutations mapping to the third chromosome. Crosses were made in both directions such that *l(3)snap* was both maternally and paternally inherited in order to control for haploinsufficient parental effects. *l(3)snap* complements fully, and is therefore not allelic to, the following mitotic mutations: *l(3)7m62*, *l(3)12m137*, *l(3)e20*, *l(3)1902*, *l(3)2004* (50.3), *l(3)2612* (48.7), *l(3)1X-14* (49.0), *l(3)K43* (51.1), *l(3)g60A*, *l(3)15m25*, *l(3)X11-10* (Gatti and Baker 1989), *rod*, Karess and Glover 1989, *polo* (Sunkel and Glover 1988), *gnu* (Freeman et al. 1986), *asp* (Ripoll et al. 1985), *l(3)ck19*^{e211} (87B6-12: Chapter 5 and Axton et al. Cell, in press), *aurora* (87A8,9), *lodestar* (47.9) Glover et al. 1989, *mgr* (51.3) González et al. 1988. The published map position is quoted here if it is within 2 m.u. of the *l(3)snap* locus. While this list is not exhaustive, these experiments suggest that *l(3)snap* is a novel mitotic mutation. In addition, *l(3)snap* does not enhance the mutant phenotype of these other mitotic mutations in that the *in trans* heterozygous condition is neither lethal nor sterile.

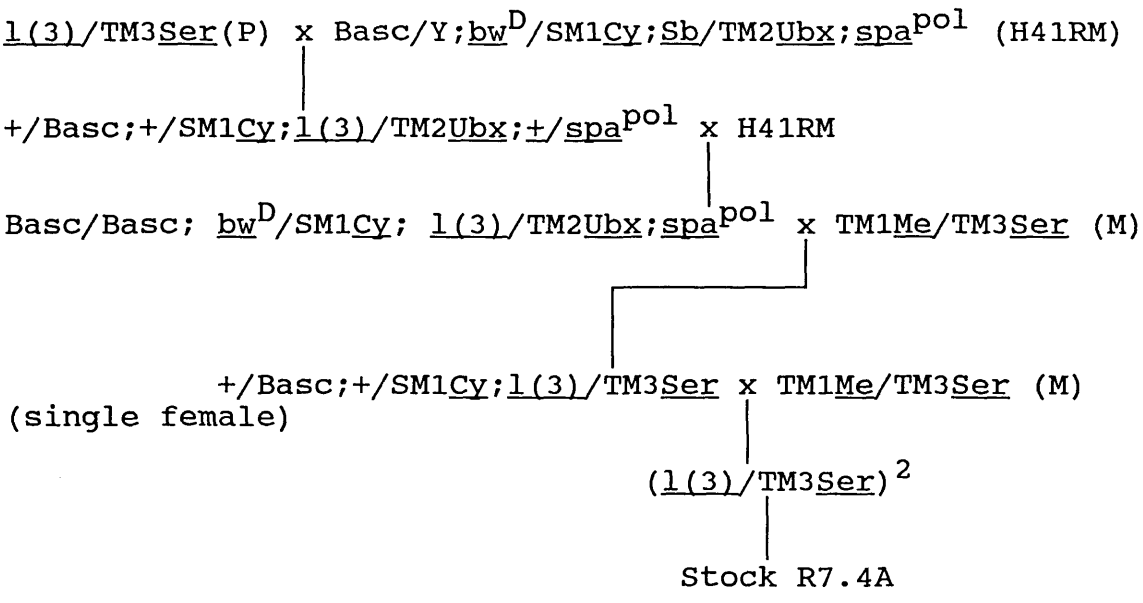
3.2.2 Mapping the *l(3)snap* mutation.

The lethal mutation on the R7.4 *red e* third chromosome was initially mapped using the dominant visible markers *Ly* and *Sb*. This procedure does not give a very accurate map position if the mutation is far from both markers. However, if dominant markers are used, fewer generations are required to map the locus responsible for the lethality of the mutagenised chromosome. The following scheme was used:



These data (Table 2a) place the lethal mutation between the two dominant markers at approximately map position 50 with a sample standard deviation of 1.9 m.u.

Subsequently, all of the mutagenised chromosomes apart from the third chromosome bearing the lethal mutation were replaced with those of a P element-free M strain. During these crosses, a single third chromosome was selected:



Homozygous mutant larvae from this lethal stock R7.4A were phenotypically identical to those from the original lethal stock before chromosome replacement. The lethal mutation was then mapped by recombination between the R7.4A chromosome and one bearing the recessive markers *ru h th st cu sr e ca* (*rucuca*). As both parental chromosomes carried the *e* marker, this could not be used in mapping and is omitted from Table 2b. A chromosome bearing the *rucuca* markers and the homozygous lethal dominant visible bristle marker *Pri* (*ruPrica*) was used to recover recombinant chromosomes:

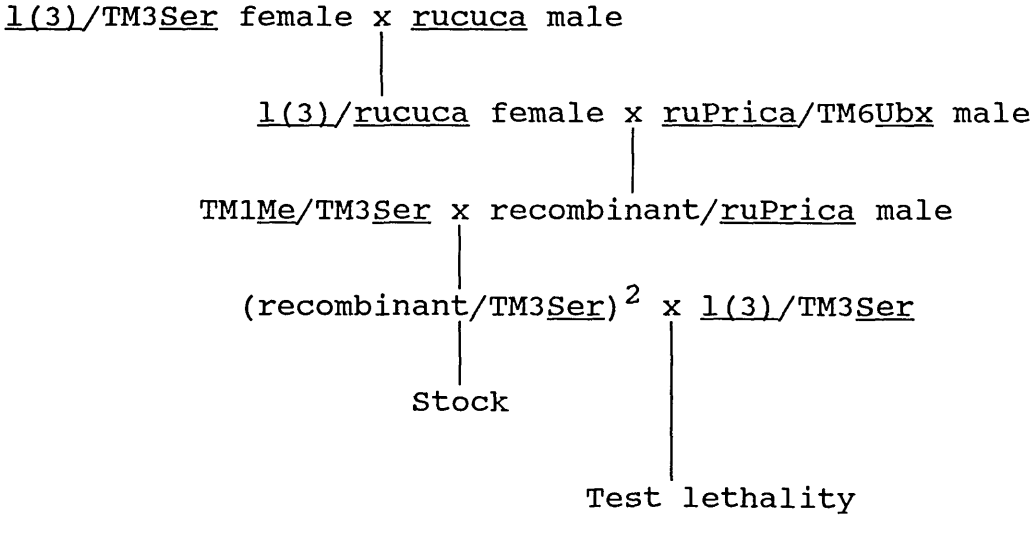


Table 2

Mapping *l(3)snap* R7.4A by recombination.

l(3)snap was mapped by recombination with chromosomes bearing (a) dominant and (b) recessive markers as shown in the text of Section 3.2.2. a) Frequency of each genotype of viable progeny recovered *in trans* with the parental R7.4 chromosome. Crossing-over between markers and the lethal mutation is also noted. Non-crossover (parental) chromosomes are denoted "NCO". Single and double crossover chromosomes are denoted "SCO" and "DCO" respectively.

b) Recombinants between *l(3)snap* R7.4A and the multiply marked rucuca chromosome were recovered and then tested for viability by backcrossing to the original R7.4A stock. The following genotypic classes were recovered with the frequencies shown. The number of recombinants which were viable or lethal when heterozygous with the parental lethal chromosome are recorded to the right of the genotype.

---

a)

Genotype	Number	Crossover
Ly Sb	299	NCO
+ +	4	DCO
Ly +	27	SCO
+ Sb	31 SCO	
	361	

b)

ru	h	th	st	cu	sr	ca	Viable	Lethal
-	-	-	-	-	-	-	15	1
+	+	+	+	+	+	+	0	10
-	-	-	-	-	-	+	12	0
+	+	+	+	+	+	-	0	22
-	-	-	-	-	+	+	7	0
+	+	+	+	+	-	-	1	4
-	-	-	-	+	+	+	0	3
+	+	+	+	-	-	-	0	0
-	-	-	+	+	+	+	0	1
+	+	+	-	-	-	-	0	0
-	-	+	+	+	+	+	0	17
+	+	-	-	-	-	-	6	0
-	+	+	+	+	+	+	0	12
+	-	-	-	-	-	-	7	1
							119	

These results indicate that the mutation responsible for the lethality of R7.4A lies 5.3 m.u. to the right of *st* at map position 49.3 with a sample standard deviation of 2.2 m.u. which is in agreement with the position obtained using the dominant markers. This position is also obtained if the recombination distance from *th* or *sr* is calculated. The sample is too small to obtain a useful measure of the distance from *cu*, the nearest visible marker. The *l(3)snap* abnormal mitotic phenotype was present in the 3 *ru h th st* lethal recombinants. 3 of the 7 viable *ru h th st cu* recombinants which I tested were all mitotically normal.

3.3.3 Reversion of *l(3)snap* during hybrid dysgenesis.

The original stocks of *l(3)snap*, before replacement of chromosomes, were used as the P strain in dysgenic genetic crosses in order to generate revertants of each allele (Table 3a). The results of F1 ovarian dysgenesis tests listed in Table 3a indicated that the R7.4 stock behaved as an M' or Q strain (Kidwell 1986) whereas the R10.1 stock was a moderate, and R7.1 a strong P strain. It was therefore necessary to use another P strain stock to attempt to mobilise the elements in the R7.4 chromosome. A TM3/TM6B balancer stock into which P elements had been introduced from the $\pi 2$ P strain was used for the crosses shown in Table 3b in this further attempt to generate viable revertants. Ovarian dysgenesis tests were not performed on the F1 of this cross, and no viable revertants of *l(3)snap* were recovered.

It was not clear whether R10.1 revertants were sterile due to gonadal dysgenic effects of P element activity at 25°C or whether the R10.1 chromosome carries a second mutation responsible for the sterility. The R7.1 revertant chromosome (r40) obtained in the first reversion experiment (Table 3a), was isolated by crossing the original *l(3)snap* heterozygote to TM3/TM6B (M) and establishing TM6B-balanced stocks derived from single red e chromosomes. The r40 homozygotes are viable and fertile; mitosis in larval neuroblasts appears normal. The isolation of a single revertant from an experiment which produced revertants at such a low frequency is not necessarily evidence that the *l(3)snap* mutation is caused by a P element insertion. In similar experiments to revert the M6.2 allele of *rod*, which was generated in the same series of mutagenesis experiments as the *l(3)snap* alleles, several phenotypic revertants were recovered which showed DNA polymorphisms characteristic of the balancer chromosomes used in the dysgenic crosses (Karess, pers. comm.). The M6.2 allele does not carry a P insertion at the *rod* locus and so these "revertants" must have been the result of some form of recombination between the mutant and balancer chromosomes either sponsored by P element activity, or spontaneous but selected during the reversion experiment.

Table 3

Two schemes for obtaining revertants of *l(3)snap* during hybrid dysgenesis.

a) The *l(3)snap* stock was used as a P strain, or source of P transposase, to mobilise the elements on the l(3)snap chromosome in the F1 generation. The F1 balanced heterozygotes were crossed inter se to give balanced heterozygotes and homozygotes. Only revertants of *l(3)snap* can survive to produce red e adults. The numbers of heterozygotes and viable red e adults recovered from crosses involving the indicated *l(3)snap* alleles are recorded in the table below. The P element activity was estimated by measuring the percentage of F1 ovaries showing gonadal dysgenesis at 29oC.

b) In these crosses, the *l(3)snap* allele was the M strain. The percentage of gonadal dysgenesis was not recorded (ND).

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a) l(3)snap red e/TM3 (P) x TM1/TM3 (M) virgin

↓

(l(3)snap red e/TM1 or TM3)^2

↓

snap$^+$red e/l(3)snap red e (viable revertant)

l(3)snap red e/TM1 (or TM3) (heterozygote)

b) l(3)snap red e/TM3 virgin X TM3/TM6B(P)

↓

as above

	%Gonadal Dysgenesis	Heterozygotes	red e adults
a)			
R7.4	4.5	4242	0
R10.1	21	3800	9(all sterile)
R7.1	75	5225	1
b)			
R7.4A	ND	2048	0
R10.1	ND	843	0

3.3.4 Cytogenetic mapping

The recombination mapping of R7.4A placed the lethality of *l(3)snap* in the proximal one fourth of chromosome 3R in the interval between *st* and *cu*. The closest visible marker, *cu*, has been localised to 86D1-4 (Ashburner et al. 1981). In order to locate the *l(3)snap* gene, the R7.4A chromosome was crossed to each of a set of deficiency chromosomes which together uncover much of the proximal region of the right arm of chromosome 3 (divisions 82-87). I have not yet found a deficiency which uncovers the mutation. Fig. 4 shows the deficiencies which fail to uncover the lethality of *l(3)snap*.

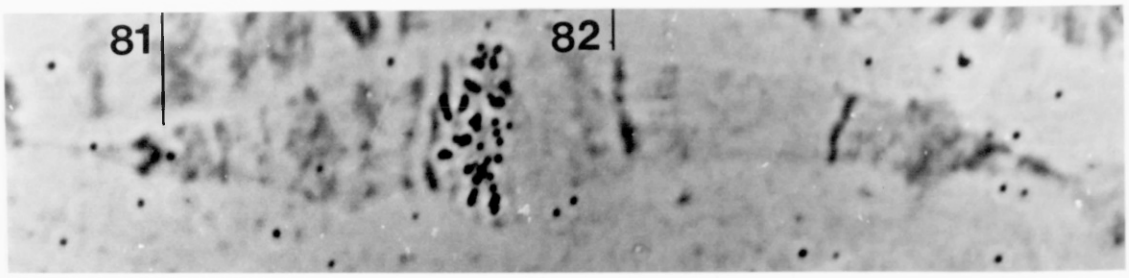
Although reversion experiments provided no conclusive evidence for P element insertions in either the R7.1 or R7.4 chromosomes, insertions of transpositionally defective P elements would not be detected in this way. For this reason, I probed heterozygous *l(3)snap* (R7.4/*red e*) salivary gland chromosomes for the presence of P element homology by *in situ* hybridisation. The original R7.4 stock and its derivative R7.4A had P elements at 82E, 86F, 98B and 100F in chromosome 3R. Two separate viable recombinants of genotype *ru h th st cu l(3)snap*$^{+}$ (Table 2) carried only the two more distal elements on 3R. However, it was not possible to correlate the *l(3)snap* phenotype and lethality with either the 82E or 86F elements. Two *l(3)snap* R7.4A (lethal) recombinants were probed. *ru h th st l(3)snap* also retained the two distal elements but no longer carried the 82E or 86F elements. *l(3)snap sr ca* retained only the 82E element.

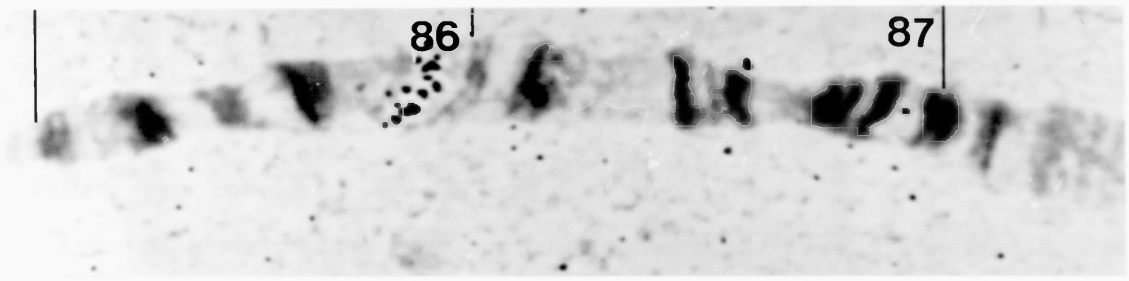
The site of P insertion at 86F is uncovered by Df(3R)T61. This deficiency uncovers no mitotically abnormal phenotype (Table 1). Indeed, *l(3)snap*R7.4/Df(3R)T61 heterozygotes are viable and fertile (Fig. 4). I concluded that a current P element insertion is not responsible for the *l(3)snap* mutation on the R7.4 chromosome. In addition, no cytological abnormality was seen on salivary chromosome 3R in these mutants.

Fig 4

P element probe pπ25.1 hybridises *in situ* to chromosomes heterozygous for *l(3)snap* allele R7.4 at two sites in the proximal region of chromosome arm 3R.

Two P elements were detected in the proximal region of chromosome 3R from *l(3)snap* allele R7.4A. These sites, at 82E and 86F, are arrowed on the conventional polytene map (Lefevre 1976) shown in the lower part of the figure. A number of cytologically visible deficiencies were tested for complementation with the *l(3)snap* mutations. These are also shown in the lower part of the figure. The horizontal bars show the extent of chromosomal region absent from the deficiency chromosomes. Although the recessive lethality of *l(3)snap* maps to the proximal one fourth of 3R it is not uncovered by any of the deficiencies shown. Thus it is most likely that *l(3)snap* falls within one of the following intervals: 85B6;85D11-14, 85F16;86C1,2 or 86D8;86E3-6.


81
82


86
87


Ki
p
82
83
84
85
86
87
J17PD107D
TPI
10
dsx+29
by62
cu40
TE61
Dfd13
p40
KarD1
AntP17
Scx4

3.3 Future prospects for isolation of the *l(3)snap* gene

I have identified and mapped the *l(3)snap* gene, represented by at least 2 independent alleles induced by P-M hybrid dysgenesis. The R7.4 allele is not suitable for use in cloning the *l(3)snap* gene by transposon tagging as there is no P element insertion associated with the *l(3)snap* mutation on this chromosome. The first priority in attempting to isolate *l(3)snap* must be to obtain deficiencies which uncover the recessive mutant mitotic phenotype. The R7.1 and R10.1 chromosomes should then be probed for P elements by *in situ* hybridisation. Only R10.1 gave an appreciable frequency of revertants under hybrid dysgenesis. Unfortunately these were all sterile and consequently of no use. Because most *l(3)snap* mutants die shortly before eclosion, it is possible that occasional "escapees" may be found which do eclose. The sterile R10.1 "revertants" detected may in fact be such rare escapees. The R10.1 allele should be subjected to recombination mapping to determine whether any other mutations causing sterility are present on the chromosome. If the sterility is due to another mutation, either recombinants or a deficiency uncovering *l(3)snap* could be used to recover revertants regardless of other mutations on the chromosome.

l(3)snap is somewhat unusual when compared with other mitotic mutations causing late lethality (Gatti and Baker 1989). *l(3)snap* homozygotes display a relatively high frequency of abnormal mitosis in neuroblasts. In spite of this the imaginal discs of third instar larvae are normally developed. Lethality of the organism occurs late in pupation and external structures appear normally differentiated. Either neuroblasts and ganglion mother cells are particularly sensitive to loss of $snap^+$ function, or $snap^+$ function is only required for mitosis in neural cells. More alleles of *l(3)snap* of different strengths of mutant effect would be useful for further analysis of the mutant phenotype.

In many squash preparations the abnormally condensed *l(3)snap* chromosomes fall into about 5-10 regularly-sized blocks linked by undercondensed threads. While these blocks may result from the mechanical forces of squashing imposed on abnormal chromosomes it is likely that these blocks represent sub-assemblies in the higher order packaging of mitotic chromosomes. Less extreme mutant alleles of *l(3)snap* may permit quantitation of the number and sizes of such blocks in each chromosome. *l(3)snap* mutations may in fact preferentially cause decondensation of particular sites distributed rather frequently throughout the chromosome arms. A conditional allele would be particularly useful as it would allow us to examine less extreme effects of $snap^+$ insufficiency on chromosome structure. In particular, aberrantly condensed chromosomes could be examined during their first abnormal mitosis without the background of chromosome fragments from the previous division.

3.4 Yield of mutations affecting mitosis

Gatti and Baker (1989) reported that about half the ethyl methane sulphonate-(EMS-) induced mutations they examined which caused larval or pupal lethality were abnormal in some aspect of mitosis. We have found a much lower frequency of mitotically abnormal mutations among P element-induced mutations causing lethality at these stages. Several factors may have contributed to this difference in yield. Firstly Gatti and Baker were selectively examining mutants from a collection of lethals whose imaginal disc proliferation had previously been investigated (Shearn et al. 1971). If we had rigorously selected only those stocks exhibiting lethality of large late larvae which however showed little imaginal disc proliferation we might have recovered more mitotically abnormal mutants. Secondly we have noted that at least two independent alleles of *l(3)snap* were obtained as well as 8 alleles of *rough deal*. P elements have been reported to exhibit locus-specific differences in insertion frequency of 10^3-fold or more (Simmons and Lim 1980, reviewed by Kidwell 1986). Thus we may have unwittingly examined multiple alleles of the same few mutant loci, relatively few of which caused mitotic abnormalities.

Thirdly, while it is theoretically possible that a zygotic null mutation may show its first mutant effects in late larval cell divisions, perhaps the majority of mitotically abnormal late larval lethals are leaky mutations in genes essential for cell division. Early larval or embryonic lethality may well be more typical of such null mutations. If so, then selecting mitotically abnormal late larval lethals will bias the screen toward the recovery of hypomorphic mutations. Insertion mutations and chromosomal rearrangements generated by transposon movement may be more likely to create null mutations than does the alkylating agent EMS. If so, individuals homozygous for these mutations might die earlier, as small larvae, or late larvae with insufficient proliferating cells to permit analysis. Another theoretical possibility is that proteins functioning both in interphase and in mitosis may be mutated to a mitotically abnormal phenotype by mis-sense mutations but that null mutations could cause early lethality due to loss of a generally required function in non-mitotic cells.

3.5 Cytological screen for X-linked mitotic mutants induced by hybrid dysgenesis.

I also examined a collection of X-linked lethal mutations induced by Simmons et al. (1984). These mutations were induced by hybrid dysgenesis using the $\pi 2$ P strain as a source of both P elements and transposase. About 50% of the mutant chromosomes are derived from $\pi 2$, the remainder from the *f* marked M strain used. Mutation-bearing chromosomes do not carry any marker visible in larvae. In order to examine the lethal stage of each mutation I exchanged the FM7 balancer chromosome for the lethal balancer DfBasc (DFB). Because this balancer is deficient for the tip of the X, distal to the breakpoint of $In(1)sc^1$, balancer-bearing males are inviable unless supplied

with a covering X;Y translocation such as T(1;Y)sc^8 (sc^8Y). Virgin females heterozygous for the lethal mutation and DFB were mated to y^1 w^1 sn^3 / Y males. Mutant male progeny could thus be identified by gonadal size, y^+ mouthparts and chromosomal sex. A limitation of this approach is that mutations which prevent the proliferation of the cells of the male gonad may be mistaken for early lethals with an embryonic or first instar lethal stage. Despite this theoretical objection to the use of this screen in isolating mitotic mutations such a screening procedure was initially used in preference to introduction of a larval marker onto each mutant chromosome by recombination.

Of 101 lethal stocks, 64 gave no mutant male larvae and were thus judged to be early zygotic lethals. These were not investigated for their effect on mitosis. The 37 stocks which produced mutant males dying as late larvae or pupae were examined in squash preparations of ganglia from third instar larvae. Two stocks were distinguishable from wild type in these preparations. Larvae from MS24 were small, with undeveloped imaginal discs and ganglia. No mitotic figures were seen. None of these larvae survived later than early (white) pupal stages. It is possible that this mutation (which was not examined further) blocks the cell cycle in interphase. MS55 were selected for further investigation because although the mutant males possessed recognisable testes, imaginal discs were very small and all mutants died as late third instar larvae or shortly after pupariation. Furthermore, chromatin condensation was grossly abnormal in nearly all of the mutant mitotic neuroblasts. This mutation is discussed in the following chapter.

Chapter 4: *mus(1)101*

Introduction

mutagen sensitive (1) 101 (mus101) mutants were originally isolated as conditional wholly recessive lethal mutations causing hypersensitivity to killing by the alkylating agent methyl methane sulphonate (MMS) (Boyd et al. 1976, Smith 1976). Alleles *mus101*^{D1} and *mus101*^{D2} also confer hypersensitivity to killing by the bifunctional DNA cross-linking agent nitrogen mustard (HN2) and the pro-carcinogen 2-acetyl aminofluorene (AAF). Repair defects were distinguished from metabolic or transport effects by characterisation of the response to radiation damage. *mus101* mutant larvae were hypersensitive to gamma radiation but were similar to wild type in their recovery after UV irradiation. Excision of thymidine dimers induced by UV is normal in *mus101* (Brown and Boyd 1981) and the lethality after gamma irradiation is unlikely to be due to single strand breaks because primary cells cultured from *mus101*^{D1} embryos exhibit normal repair of such breaks induced by X-rays.

These primary cultures are deficient in their ability to synthesise DNA on a UV-damaged template (Boyd and Setlow, 1976). By analogy with a form of repair synthesis found in *E. coli* this synthetic ability is termed post-replication repair (Rupp and Howard-Flanders 1968). The use of ^{3}H thymidine pulse labelling at different times after UV irradiation reveals that while *mus101*^{D1} mutant cells synthesise normal amounts of DNA, the length of the strands synthesised is abnormally short (Brown and Boyd 1981). In unirradiated cells, the rate of increase in molecular weight of DNA immediately after labelling with a pulse of ^{3}H thymidine is normal but the subsequent increase is slowed with respect to wild type. This effect may be due to failure to repair damage caused by ^{3}H decay (Farmer, J. pers. comm. in Boyd and Setlow, 1976).

mus101^{+} product is also active in early embryos. For a DNA modification caused by a mutagen to become heritable, it must be converted by the cell's DNA repair enzymes into a sequence which may be replicated. If functional DNA is disrupted by this process, the lesion is fixed as a heritable mutation. Untreated *mus101*^{D1} females exert a maternal effect on the fixation of lesions present in sperm of mutagenised wild type males (Graf et al. 1979). These authors concluded that the mutant exhibits altered repair of lesions caused by X-ray and ethyl methane sulphonate (EMS) treatment. Very few X-linked recessive lethal mutations were recovered from sperm from males treated with HN2, so *mus101*^{D1} is almost completely deficient in repair of DNA crosslinking.

Non-mutagenised homozygous *mus101*^{D1} females at 23°-25°C have spontaneously elevated levels of meiotic X chromosome loss and non-disjunction. This could be the result of any of several aberrant processes, one of which might be failure of crossing over by homologous recombination (reviewed by Baker and Hall 1976). The frequency of exceptional male progeny is greater than wild type by a factor of about 10^{2}. The

frequency of spontaneous meiotic non-disjunction assayed as exceptional female progeny is elevated about tenfold (Boyd et al. 1976). Oddly, these authors give similar figures for *mus101*^{D2} homozygotes rather inconsistently described in the same work as being "sterile". Baker and Smith (1979) described *mus101*^{D1} and *mus101*^{D2} as showing "extreme female infertility" and demonstrated that the mutations cause chromosomal instability in somatic mitoses of the wing disc at 25°C. Three viable alleles could be ranked by mutant effect. *mus101*^{D1} homozygotes are indistinguishable from wild type, *mus101*^{A1} shows an increase in somatic clone frequency. *mus101*^{D2} has the strongest effect with a nearly tenfold increase in *mwh* clones . Size distribution analysis indicates these may all be aneuploid clones derived from chromosome breakage. Somatic instability was characteristic of all three *mus101* alleles tested when homozygous, or hemizygous with Df(1)HA92 (12A6,10-D3). These mutations are hypomorphic, the mutant phenotypes are more strongly expressed in hemizygous condition.

Gatti (1979) found *mus101*^{D1} , *mus101*^{D2} males and homozygous females similar to wild type in frequency of abnormal mitoses in ganglion squashes. However, direct cytological evidence that *mus101*$^{+}$ expression is required to maintain the integrity of mitotic chromatin was obtained using a temperature sensitive allele *mus101*^{ts1} lethal at temperatures above 22°C in both sexes (Gatti et al. 1983). Within 2h of being shifted to 29°C most cells in male ganglia have abnormally undercondensed or broken Y chromosomes. With increasing time at the non-permissive temperature all of the major heterochromatic regions of the genome become undercondensed. A high frequency of Y chromosome breakage is also evident from analysis of somatic clones generated under semi-restrictive conditions. At 22°C, *mus101*^{ts1}/*mwh*$^{+}$Y; *mwh* males produce tenfold more *mwh* clones than *mus101*^{ts1}/Y; *mwh*$^{+}$/*mwh* males (Smith et al. 1985).

By shifting ganglia incubated in ^{3}H thymidine to a nonpermissive temperature (29°C) for various time periods and analysing squash preparations after autoradiography, Gatti et al. (1983) found firstly that cells which had replicated at either temperature were equally likely to exhibit decondensation on reaching mitosis at the nonpermissive temperature. Secondly, those mitotic cells which had incorporated labelled nucleotides at the nonpermissive temperature also incorporated label into heterochromatic regions. This was independent of whether or not these regions were undercondensed in mitosis. The authors therefore concluded that *mus101*$^{+}$ is required in G2 for the mitotic condensation of heterochromatic regions and that mutagen-sensitivity and chromosome instability were probably a consequence of a failure of proper chromatin packaging.

However, a "subtle" effect of these hypomorphic mutations on DNA replication cannot be excluded from consideration in view of several studies indirectly implicating *mus101* in processes of replicative DNA amplification. A female sterile allele of *mus101* was shown to be defective in a factor required *in trans* for chorion protein gene amplification, a form of differential DNA replication which occurs during

oogenesis in follicular cells (Orr et al. 1984, Baker, B.S., pers. comm. (in) Snyder et al. 1986). This allele, *fs(1)K451*, is also hypomorphic, showing a stronger defect when uncovered by Df(1) HA92. Orr et al. (1984) suggest that chorion amplification may involve unusually rapid DNA synthesis for which the mutants are poorly equipped. Alternatively, the amplified DNA strands may require a particular interphase chromatin structure involving *mus101*$^{+}$ protein.

In common with other mutants defective in post-replication repair, *mus101*^{D1} and *mus101*^{D2} suppress the "magnification" of rDNA of Y*bb*$^{-}$ chromosomes which bear large deletions of the tandemly repeated 18S and 28S ribosomal genes, with *mus101*^{D1} having the stronger mutant effect (Hawley et al. 1985). The mechanism of magnification in the male germ line is unclear but may be due to reciprocal meiotic exchange or arise pre-meiotically by unequal sister chromatid exchange. rDNA amplification does occur in the testes of Y*bb*$^{-}$ bearing males and may be involved in pre-meiotic magnification (Locker and Marrakechi 1977). In attempting to explain the effect of *mus101* mutations on rDNA magnification it is necessary therefore to consider not only the defective DNA repair in *mus101* mutants but also the possible roles of *mus101*$^{+}$ in DNA replication and maintenance of the peculiar organisation of amplified chromosomal loci mentioned above in connection with chorion amplification.

This historical account of the phenotypic analysis of *mus101* mutations emphasises the need for molecular analysis of the gene in order to clarify the role of its product in each of these aspects of chromatin function.

4.1 The MS55 chromosome bears lethal allele *mus101*^{lcd}.

The MS55 stock described in the previous chapter was examined in more detail. The lethality and mutant mitotic phenotype of the MS55 chromosome are covered by Dp(1;f)LJ9 (12A6-10;13A2-5) (Table 4, and Fig. 10) and $y^{+}g^{+}na^{+}$Y (of Schalet) (12A8;12F) but not Dp(1;f)LJ8 (12B6,7;13A2-5). Using *y* marked lethal recombinants derived from MS55 and these y^{+} duplications I generated *in trans* heterozygous combinations of MS55 and mitotic mutations *l(1)d.deg9*, *10*, *12* (Gatti and Baker 1989). All of these combinations are viable and fertile females.

MS55 fails to complement the larval lethality of *mus(1)101*^{SM}. The MS55 chromosome thus bears an allele of *mutagen-sensitive(1)101* which I refer to as *mus101*^{lcd} (*lethal-compact-disc*).

4.1.1 *mus(1)101* is essential for mitosis

The proportion of abnormal cells in mitosis in the ganglia of third instar larvae bearing heterozygous combinations of *mus101* alleles was measured. The viability and fertility of these genotypes were also tested. The results are recorded in Table 4. Many chromosomes are regionally under-condensed in the pericentric heterochromatin Fig. 5b. However, most of the mitotic neuroblasts in *mus101*^{lcd} ganglia show more

extreme effects on chromosome integrity, characteristically grossly irregular chromatin condensation or even chromosome fragmentation, a typical example of the majority of *mus101*^{lcd} cells is shown in Fig. 5c.

As ganglia were routinely incubated for 1h in 10^{-5}M colchicine and hypotonic saline before fixation and squashing it was important to demonstrate that the mitotic abnormalities seen were not due to hypersensitivity of the mutants to colchicine. Ganglia from both *l(1)d.deg4* and *mus101*^{lcd} larvae fixed and squashed without colchicine or hypotonic treatment showed aberrant mitoses similar to those of treated cells. Viability and fertility of mutant combinations are recorded in Table 4, it is clear that a high (>60%) frequency of aberrant mitotic cells in neuroblast squashes correlates with lethality. *mus101*^{lcd} heterozygotes with mutagen-sensitive alleles *A1*, *D1*, *D2* (c) have a frequency of abnormal mitoses slightly elevated above the low frequency seen in viable fertile balanced heterozygous controls (d). However, the mutant combinations in (c) are all female sterile.

Fig. 5

Acetic orcein-stained ganglion mitoses typical of *mus101*^{lcd} and *l(1)d.deg4* mutants.

a) Wild type XX metaphase.

b)*mus101*^{lcd}/Y chromosomes showing undercondensed pericentric heterochromatin. This pattern of preferential decondensation of heterochromatic regions is similar to that reported by Gatti et al. (1983) in neuroblasts of *mus101*^{ts1} shortly after they were shifted to the non-permissive temperature. This phenotype may be the earliest defect to appear in *mus101*^{lcd} mutant cells.

c) Extreme phenotype more typical of *mus101*^{lcd}/Y, with irregularly condensed or broken chromosomes. Blocks of chromatin are probably chromatid arms.

d) *l(1)d.deg4*/Y overcondensed, metaphase-arrested chromosomes. The chromatid arms are characteristically widely splayed.

e) *l(1)d.deg4*/Y hyperploid cell with overcondensed chromosomes. Despite resemblance to c) these blocks are probably whole overcondensed chromatids.

Colchicine and hypotonic treatment were used in preparation of a,b but not of c-e. The bar represents 10μM.

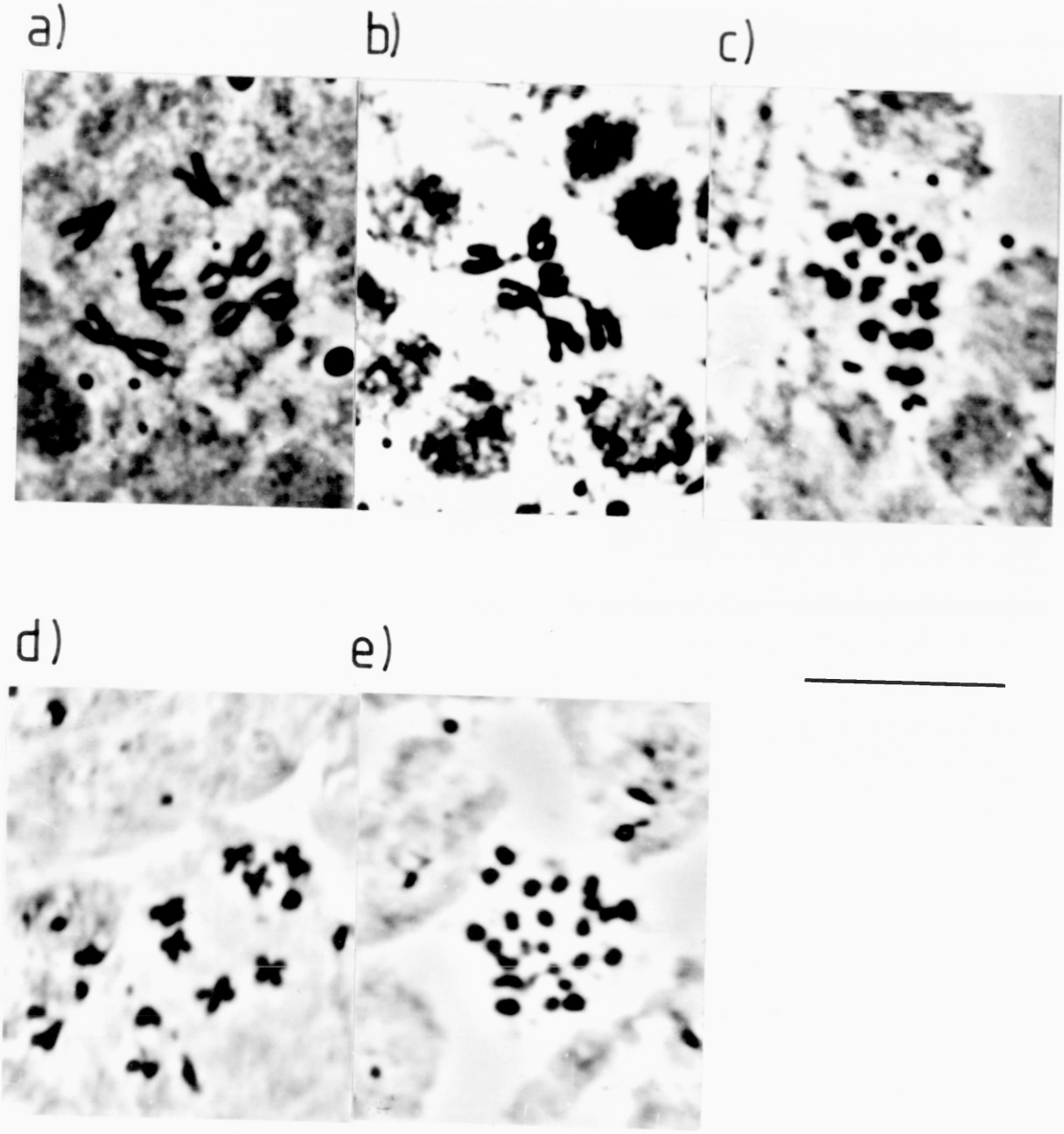

a)
b)
c)
d)
e)

Table 4

a-d) Neuroblast mitosis and viability of *mus101* mutants.

This table records the frequencies of normal and aberrant mitotic figures in acetic orcein squashes of neuroblasts from mutant larvae of different genotypes. Aberrant cells of *mus101* ganglia are those exhibiting abnormal condensation of at least one chromosome (Fig. 5b,c). Results obtained using colchicine plus hypotonic treatment are denoted by (+) under the "Col." heading. Results from untreated ganglia are marked (-). The appearance of abnormal mitotic figures in mutant brains was similar with and without the colchicine treatment. The viability and fertility of mutant combinations are recorded in the column labelled "Fert." in the table. (l) denotes lethality in third instar larvae, (m+) males were fertile, (f+) females were fertile, (fs) females were sterile. The lethal allele, $mus101^{lcd}$, has a stronger mutant effect on mitosis than the viable, female sterile alleles $mus101^{A1}$, $mus101^{D1}$ and $mus101^{D2}$. $mus101^{lcd}$ was uncovered by Df(1)HA92 and covered by Dp(1;f)LJ9 but not by Dp(1;f)LJ8.

e) Neuroblast mitosis and viability of *l(1)d.deg4* mutants.

Aberrant *l(1)d.deg4* cells had strongly overcondensed chromosomes or were polyploid or hyperploid (Fig. 5d,e). The *l(1)d.deg4* phenotypes of lethality and abnormal mitosis are also uncovered by Df(1)HA92 but *l(1)d.deg4* is not allelic to $mus101^{lcd}$.

Genotype	Aberrant(%)	Wild type	Ganglia	Col.	Fert.
a)					
mus101^{lcd}/sc^{8}Y	82 (80)	21	12	+	l
mus101^{lcd}/sc^{8}Y	150 (72.4)	57	12	-	l
mus101^{lcd}/mus101^{lcd}	160 (95)	8	13	+	l
mus101^{lcd}/Df(1)HA92	50(100)	0	3	+	l
b)					
mus101^{lcd}/sc^{8}Y /Dp(1;f)LJ8	117 (63)	68	12	+	l
mus101^{lcd}/sc^{8}Y /Dp(1;f)LJ9	0 (0)	128	10	+	m+
c)					
mus101^{lcd}/mus101^{A1}	10 (5.4)	183	5	+	fs
mus101^{lcd}/mus101^{D1}	8 (3.9)	204	6	+	fs
mus101^{lcd}/mus101^{D2}	9 (5.5)	156	9	+	fs
d)					
mus101^{A1}/FM6	3 (1.2)	257	9	+	f+
mus101^{D1}/FM6	0 (0)	139	5	+	f+
mus101^{D2}/FM6	2 (1.4)	138	5	+	f+
e)					
mus101^{lcd}/d.deg4	0 (0)	383	12	+	f+
d.deg4/Df(1)HA92	276 (82)	60	7	+	l
d.deg4/Y	303(100)	0	6	+	l
d.deg4/Y	306(100)	0	6	-	l

4.1.2 Embryonic nuclear division requires maternal *mus(1)101*$^{+}$ product.

To attempt to explain the female sterility of these mutants, I then examined the embryos they produced. One allele of *mus101*, *fs(1)K451*, has been shown to be defective in a factor required *in trans* for amplification of the chorion protein genes (Orr et al. 1984). The original mutagen sensitive alleles were isolated as male fertile conditional lethal mutations and maintained for ten years in compound-X stocks which rarely produce homozygous mutant females. I therefore established balanced stocks of *mus101*^{D1}, *mus101*^{D2}, and *mus101*^{A1} and investigated adult viability and fertility of female homozygotes at 25°C. I also examined chorion integrity under the highest power of the dissecting microscope. The results are recorded in Table 5. Two classes of female sterility are distinguishable, *mus101*^{K451} and *mus101*^{tsl} which lay eggs with fragile or incomplete chorions often lacking dorsal appendages, and *mus101*^{D1}, *mus101*^{D2}, and *mus101*^{A1} which all produce eggs with superficially normal chorions. I was stimulated by this observation to look for another possible cause of *mus101* female sterility.

At 18°C, *mus101*^{D2} homozygotes lay fertilised eggs, some of which develop into fertile adults. If, however, the homozygotes grown at 18°C are shifted to 25°C they become sterile after 2 days. Fertility is regained after a further 2 days at 18°C. I concluded from this that the *mus101*$^{+}$ product is essential for oogenesis, fertilisation or early embryonic development. In order to investigate whether maternally-contributed *mus101*^{D2} product causes a temperature sensitive effect on embryonic development I then investigated the fate of embryos laid by *mus101*^{D2} homozygous mothers grown at 18°C. Embryos laid at 18°C and shifted to 25°C or 29°C did not hatch. 2h collections of these embryos were incubated for a further 30min at either 18°C or 29°C and then fixed and stained with the fluorescent compound H33258 to reveal chromatin. This procedure yielded wild type and mutant embryos undergoing nuclear cycles 9-12. Figs. 6a and 7 show that wild type embryos incubated at both temperatures have normally condensed mitotic chromosomes. Tubulin and centrosomes were visualised by indirect immunofluorescence using antibodies YL1/2 and Rb188 respectively. Wild type embryos shifted to 29°C have regularly-spaced mitotic spindles of normal morphology, and at anaphase, only one Rb188-stained centrosomal structure at each spindle pole (Fig. 6c).

Embryos from *mus101*^{D1} and *mus101*^{A1} mothers show no sign of embryonic nuclear division and may not even be fertilised (not shown). Embryos from *mus101*^{D2} mothers, incubated at 18°C for 30 min are indistinguishable from those from wild type mothers. However, those shifted to 29°C reveal a temperature sensitive defect in the appearance of condensed chromosomes at anaphase (Fig. 6c).

Typically the chromosomes take the form of a mass of chromatin remaining on the metaphase plate while relatively thin streaks of chromatin are seen extending to the poles (Fig. 6b). This mutant phenotype is consistent with regional decondensation of chromosomes in the pericentric heterochromatin which, while permitting the kinetochores to segregate to the poles, would not transmit segregational forces to the chromosome arms, thus leaving them at the metaphase plate. Such extended chromatin might easily become tangled, further impairing chromatid segregation. Mitotic spindles, by contrast, are not significantly different in morphology to those of the wild type.

The fixation protocol for immuno-staining requires the use of 5μM taxol in order to preserve the structure of mitotic microtubules (Frasch et al. 1986), however, visualisation of chromatin with H33258 may be performed in the absence of the drug. The *mus101*^{D2} embryos still exhibited the same temperature sensitive chromosomal phenotype in embryos fixed without taxol (Fig. 7). The appearance of mitotic chromatin was also unchanged in wild type embryos at both temperatures. This indicates that the mutant mitotic phenotype is not due to any hypersensitivity to taxol at the nonpermissive temperature.

To test the paternal contribution of *mus101* product to embryonic mitosis, virgin *mus101*^{D2} female were mated to mutant and wild type (Canton S) males (Fig. 8). Paternal rescue was perhaps not to be anticipated because little of the zygotic genome is transcribed until cell formation in interphase 14 (Edgar et al. 1986). This experiment showed that paternal *mus101* does not influence the mitotic phenotype or viability of syncytial embryos derived from *mus101*^{D2} females.

Linked interphase nuclei are also seen in embryos incubated at 29°C, so the chromatin cycle continues without resolution of these aberrant mitoses (not shown). Fig. 8 also shows that the impediment to nuclear division in the mutant does not stop the cycle of proliferation of centrosomes. Two Rb188-stained centrosomes are visible at the poles of the aberrant mitoses in many mutant embryos. Centrosomes, detached from nuclei and nucleating free asters are also seen. This excess of free centrosomes can result from continued centrosome replication in the absence of nuclear division (Freeman et al. 1986).

Despite the superficially normal appearance of the chorions of eggs laid at 18°C by *mus101*^{D2} mothers there might nevertheless be some structural defect which would render embryos more susceptible to desiccation than their wild type counterparts. To control humidity, all collections were made on moist grape agar plates, and soaked filter paper included with the embryos during the 30min incubation. Furthermore, wild type embryos removed from their chorions may be desiccated over silica gel and lose about 10% of their volume without lethal effect (Section 2.4). It is therefore unlikely that the abnormal mitotic phenotype observed in *mus101*^{D2} embryos results from desiccation. Thus the temperature sensitivity of mitosis in these embryos probably results from thermosensitive maternal *mus101*^{D2} product or from a process rendered temperature sensitive by mutation in the *mus101* gene. I conclude that perhaps not all viable *mus101* alleles are defective in chorion amplification, and that defects in embryonic mitosis account for the sterility of the female sterile alleles producing eggs with intact chorions.

Table 5

Fertility and chorion integrity of *mus101* mutant alleles.

Different combinations of *mus101* alleles were tested for adult viability and, where appropriate, for male and female fertility. "ts" indicates that the mutation tested was similar to wild type (+) at 18°C but abnormal (-) when grown at 25°C. Chorions of eggs laid by females were examined for integrity. Chorions with holes or malformed dorsal appendages were laid by some mutant females (-). Gaps have been left in the table where larval lethality prevented further investigation.

mus101 allele	Viability	Fertility		Chorion appearance
		Male	Female	
D1/D1	+	+	-	+
D2/D2	+	+	ts	+
A1/A1	+	+	-	+
K451/K451	+	+	-	-
ts1/ts1	ts	+	-	-(18°C)
lcd/lcd	-			
ts1/lcd	ts		-	-(18°C)
ts1/Df(1)HA92	ts		-	-(18°C)

Fig. 6a,b

Temperature sensitivity of condensed mitotic chromosomes in syncytial embryos from *mus101*^{D2} mothers.

Close-up view of anaphase chromosomes of syncytial embryos fixed at nuclear cycle 10 in the presence of 5μM taxol and stained with the fluorescent dye H33258 (*bis*-benzimide). Embryos were laid and grown for 2h at 18°C and experimentally shifted to 29°C for 30min In the wild type embryo (a), chromatids are discrete and well-formed. However, in the embryo from *mus101*^{D2} parents (b), condensation is extremely abnormal. The bulk of the chromatin remains on the metaphase plate but centromeres are moved to the poles. Intervening chromatin is thin and stretched. The small bright dots are endosymbiotic micro-organisms (Wolstenholme 1965).

a) Wild type.

b) Embryo from *mus101*^{D2} parents.

b
a

Fig 6c

Temperature sensitivity of condensed mitotic chromosomes in syncytial embryos from *mus101*^{D2} mothers. Examination of the mitotic apparatus using indirect immunofluorescence microscopy.

Embryos from parents homozygous for *mus101*^{D2} (D2), or wild type (CS), were laid and grown for 2h at 18°C, and then shifted for 30min to the indicated temperature before fixation in the presence of 5μM taxol. The same microscope field is shown in each column. The rows indicate the stain used: H33258, YL1/2 and fluorescein-conjugated second antibody, Rb188 and rhodamine-conjugated second antibody were used to reveal DNA, tubulin, and a centrosomally-associated antigen, respectively. Maternally supplied *mus101*^{D2} product results in the appearance of abnormal anaphase chromatids at 29°C but not at 18°C. In contrast, the morphology of mitotic spindles does not appear to be noticeably abnormal at either temperature in the mutant embryos.

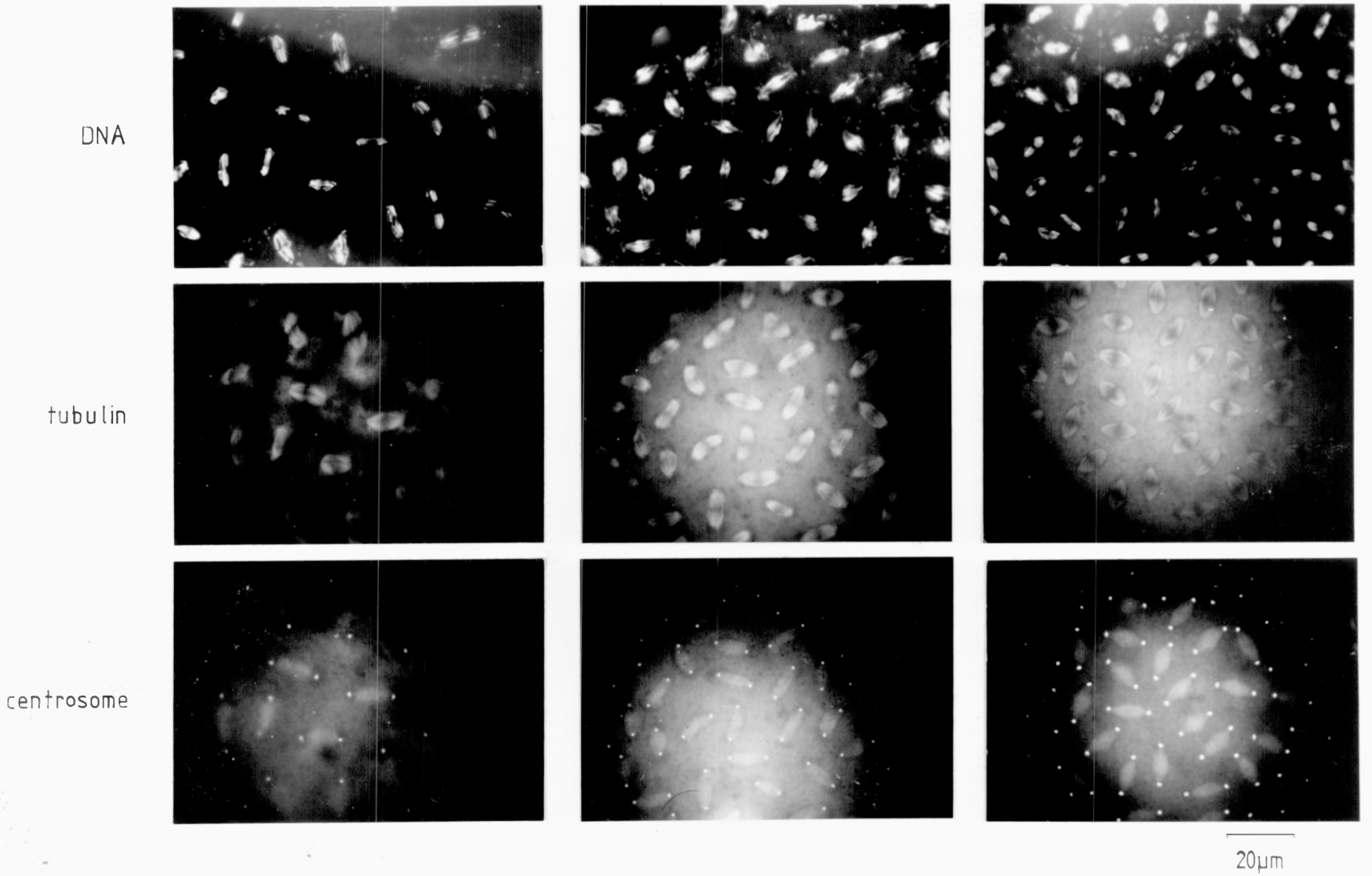
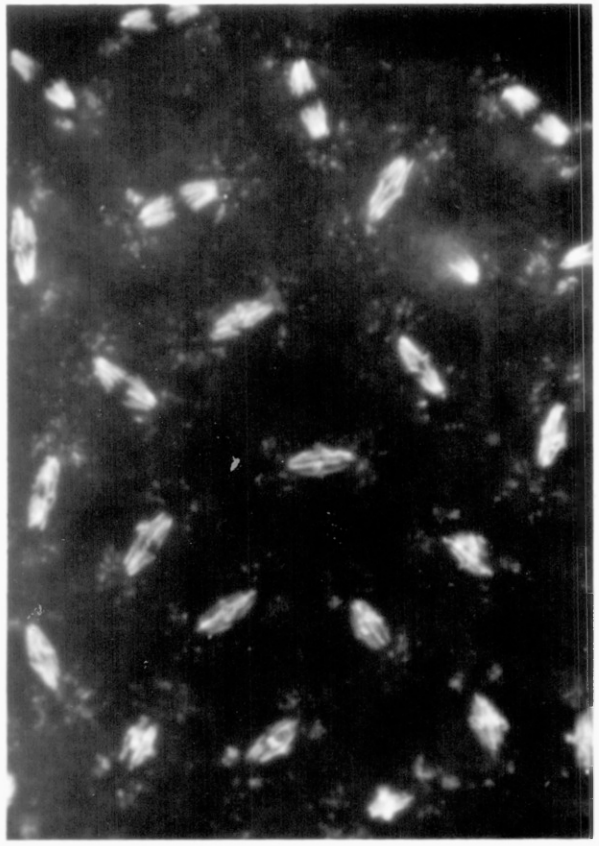
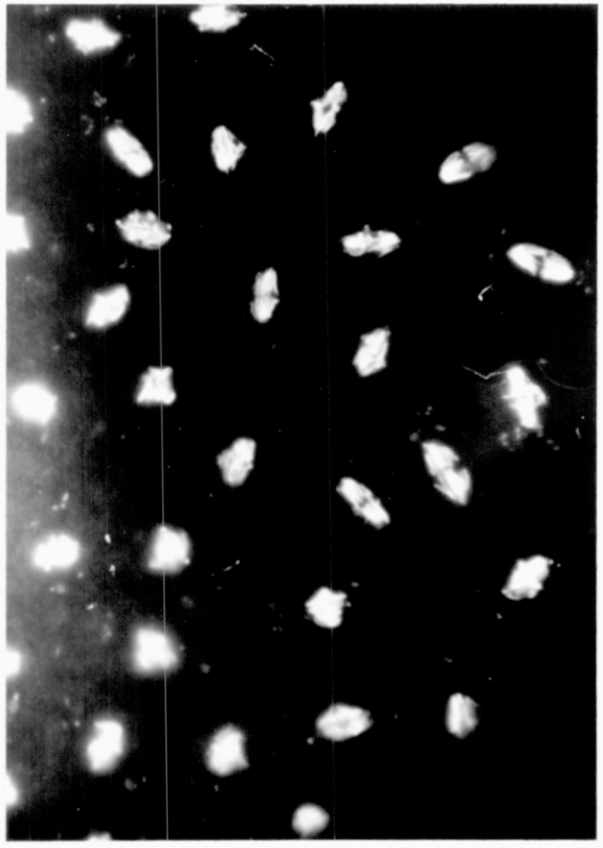

DNA
tubulin
centrosome
20µm
mus 101 18°C
D2xD2
mus 101 29°C
D2xD2
Canton S 29°C

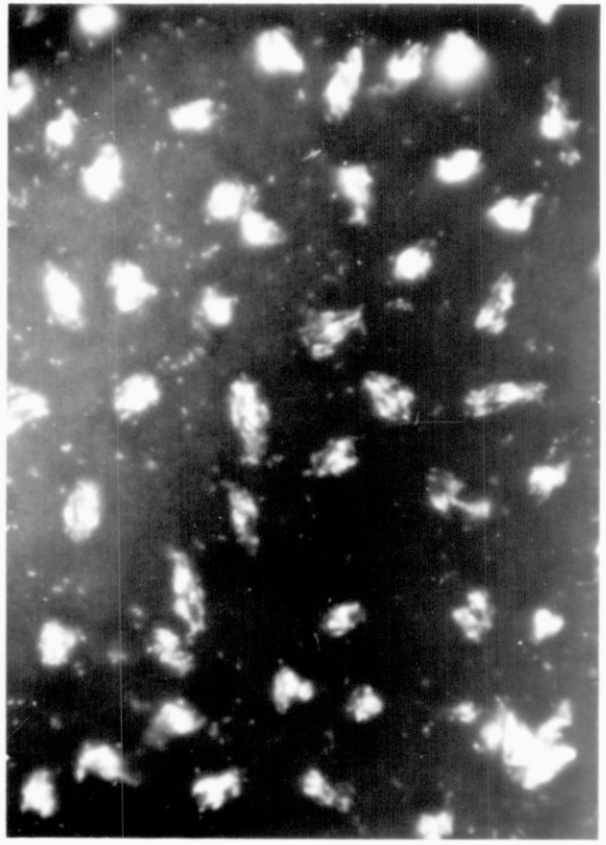
Fig. 7

Presence of taxol during fixation does not influence the appearance of abnormally condensed chromosomes in *mus101*^{D2} embryos.

This figure illustrates the results of several control experiments performed during the investigation of the temperature sensitivity of chromosomes in *mus101*^{D2} embryos. The presence or absence of the microtubule-stabilising drug, taxol, during fixation does not alter the condensation or morphology of wild type anaphase chromosomes, nor does the drug cause the characteristic defect seen in *mus101*^{D2}-derived embryos after shift to 29°C.







20µm

Canton S
+taxol 18°C

Canton S
-taxol 29°C

mus 101(D2xD2)
-taxol 29°

Fig. 8

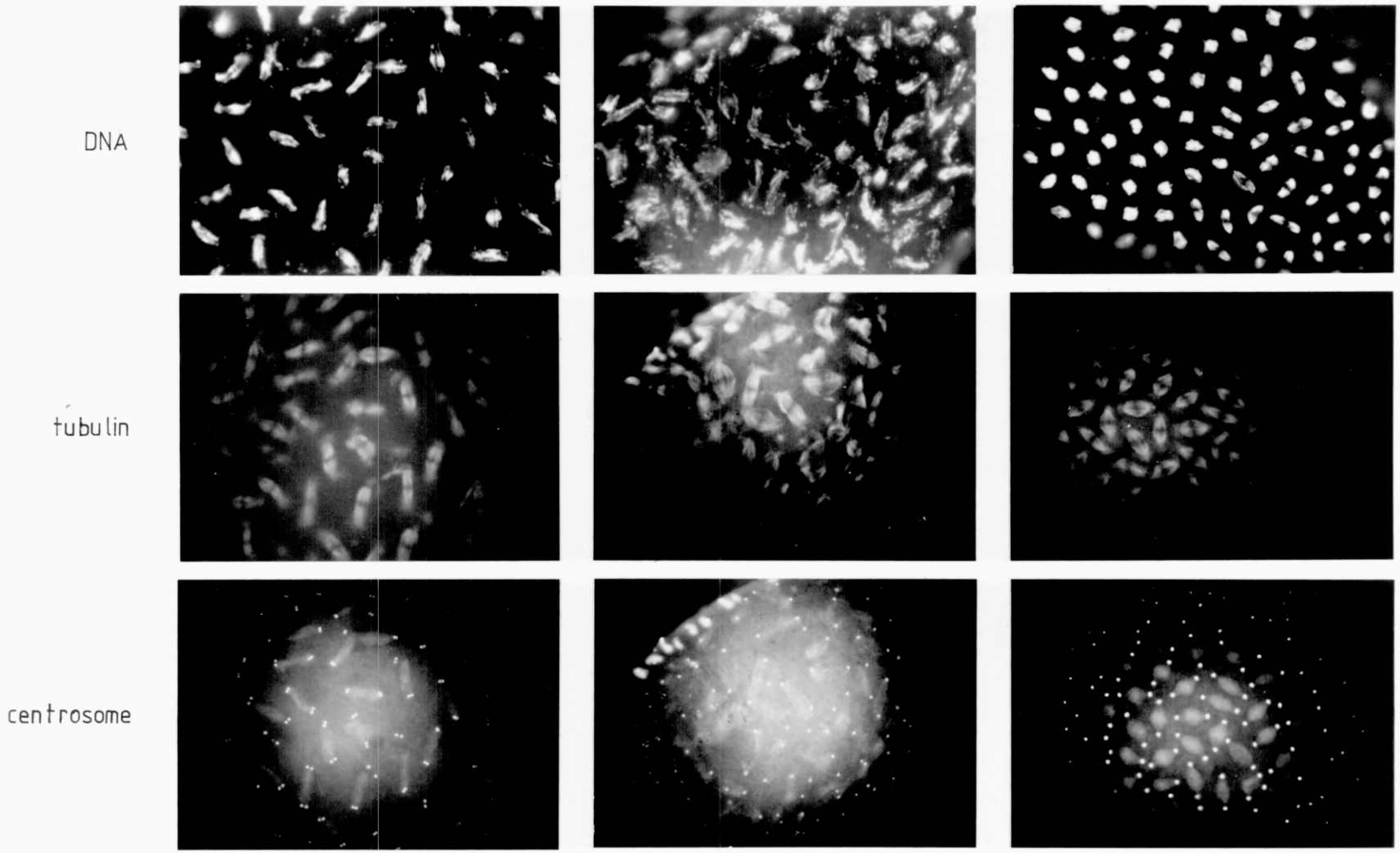
Table and figure.

Test of paternal rescue of the temperature sensitive defect in chromatin condensation in embryos from *mus101*^{D2} mothers.

Syncytial embryos from *mus101*^{D2} mothers were shifted from 18°C to 29°C. No difference in the frequency of mutant embryos with abnormally condensed chromatin was observed whether *mus101*^{D2} virgins were mated to *mus101*^{D2} or to Canton S (wild type) males (below). There is therefore no paternal rescue of the temperature sensitivity of anaphase chromatin in embryos from *mus101*^{D2} mothers. Embryos from the two matings, shifted to the restrictive temperature and stained as Fig. 6, are shown opposite. Note that in the embryo from *mus101*^{D2} parents, the centrosomes have divided in spite of the failure of nuclear separation (opposite page, lower left panel). This defect is common in mutant embryos at the non-permissive temperature and indicates the lack of coupling between cytoplasmic and nuclear cycles in syncytial embryos.

Shifted from 18°C to:

	18°C		29°C	
Embryos	abnormal	+	abnormal	+
Canton S	0	39	0	84
mus101^{D2} x mus101^{D2}	4	17	28	9
mus101^{D2} x Canton S	4	14	25	5


DNA
tubulin
centrosome
mus 101 29°C
D2♀xD2♂
mus 101 29°C
D2♀xCS♂
Canton S 29°C
20µm

4.1.3 *mus101* and *l(1)d.deg4* are closely linked but distinct genes.

Complementation analysis of *mus101*^{lcd} with the mitotic mutation *l(1)d.deg4* was of particular interest. Despite close proximity to *mus101* on the X chromosome (Fig. 10) *l(1)d.deg4* mutations form a different complementation group. Two larval lethal alleles in each complementation group (*mus101*^{lcd} and *mus101*^{sm}, *l(1)d.deg4* and *l(1)d.deg4*^{820}) were tested for viability in all four heterozygous combinations to arrive at this conclusion. Further evidence that the two genes are distinct is provided by the fact that *l(1)d.deg4* complements the abnormal lethal mitotic phenotype of *mus101*^{lcd} (Table 4e) and the female sterility of *mus101* allele *fs(1)k451*. Although some of the abnormal mitotic cells of *l(1)d.deg4* larvae (Fig. 5d) resemble those seen in *mus101*^{lcd} mutants (Fig. 5c) it is probable that these arise by different mechanisms. Most of the abnormal figures in *l(1)d.deg4* ganglia resemble extreme colchicine metaphases, with overcondensed chromosome arms widely separated but without sister chromatid disjunction. Hyperploid and polyploid (4N, 8N) cells are common in *l(1)d.deg4* but rare in *mus101*^{lcd} ganglia.

4.1.4 *mus(1)101* shows no heterozygous interaction with mitotic mutations on the third chromosome.

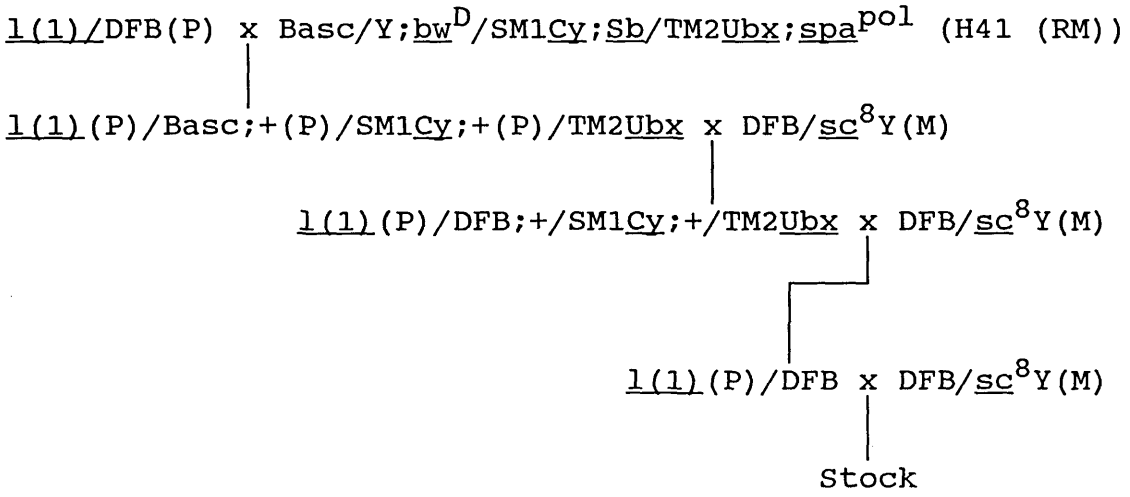
Many mutations have been discovered which produce abnormally condensed mitotic chromosomes or which affect the integrity of mitotic chromosomes. This is hardly surprising, given that chromosomes are complex structures. The products of many genes must be required, both within this structure, and also in the regulation of the changes in chromatin structure which occur during the cell cycle. Interactions might therefore be expected between mutations in genes encoding proteins which interact. Indeed, use has been made of such an approach in analysis of other macromolecular assemblies. Failure of mutations in the heterozygous state to complement the male sterility of β2 tubulin mutations has been employed to identify interacting microtubular proteins (Fuller et al. 1987). In order to investigate whether the *mus101* product interacts with any of the products of loci on the third chromosome known to be required for normal mitotic chromosome condensation I crossed *mus101*^{lcd} balanced heterozygous females to males of genotype *l(3)*/TM6B. This cross permits detection of mutations on the third chromosome which suppress the lethality of *mus101* to produce male progeny bearing the *mus101* chromosome. It would also be possible in the same cross to detect if the (normally recessive) mutant phenotype of the heterozygous *mus101* mutation is enhanced by the presence of the heterozygous mutation on the third chromosome. If such enhancement were to occur, the effect could be "dominant" lethality or sterility.

Mutations affecting mitotic chromosome condensation *12m137, e20, 1902, 2004, 2612, 1X-14, K43, g60A, 15m25, X11-10* and *l(3)snap* were all tested in this way but

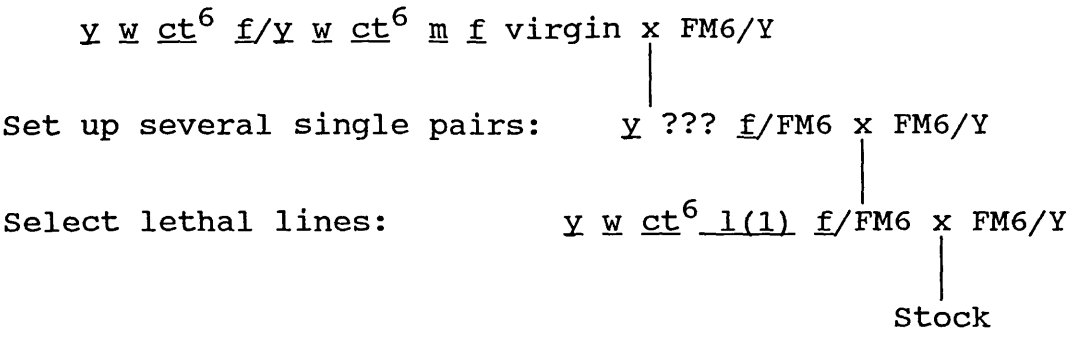
none of these altered the recessive lethality of *mus101*^{lcd}. This negative result is not of course evidence against an interaction between the products of these genes and it may be worthwhile to repeat these experiments using one of the "weaker" *mus101* female sterile alleles.

4.2 Mapping the *mus101*^{lcd} mutation by recombination.

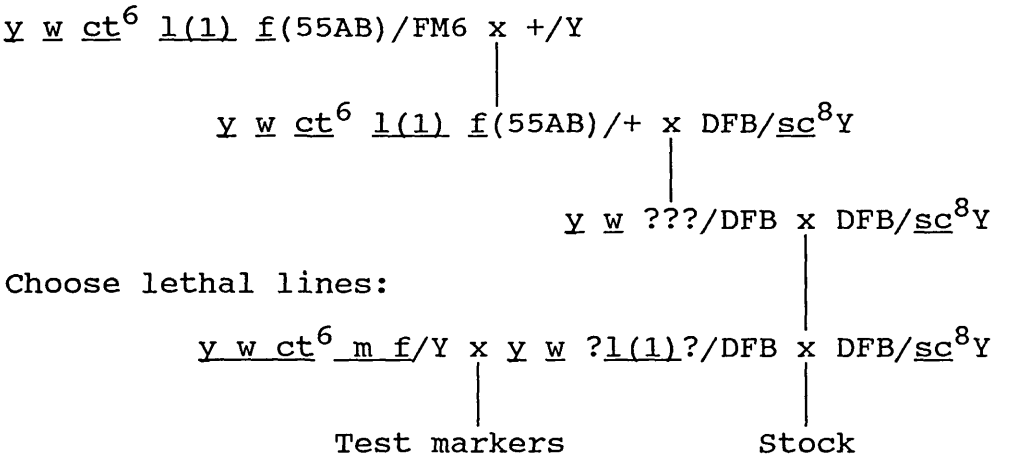
In order to be able to correlate the genetic localisation of *mus101*^{lcd} with the presence of P elements detected in DNA blotting experiments, I first sought to replace the autosomes of the MS55 stock with chromosomes which were free from P elements. The lethality and mutant mitotic phenotype were unchanged after the autosomes were replaced with those from the DFB / *sc*^{8}Y (M) strain. Replacement of the second and third chromosomes was achieved by crossing balanced mutant females to the multiple balancer stock H41(RM). The fourth chromosome comprises less than 2.5% of the genome and I considered for that reason it was relatively unlikely either to complicate genetic analysis by contributing mutant modifier loci or to contribute enough extra P elements to confuse molecular analysis of the MS55 mutant chromosome.



The lethal lesion was then mapped by recombining the MS55 chromosome with one bearing the visible markers *y w ct*^{6} *m f* and backcrossing the heterozygous F1 females in which recombination occurred to *y w ct*^{6} *m f* males. The F2 progeny were scored and are recorded in Table 6. The relative viability of male and female progeny in each recombinant class was used to locate the *mus101*^{lcd} lethality 8.7 m.u. proximal to *m* at map position 44.8 with a sample standard deviation of 0.18 m.u. Homozygous lines were established from viable male recombinants. Lethal recombinant lines were recovered by crossing single virgin heterozygous females from the mapping cross described above to balancer males as shown:



The markers were confirmed by backcrossing females from the lethal lines to *y w* ct^6 *m f* males. One representative lethal line *y w* ct^6 $mus101^{lcd}$ *f*(55AB) was chosen for further use. Next, the proximal portion of the X chromosome was replaced with P element-free wild type (Canton S) chromosome in the following crosses to generate recombinant lines that were f^+ but still $mus101^{lcd}$.



5 recombinant lines: *y w* ct^6 $mus101^{lcd}$ (55ABD, ABF, ABJ, ABL, ABT) were kept for further analysis. All are lethal in third instar larvae and exhibit the $mus101^{lcd}$ mutant mitotic phenotype in larval ganglion squashes.

Table 6

Frequencies of progeny obtained by recombining the MS55 chromosome with *y w ct*^{6} *m f*.

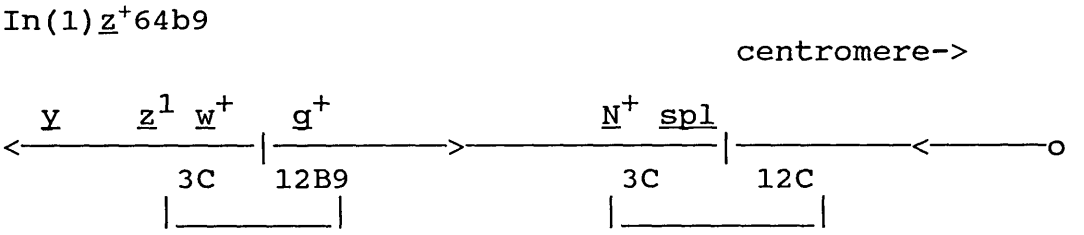
Parental	Female	Male
y w ct^{6} m f	419	401
+ + + + f	621	40
Recombinant		
y w ct^{6} + f	100	1
+ + + + f	113	129
y w + + f	108	13
+ + ct^{6} m f	101	84
y + + + f	7	0
+ w ct^{6} m f	3	3
y w + m f	6	3
+ + ct^{6} + f	9	0
y + + + f	1	0
+ w ct^{6} m f	0	0
y + + m f	1	0
+ w ct^{6} + f	0	0
	1489	674
	2163	

4.3 Use of deficiencies and duplications to locate the *mus101* gene.

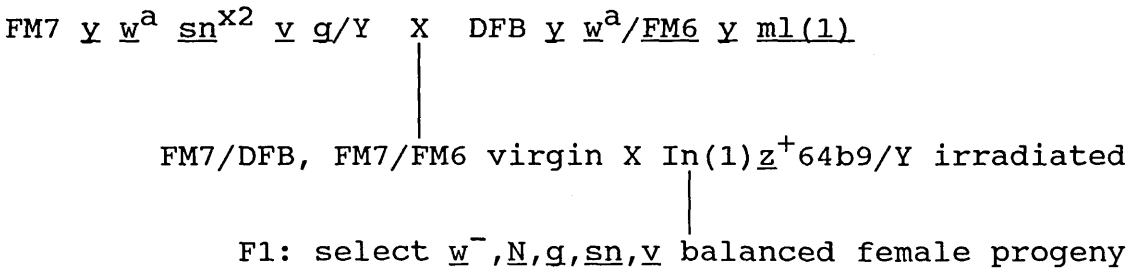
To improve the resolution available using existing duplications and deficiencies, I created new mutations by γ-irradiation and imprecise excision of a marked P element. I also made synthetic interstitial deficiencies (segmental aneuploids) using segments of appropriately marked T(1;Y) translocations.

4.3.1 γ-ray mutagenesis

To generate further rearrangements in the interval 12A6-D3 for use in mapping the *mus101* and *l(1)d.deg4* mutations, I created new mutations by γ-irradiation of In(1)z^{+}64b9 (Sorsa et al. 1973), a rearrangement which inverts a segment of the X chromosome between 3C1 and 12B9. The inversion is represented below, the wild type order of polytene chromosome banding is shown by arrowheads. The terms "proximal" and "distal" refer to the wild type order, not that of the In(1)z^{+}64b9 chromosome. Newly induced deficiencies extending from 12B9 distally may be detected as *g* and possibly *w* mutations. Mutations with a *N* phenotype may be deficiencies extending from 12C in a proximal direction. Examples of such deficiencies are shown below.



The following breeding scheme was used to generate large numbers of balancer-bearing virgin females to which irradiated males were mated. Males were subjected to 4500 rad from an 1800Ci ^{60}Co source and mated to a fourfold excess of virgins in groups of 50 males.



Several independently-derived visible mutations were recovered in balanced heterozygous females (Table 7). Because mutagenised chromosomes were balanced in F1

females and the F1 males were entirely FM7, balanced lines were easily established from non-virgin F1 females. *w* and *g* mutations would have been missed if carried in an FM6 heterozygous female (about 25% of the F1), so several bottles of these females were crossed to their FM7 brothers. The F2 generation was then examined for visible mutations.

These new mutations were tested for complementation of *mus101*^{lcd}, subsequently, salivary gland chromosomes of several of the mutants were examined as heterozygotes with In(1)*z*+64b9. Seven of the *N* lethals complement *mus101*^{lcd} lethality fully. A small deficiency, Df(1)w^{LCD}, was obtained which is lethal and deficient for *white* and *garnet*. This chromosome lacks the cytological region 12B2-9 (Fig. 10). Df(1)w^{LCD} fails to complement the lethality of *mus101*^{lcd} and *l(1)d.deg4*. Further, the lethality of Df(1)w^{LCD} is not covered by Dp(1;f)LJ9 suggesting that the deficiency also extends to one or more lethal loci within 3C. Indeed, Df(1)w^{LCD} fails to complement the lethality of the LS allele of *zw9*, a locus reported to be the next gene distal to *white* (Sorsa et al. 1973). Df(1)w^{LCD}/In(1)z^{+}64b9 females are red eyed, indicating that w^{LCD} is probably deficient for the whole *white* locus.

Breakpoints in the *garnet* gene at 12B6,7 would also be of use as landmarks in a molecular walk in the *mus101* region. *g* lies only 0.1 m.u. proximal to *mus101* (A. Schalet, pers. comm.). Two *garnet* alleles were isolated and identified by their failure to complement g^{S3}. T(1;2)g^{R1} is a viable but male sterile translocation. The heterozygous polytene chromosomes in Fig. 9 show the new order 1A-3C1|12B9|58C1,2-30A; 60A-58D1,4|12B5,6-20. The other allele, g^{J1} retains the original structure of the In(1)z^{+}64b9 chromosome. The g^{J1} lesion does not affect viability or fertility and is not visible in polytene chromosomes (not shown).

Table 7

Results of two schemes to recover cytologically visible deficiencies in the 12BC region.

a) Visible mutations were recovered from γ-irradiation of In(1)z^{+}64b9 (Section 4.3.1). This inversion places the w^{+} and g^{+} loci near to *mus101*. The progeny of irradiated male flies were screened for mutation of either or both of these eye colour markers.

b) Viable and lethal dysgenic revertants of P[ry^{+}]R301.2 were recognised by their *ry* phenotype (Section 4.3.2). Lethal revertants were presumably generated by imprecise excision of the marked P element. Two sources of transposase were used to mobilise the element: a P strain, π2, and the immobilised single P element Δ2-3 99B. The numbers of viable and lethal revertants were recorded. The greater proportion of lethals recovered using π2 transposase is probably due to new P insertions on the X chromosome. Each food vial contained the descendants of a single dysgenic male. the proportion of vials containing one or more revertants is also noted.

---

a)

Notch lethals	12
white viable and fertile	5
white, garnet lethal	1
garnet	2
vermilion lethal	1
singed female sterile	1
F1 recovered	11,306
F1+F2	3,046

b)

Transposase	DELTA2-3 99B	π2;ry^{506}
Revertants	83 (40 vials >=1)	24 (16 vials>=1)
Total F2	4709 (80 vials)	3298 (53 vials)
Viable revertants	67	17
Lethal revertants	5	3
Not tested	11	4 (1 sterile)
Percent revertants	(1.76)x2=3.5	(0.7)
% of revertants lethal	7	15
Examined cytologically	5	2

Fig. 9

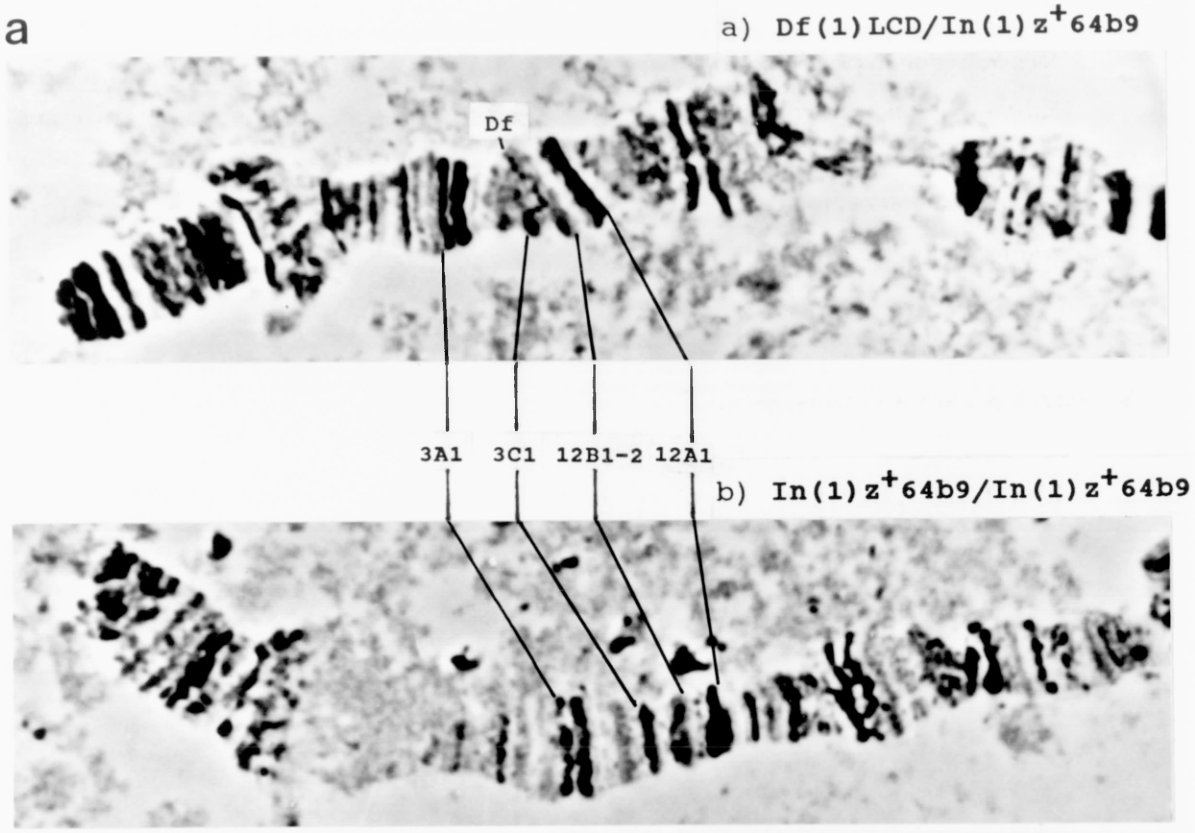
Rearrangements generated by gamma ray mutagenesis of In(1)z^{+}64b9

Salivary gland polytene chromosomes were prepared by the lactic propionic orcein method (Section 2.3.2) from larvae of the indicated genotypes. The numbered divisions of Bridges (1938) and Bridges and Bridges (1939) are indicated on the photographs.

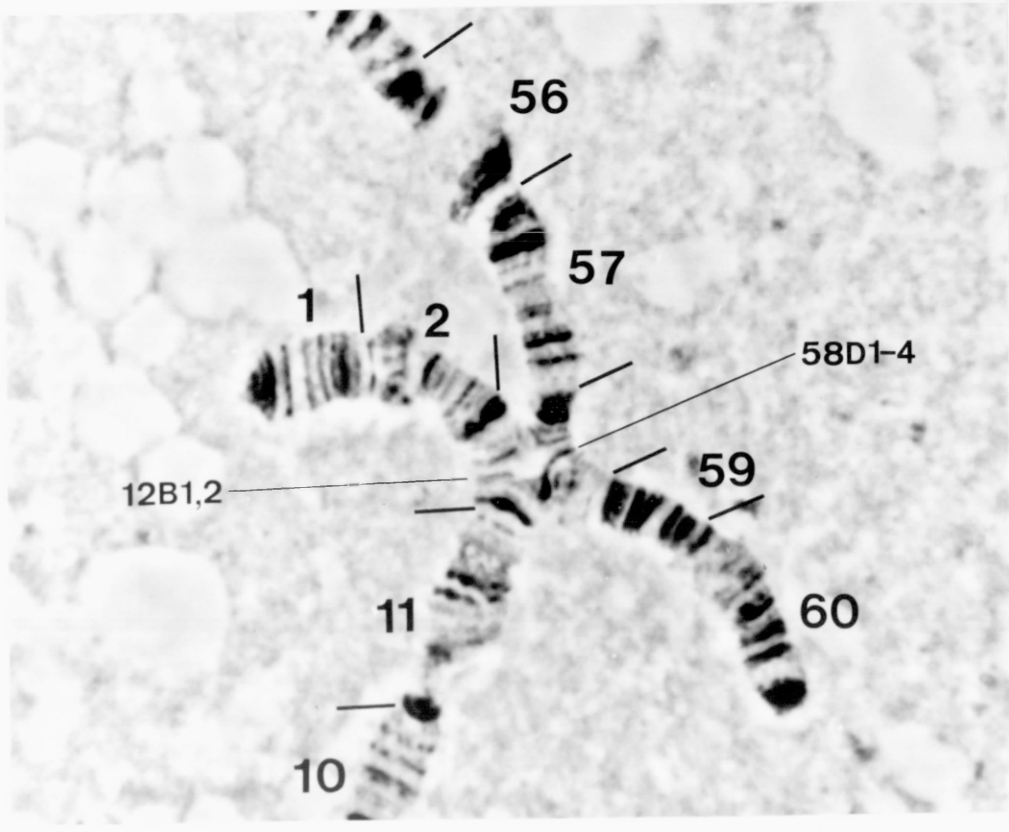
a) Comparison of Df(1)w^{LCD}/In(1)z^{+}64b9 heterozygote with the In(1)z^{+}64b9 homozygous parent chromosome.

b) T(1;2)g^{R1}/In(1)z^{+}64b9 heterozygote.

a


a) Df(1)LCD/In(1)z+64b9
Df
3A1
3C1
12B1-2
12A1
b) In(1)z+64b9/In(1)z+64b9

b


56
57
1
2
58D1-4
12B1,2
59
60
11
10

4.3.2 Use of marked P elements to create mutations by imprecise excision.

I have also attempted to generate lethal mutations including deficiencies within a defined chromosomal region by making use of imprecise excision of a transposable element within that region. Tsubota and Schedl (1986) used hybrid dysgenesis to generate revertants of *rudimentary* mutations caused by insertion of a P element at the 5' end of the gene. Using the $\pi 2$ P strain as a source of transposase and scoring revertants by their *r* phenotype, they recovered revertants at a frequency of 0.2% including 3 precise excisions, 6 inversions, 11 insertions ranging from 8bp to a whole P element, and deficiencies from 1.4-6.5kb. However, their scheme would not have recovered lethal revertants. Salz et al. (1987) recovered revertants of a P element insertion in the *Sxl* gene at a frequency of 7%. Deletions, the smallest of which was 5kb in length, were recovered at a frequency of 0.1%.

I attempted to mobilise the single marked P element insert P[ry^+]R301.2 at 12C8-D1 (deCicco and Spradling 1984) with the intention of recovering a series of cytologically visible deficiencies. Two sources of transposase were used. $\pi 2;ry^{506}$, a wild type P strain which has been made homozygous for the ry^{506} marker, and the single, modified element Δ2-3 99B.

The element Δ2-3 99B (Robertson et al. 1988) produces transposase constitutively in somatic tissues as well as germline. The element itself is mobilised at a remarkably low frequency. As a source of transposase, it has the advantage that it may be introduced and removed in two genetic crosses leaving stable mutations in a background free from active P elements. In addition, the Δ2-3 99B element is, like the R301.2 target, marked with ry^+ so that ry^- revertants generated in the second series of experiments should be completely free from P elements and, it is hoped, free from mutations in regions other than the immediate vicinity of the original P element insertion. The breeding schedule I used is shown below.

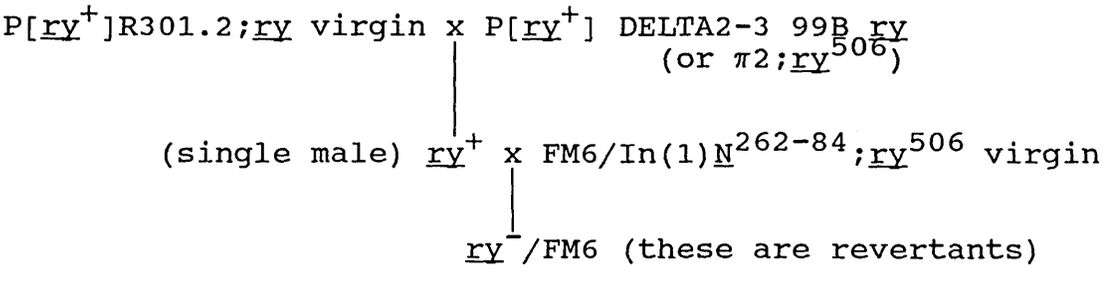
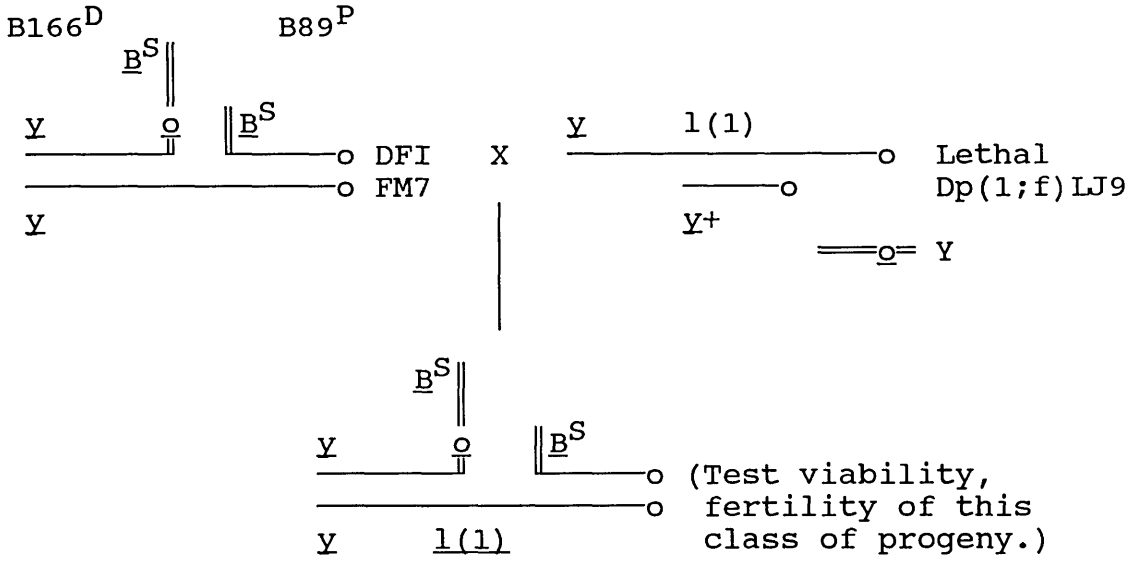


Table 7b summarises the results obtained. None of 7 lethal revertants examined was allelic to either *mus101*^{lcd} or *l(1)d.deg4* or exhibited any cytologically visible lesion in salivary chromosome squashes. The lethality of the Δ2-3 99B-sponsored revertants must result from the disruption or deletion of a gene (or genes) adjacent to the R301.2 insertion site. Larger lesions might be recoverable in a larger experiment However, it is clear that only a small proportion of R301.2 ry^- revertants are lethal. A

more certain way to make use of the R301.2 insertion would be to use the ry^+ gene it bears as a dominant marker to recover X-, or γ-ray induced deficiencies.

4.3.3 Use of T(1;Y) segments to create interstitial deficiencies.

A considerable collection of T(1;Y) stocks are available which permit the construction of complementary interstitial duplications and deficiencies (Stewart and Merriam 1975). While the breakpoints of deficiencies synthesised from T(1;Y) segments are more difficult to ascertain with confidence than those of conventional deficiencies, the translocation breakpoints are very useful as landmarks with which to connect mapping of a genetic function to a chromosomal walk. I have synthesised 5 interstitial deficiencies (DFIs) from pre-existing T(1;Y) stocks with different X breakpoints and have tested these for complementation of the lethality of *mus101*^{lcd} and *l(1)d.deg4*. An example of such a test cross in which the *y* and B^S markers are used to follow the various chromosomes is shown below:



Results of these experiments are shown in Table 8

4.3.4 The cytological location of *mus101* and *d.deg4* in polytene chromosomes.

The results of the cytological analysis of the duplications and deficiencies synthesised above, as well as the confirmed positions of previously published rearrangements used in mapping these two lethal mutations are summarised in Fig. 10. *mus101*^{lcd} is uncovered by Df(1)HA92, placing it in 12A6-D3, it is also uncovered by Df(1)w^{LCD}, refining the location to 12B2-9. The X breakpoint of T(1;Y)B166 is in 12AB, however, the translocation itself has a *garnet* phenotype indicating that the translocation breakpoint is within the *garnet* locus at 12B6,7 or close enough to inactivate the gene by heterochromatic position effect. Because none of the interstitial

deficiencies with the distal element of B166 uncovers the lethality of *mus101*^{lcd}, the mutation must be located distal to *garnet*. This conclusion is strengthened by the observation that *mus101*^{lcd} is not covered by Dp(1;f)LJ8 which variegates for *garnet*. Thus I conclude that *mus101* lies in the interval 12B2-6. *l(1)d.deg4* is nearby and has not been separated from *mus101* using any of these deficiencies and duplications. However, as mentioned above, female *in trans* heterozygotes for the two mutations are viable and fertile so *mus101* and *l(1)d.deg4* are not allelic.

Table 8

Mapping *mus101* and *l(1)d.deg4* with interstitial deficiencies generated from T(1;Y) segments.

a) Interstitial deficiencies were constructed by combining proximal "^{P}" and distal "^{D}" X chromosome segments of T(1;Y) translocations. "+" denotes that the interstitial deficiency complements the mutation tested and therefore carries the wild type locus, "ND" denotes "result not determined". "Viability" refers to the phenotype of males bearing the interstitial deficiency, "ms" indicates male sterility.

b) The following 3 interstitial deficiencies were also synthesised but were not tested for complementation of *mus101* or *d.deg4* lethality because the markers borne on the T(1;Y) segments did not permit unambiguous identification of all classes of progeny.

---

a)

Stock	Dfi	Phenotype	Viability	mus101	d.deg4
$B166^{D}B89^{P}$	12AB;12B9,C1	$\underline{B}^{S}\underline{B}^{S}$ $\underline{g}$	-	+	+
" $B32^{P}$	" ;12DE	"	-	+	ND
" $B24^{P}$	" ;12E	"	-	+	ND
$B136^{D}B32^{P}$	12C;12DE	"	-	+	+
" $B24^{P}$	" ;12E	"	-	+	ND
b)					
$B89^{D}$ $B1^{P}$	12B9,C1;12C	$\underline{y}^{+}\underline{y}^{+}$	ms		
" $B54^{P}$	" ;12E	"	-		
$B32^{D}$ "	12DE; "	"	+		

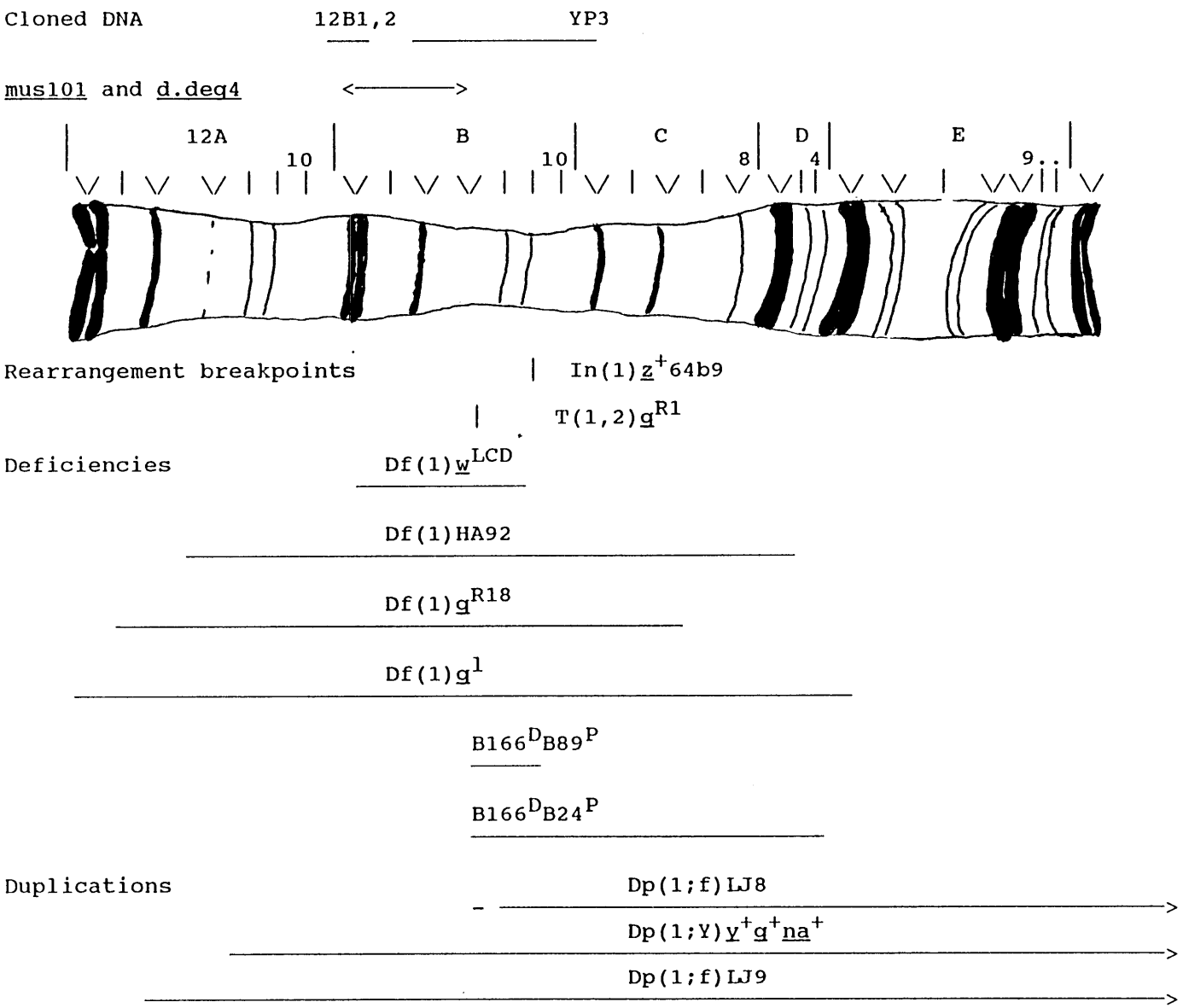
Fig. 10

Cytological mapping of *mus101* and *d.deg4*.

The positions of cytologically visible rearrangements used in mapping these loci are shown below the standard salivary gland polytene chromosome map drawn after Bridges (1938). Horizontal bars show the regions deficient in deficiencies and present in the duplications.

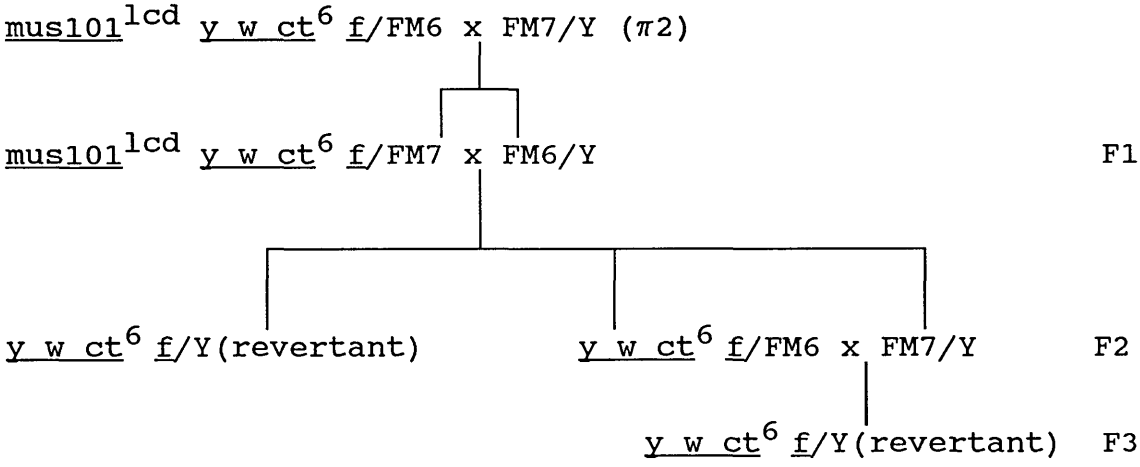
Both *mus101* and *l(1)d.deg4* lie within the cytological interval 12B1,2;12B6,7. These lethal mutations are uncovered by Df(1)w^{LCD}, Df(1) HA92, Df(1)g^{R18}, Df(1)g^{1} but not by deficiencies extending in a proximal direction from the breakpoint of T(1;Y)B166 (Table 8). Both mutations are covered by Dp(1;Y)$y^{+}g^{+}na^{+}$ and Dp(1;f)LJ9 but not by Dp(1;f)LJ8. The position of the *garnet* gene (*g*) at 12B6,7 is a useful landmark in the cytogenetic analysis of this region. The breakpoints of T(1,2)g^{R1} and T(1,Y)B166 are associated with *g* mutant phenotypes. Dp(1;f)LJ8 variegates for *g*.

The positions of *in situ* hybridisation of probes from the *YP3* walk and of clones isolated with microdissected probes are shown above the chromosome.


Cloned DNA
12B1,2
YP3
mus101 and d.deq4
12A
B
C
D
E
10
10
8
4
9
Rearrangement breakpoints
In(1)z+64b9
T(1,2)gR1
Deficiencies
Df(1)wLCD
Df(1)HA92
Df(1)gR18
Df(1)gl
B166DB89P
B166DB24P
Duplications
Dp(1;f)LJ8
Dp(1;Y)y+g+na+
Dp(1;f)LJ9

4.4 What is the *mus101*^{lcd} lesion?

During PM hybrid dysgenesis P elements are mobilised resulting in mutations caused by new insertions. Imprecise excisions, inversions and other rearrangements may also result. Other types of transposable element-induced hybrid dysgenesis such as the IR (e.g. Fawcett et al. 1986) and *hobo* (e.g. Yannopoulos et al. 1987) systems may be transpositionally active during mutagenic crosses designed to mobilise P elements. P element insertions may often be distinguished by their relatively high rate of reversion in dysgenic crosses. For this reason, I attempted to generate revertants by re-exposing the *mus101*^{lcd} *y w ct*^{6} *f* recombinant chromosome to P elements from a derivative of the active P strain π2 bearing the FM7 balancer:



The main reversion experiment was performed at 25°C but several vials of F1 first instar larvae were transferred to 29°C until eclosion, females from these vials were mated to an excess of wild type males at 25°C on well yeasted medium and dissected after 5 days to measure the proportion of dysgenic ovaries (%GD). This P-M dysgenic cross gave 80% ovarian dysgenesis but yielded no viable male revertants despite 9513 F2 chromosomes and 1233 of the F3 recovered in heterozygous females. There are several possible explanations why *mus101*^{lcd} fails to revert during hybrid dysgenesis. The mutation might be a chromosomal rearrangement sponsored by P element transposition. However, salivary gland polytene chromosomes from *mus101*^{lcd}/+ heterozygous larvae reveal no cytologically visible lesion in division 12. Secondly, The mutation might be caused by the insertion of a P element which had subsequently been damaged, perhaps in its terminal repeats, such that it was no longer a substrate for the transposition reaction. Thirdly, the mutation might be a spontaneous mutation selected during P mutagenesis or a relic of a former P insertion which had excised imprecisely, damaging the *mus101* gene. If the third reason is the case, the *mus101*^{lcd} allele would be unsuitable to use to isolate the *mus101* gene by transposon tagging. To investigate these possibilities further, the *mus101*^{lcd} chromosome was probed for P elements by *in situ* hybridisation.

4.5 Molecular cloning of a P element insertion site at 12C1 in the *mus101*^{lcd} chromosome

4.5.1 *In situ* hybridisation reveals a P element at 12C1

Within the region of the X chromosome which is absent in Df(1)HA92 (Fig. 11a) a P element probe (pπ25.1, O'Hare and Rubin 1983) hybridised with *mus101*^{lcd}/*y w ct*^{6} *m f* salivary gland chromosomes reveals a single site of hybridisation *in situ* at 12C1 (Fig. 11 b,c). The molecular cloning of this element provided the first molecular step toward isolation of the *mus101* gene.

4.5.2 Isolation of clones of genomic DNA

Two reasons justified the isolation of the chromosomal DNA, containing the P element at 12C1, from the *mus101*^{lcd} chromosome. Firstly, I was not convinced that the level of resolution provided by *in situ* hybridisation and deficiency mapping was sufficient to exclude this P element as a possible cause of the *mus101*^{lcd} mutation. Also, were this a defective P element it would not necessarily have reverted during hybrid dysgenesis. Secondly, if not directly responsible for the *mus101*^{lcd} mutation this P element would still provide a convenient starting point from which to proceed to the *mus101* genomic locus by chromosome "walking".

In order to isolate the 12C1 P element and sequences flanking the insertion site, a genomic library was prepared from mutant flies using a restriction enzyme which does not cleave within the 2.7kb sequence of the complete P element (O'Hare and Rubin 1983). *y w ct*^{6} *mus101*^{lcd} *f* (55AB)/FM6 DNA was cleaved with the restriction enzyme *Bam*HI and ligated into the lambda replacement vector EMBL4 (Frischauf et al. 1983) which had also been digested with *Bam*HI. The ligation products were packaged *in vitro* (Hohn and Murray 1977) and introduced into *E. coli* Q358. An aliquot of this library was grown on the P2 lysogen *E. coli* Q359 which supports the growth of *spi*$^{-}$ recombinant phage but not that of the wild type vector. By counting the total plaque forming units (p.f.u., or viable phage particles) and comparing the number of plaques produced on the two strains I determined that 58% of the phage were recombinants, a total of 4.5×10^{4}. The haploid *Drosophila* genome is approximately 1.7×10^{5}kb (Rasch et al. 1971) so if the average insert size of the clones is 10kb, the library would be the equivalent of 1.3 diploid genomes.

The entire library was plated on *E. coli* Q358 because the plaque size was larger than that on the selective host. Two positive clones ϕAB1 and ϕAB3 were selected by hybridisation with the pπ25.1 P element probe (O'Hare and Rubin 1983) and purified to homogeneity following replating and re-screening. DNA from these clones was mapped using restriction enzyme digestion. DNA blots of these digests were hybridised with the P element probe used to select the phage. This enabled flanking restriction

fragments with no P element homology to be identified and subsequently radiolabelled for *in situ* hybridisation to polytene chromosomes. A 2kb *Bam*HI-*Xba*I fragment of ϕAB1 hybridised to 14B and a 1.35kb *Ava*II-*Hind*III fragment of ϕAB3 to 12C1 (data not shown). The 6.02kb *Bam*HI insert from ϕAB3 was subcloned into the plasmid vector Bluescribe (pBs^+, Stratagene) to form the plasmid pAB135.

The wild type sequence corresponding to the 12C1 insertion site was then obtained from the Canton S (M) genomic library prepared in the laboratory of Dr V. Pirrotta (Baylor Coll. Med. Houston Tx. USA) by ligation of partially Sau3AI digested DNA into the *Bam*HI site of EMBL4 vector. Of 6 positive clones isolated, 2: ϕCS2, ϕCS4 were chosen for further analysis. ϕCS2 was digested with *Eco*RI and the fragments ligated into pBS^+ to form subclones pCS2R-1, 7, 8. The 5.5kb *Eco*RI insert of pCS2R-8 contains the wild type 5.4kb *Bam*HI fragment homologous to the *Bam*HI insert of pAB135.

Fig. 11

Molecular cloning of a P element insertion site at 12C1 from the *mus101*^{lcd} stock 55AB.

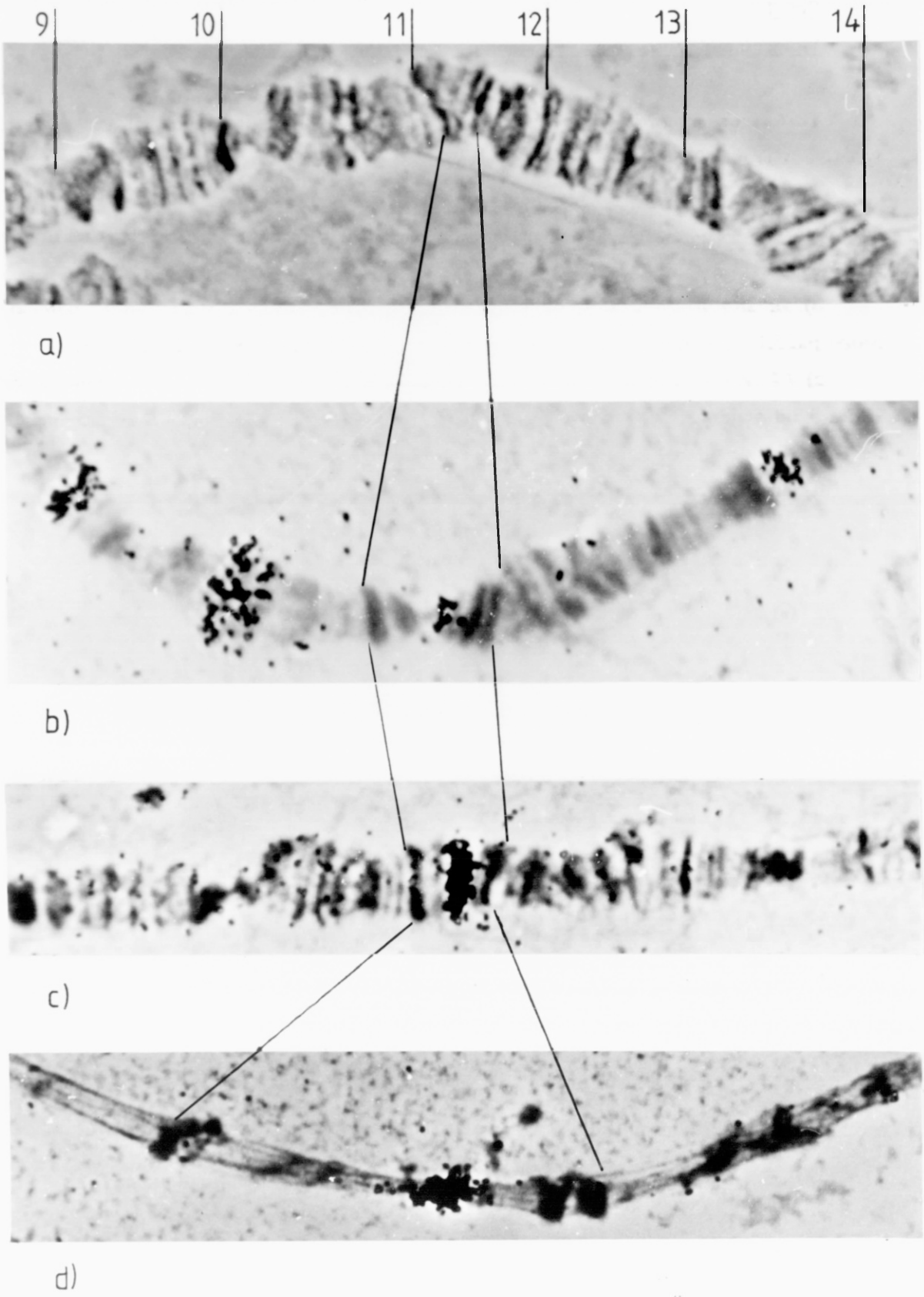
This figure records the progress from cytological mapping to isolation of wild type DNA from 12C.

a) Propionic lactic orcein squash of polytene chromosomes from a Df(1)HA92/+ heterozygous larva. Lines show the extent of the deficiency relative to the other chromosomes shown.

b) *In situ* hybridisation of *mus101*^{lcd}(55AB) heterozygote with the P element probe pπ25.1. A single signal was found within the region defined by Df(1)HA92.

c) After isolating DNA corresponding to the insertion site of this P element at 12C1, wild type polytene chromosomes were probed with a flanking DNA fragment, the 5.5kb insert of pCS2R-8 (Fig. 13).

d) The same as c), but a stretched chromosome.


9
10
11
12
13
14
a)
b)
c)
d)

4.5.3 The 12C1 P element is adjacent to, but does not disrupt the *YP3* transcription unit.

To determine if the *mus101*^{lcd} P element was inserted within a transcription unit, blots of developmentally-staged *Drosophila* RNA were probed with the insert of pCS2R-8. A single 1.5-1.7kb transcript was detected which was extraordinarily abundant in adult female polyadenylated RNA but undetectable in other developmental stages from embryo to mid-pupation (Fig. 14). This specific expression pattern and the cytological location of the pCS2R-8 sequence *in situ* at 12C1 (Fig. 11d) were similar to those of the *Yolk Protein 3* (*YP3*) gene. *YP3* was first isolated by Barnett et al. (1980). Subsequently, the structure of the transcription unit (Hung et al. 1982) and the nucleotide sequence of the genomic locus and of cDNAs have been determined (Yan et al., Garabedian et al. 1987). To confirm the identity of the female-specific transcription unit homologous to pCS2R-8 I probed restriction digests of the pAB135 and pCS2R-8 clones with a clone pGEM.YP3 containing a 1.2kb *Bam*HI-*Hind*III-cut genomic DNA fragment including the 3' end of the *YP3* gene, obtained from the laboratory of Dr Mary Bownes. Fig 13a shows a comparison of the restriction maps of the pAB135 and pCS2R-8 clones and a summary of results of Southern blot analysis of these clones probed with pπ25.1 and pGEM.YP3. The figure shows that pAB135 contains a 620bp defective P element inserted approximately 1.6kb 3' to the position of the 3' end of the YP3 transcription unit. Because no other transcripts homologous to pCS2R-8 are detected in blots of wild type RNA the 620bp P element is unlikely to be inserted within any other transcription unit (Fig. 14d).

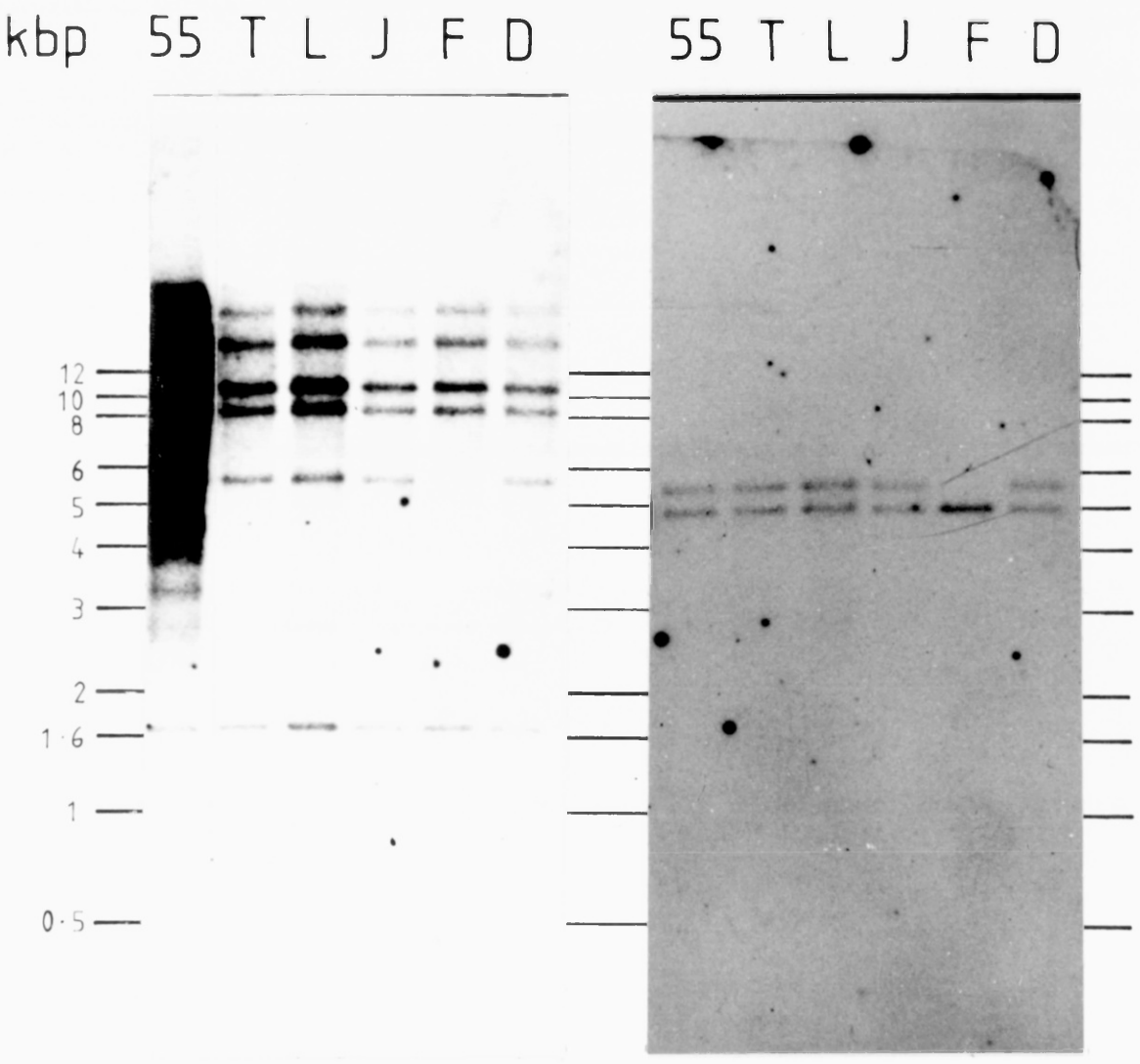
4.5.4 The *mus101*^{lcd} mutation is not due to a current P element insertion.

As the above conclusion is however limited by the sensitivity of RNA blotting, I chose a genetic means to confirm that the 620bp P insertion was not responsible for the *mus101*^{lcd} mutation. *mus101*^{lcd} recombinant lethal chromosomes were probed by genomic DNA blotting for the presence of P elements. Fig. 12 shows *Bam*HI digests of DNA from DFB-balanced heterozygotes of the original *mus101*^{lcd} (MS55) stock and *ywct*^{6}*mus101*^{lcd} recombinants 55ABD, F, J, L, T (Section 4.2) probed with P element probe pπ25.1 and flanking probe pCS2R-8. All of these recombinants retain the lethality and mutant mitotic phenotype of *mus101*^{lcd} but the 55ABF recombinant has lost the 12C1 P element. Thus the 620bp element is not responsible for the *mus101*^{lcd} mutation. Although the option of cloning *mus101* by P element tagging was not available, the P element had provided an entry point to the region from which to isolate the gene by chromosome walking.

Fig. 12

The *mus101*^{lcd} mutation is not caused by a current P element insertion.

The aim of this experiment was to attempt to correlate the lethality of *mus101*^{lcd} with the P element at 12C by probing the recombinants described in Section 4.2. A Southern blot of *Bam*HI-cut genomic DNA from DFB-balanced heterozygotes bearing the original chromosome or its recombinant derivatives 55AB,D,F,J,L,T were probed with the P element pπ25.1. After washing, the blot was reprobed with the insert of a plasmid, pCS2R-8, containing DNA flanking the 12C P element insertion site (Fig. 13a). Note that while 55ABF has lost the ≈6kb band characteristic of the 620bp insertion at 12C, this recombinant chromosome still carries the *mus101*^{lcd} mutation. Therefore the P elements at 12C is not responsible for the mutation.


P element
flanking
kbp
55 T L J F D
55 T L J F D
12
10
8
6
5
4
3
2
1·6
1
0·5

4.5.5 Chromosome walking and orientation of genomic clones

This technique (Bender et al. 1983) consists of isolating a series of overlapping clones by repeated rounds of hybridisation-selection using previously isolated clones as probes. The molecular landmarks which would form the endpoints of this walk were the inversion breakpoint of In(1)z^{+}64b9 at 12B9 and the distal breakpoint of Df(1)w^{LCD} at 12B1,2. The first objective was to identify clones extending in a centromere-distal direction from the *YP3* gene. As the orientation of the phage clones with respect to *Drosophila* chromosomes was not clear at this time, it was necessary therefore to extend the chromosomal walk outwards in both directions and then to hybridise probes made from opposite ends of the walk to polytene chromosomes in order to orient the clones with respect to the centromere. To obtain such a walk, duplicate filters obtained from plates of the Canton S library were reprobed with pCS2R-1 and pCS2R-8. 4 clones hybridising only with pCS2R-8 (Class R) and 6 hybridising only with pCS2R-1 (Class L) were obtained. 7 clones were also obtained which hybridised with both probes. Class R clones were arbitrarily defined as extending rightward from the P insertion site, Class L, leftward. In addition, Mart Speek used the pCS2R-8 probe to isolate a 35kb cosmid clone pSM2-41 from a library he created in the vector SMART2 (Speek et al. 1988) by *Bam*HI partial digestion of wild type genomic DNA. This cosmid extends the walk in a rightward direction. the 51kb covered by these clones is shown in Fig. 13b. The position of *Eco*R (R), *Bam*HI (B) and *Sal*I (S) sites in Canton S genomic DNA was confirmed by genomic DNA blotting using as probes gel-purified fragments taken from the clones in the walk (data not shown). The orientation of the walk was obtained by probing In(1)z^{+}64b9 polytene chromosomes with a 13kb *Eco*RI fragment of ϕCS22 (ϕCS22R13) which is the leftmost end of the walk. This hybridised to 12C1, centromere-proximal to the inversion breakpoint (not shown). The 2.3kb *Eco*RI fragment of pSM2-41 which is at the rightmost end of the walk, some 36kb from the ϕCS22R13 fragment, hybridised to 12B2-6, distal to the inversion breakpoint (Fig. 15).

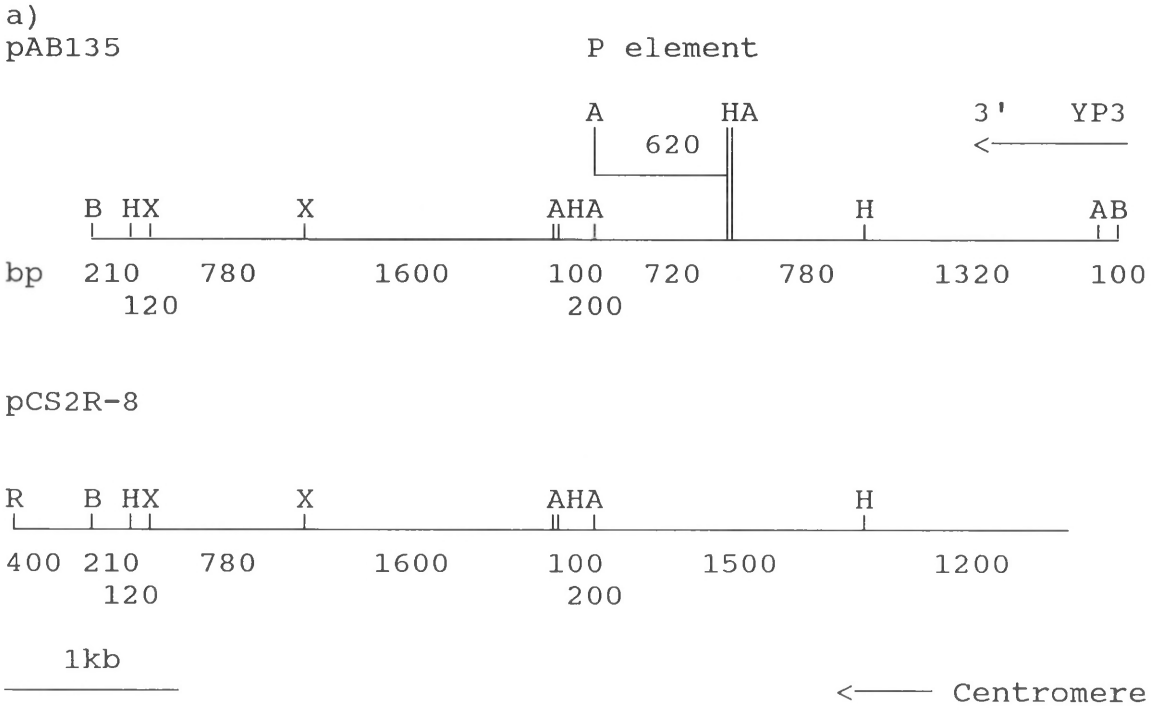
Transcription units were located within the walk by probing blots of developmentally-staged RNA. These transcription units are described in Fig. 14.

Fig. 13

Isolation of clones of genomic DNA from 12BC

a) Restriction maps of the original P element-containing plasmid pAB135 obtained from the *mus101*^{lcd} stock MS55AB. Also shown is its wild type counterpart pCS2R-8, isolated by hybridisation with pAB135. The distal end of pAB135 (at right) contains about 600bp of the 3' end of the YP3 transcription unit. The position of this transcription unit was obtained by hybridisation of pCS2R-8 to Southern blots of a number of cDNAs derived from YP3 transcripts, all of which include the *Bam*HI site which marks the distal end of pAB135 (not shown). Restriction enzyme recognition sites are coded as follows: H: *Hind*III, R: *Eco*RI, B: *Bam*HI, X: *Xba*I, A: *Ava*II. *Sal*I and *Xho*I do not cut within the insert DNA of these plasmids. Sizes of restriction fragments are shown below the map in base pairs (bp).

b) The figure on the facing page shows the overlapping clones which comprise the molecular walk extending from YP3 in the distal direction. Probes used in isolating further clones and for RNA blots (Fig. 14) are shown above the restriction map. Restriction enzyme recognition sites are coded as follows: R: *Eco*RI, B: *Bam*HI, S: *Sal*I. Sizes of restriction fragments are shown below the map in kilobase pairs (kb).



b)

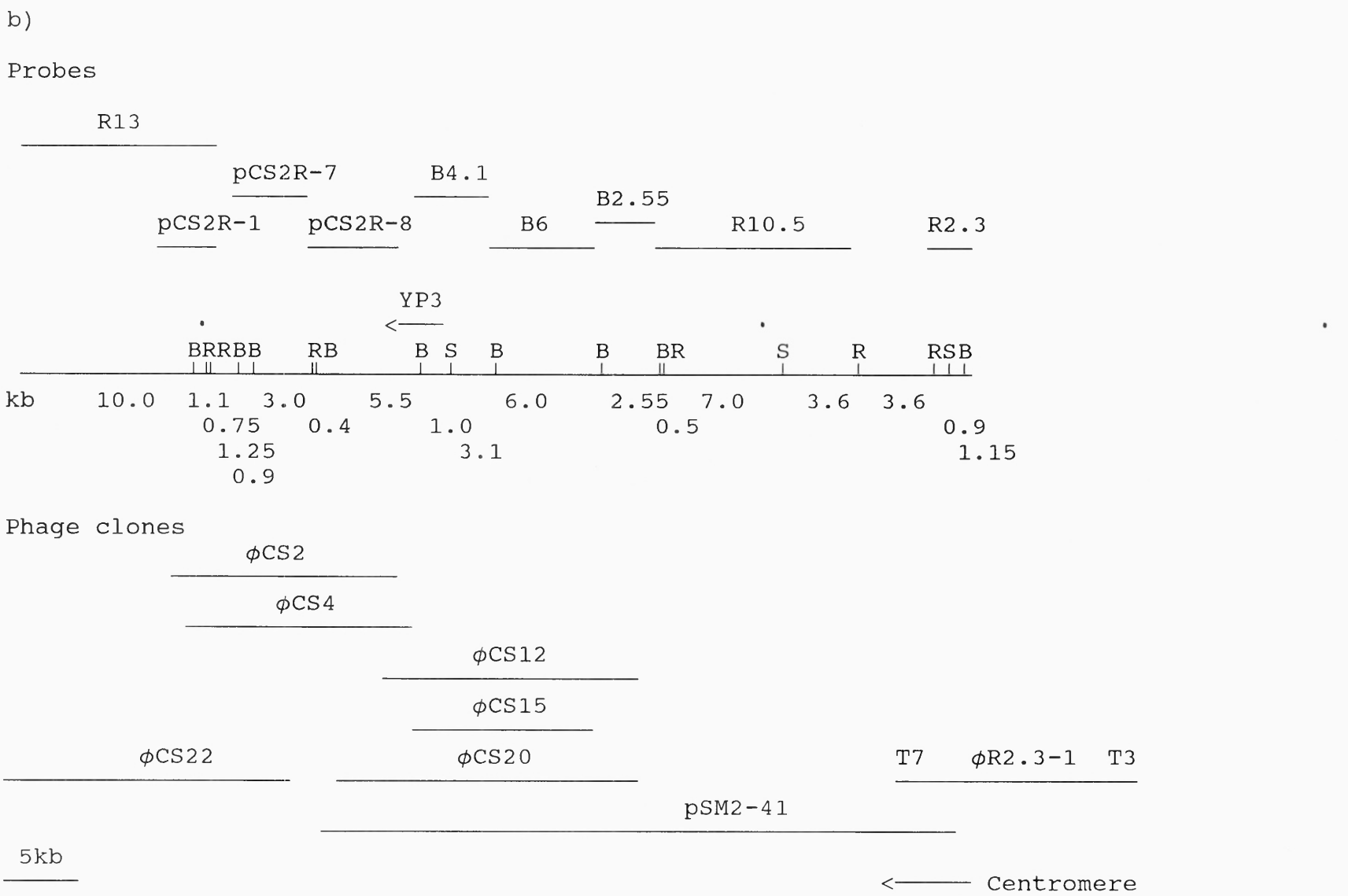

Probes
R13
pCS2R-7
B4.1
B2.55
pCS2R-1
pCS2R-8
B6
R10.5
R2.3
YP3
BRRBB
RB
B S B
B
BR
S
R
RSB
kb
10.0 1.1 3.0 5.5 6.0 2.55 7.0 3.6 3.6
0.75 0.4 1.0 0.5 0.9
1.25 3.1 1.15
0.9
Phage clones
φCS2
φCS4
φCS12
φCS15
φCS22
φCS20
T7 φR2.3-1 T3
pSM2-41
5kb
Centromere

Fig. 14

Transcription from the region of the chromosome walk adjacent to *YP3*.

The chromosomal region in the vicinity of the *YP3* is actively transcribed, with 5 different developmentally regulated transcripts derived from the ≈40kb I have investigated. At the bottom of the figure is the restriction map of the region shown in Fig. 13. The right end of the walk is centromere-distal. The P element insertion is indicated by an open triangle at the 3' end of the YP3 transcription unit. Restriction fragments used to probe RNA blots are indicated above the map. Recognition sites for restriction enzymes are coded as follows: B=*Bam*HI, R=*Eco*RI, S=*Sal*I. At the top of the figure are autoradiographs of the same RNA blot reprobed with each of the fragments indicated. The developmental stages are: E: 0-12h embryos, 1,2,3,: 1st, 2nd, 3rd larval instars. P: mid pupae, D: late (dark) pupae, female and male adults.

a) Shows the blot probed with a ≈0.5kb *Hind*III-*Sal*I fragment of pDmRas64B (Mozer et al. 1985) as a control for loading. The 1.6-1.7kb transcript is present as a constant proportion of polyadenylated RNA in all developmental stages.

b) The 13kb *Eco*RI fragment of ϕCS22, R13, hybridised with a ≈1.5kb RNA which was most abundant in embryos and third instar larvae.

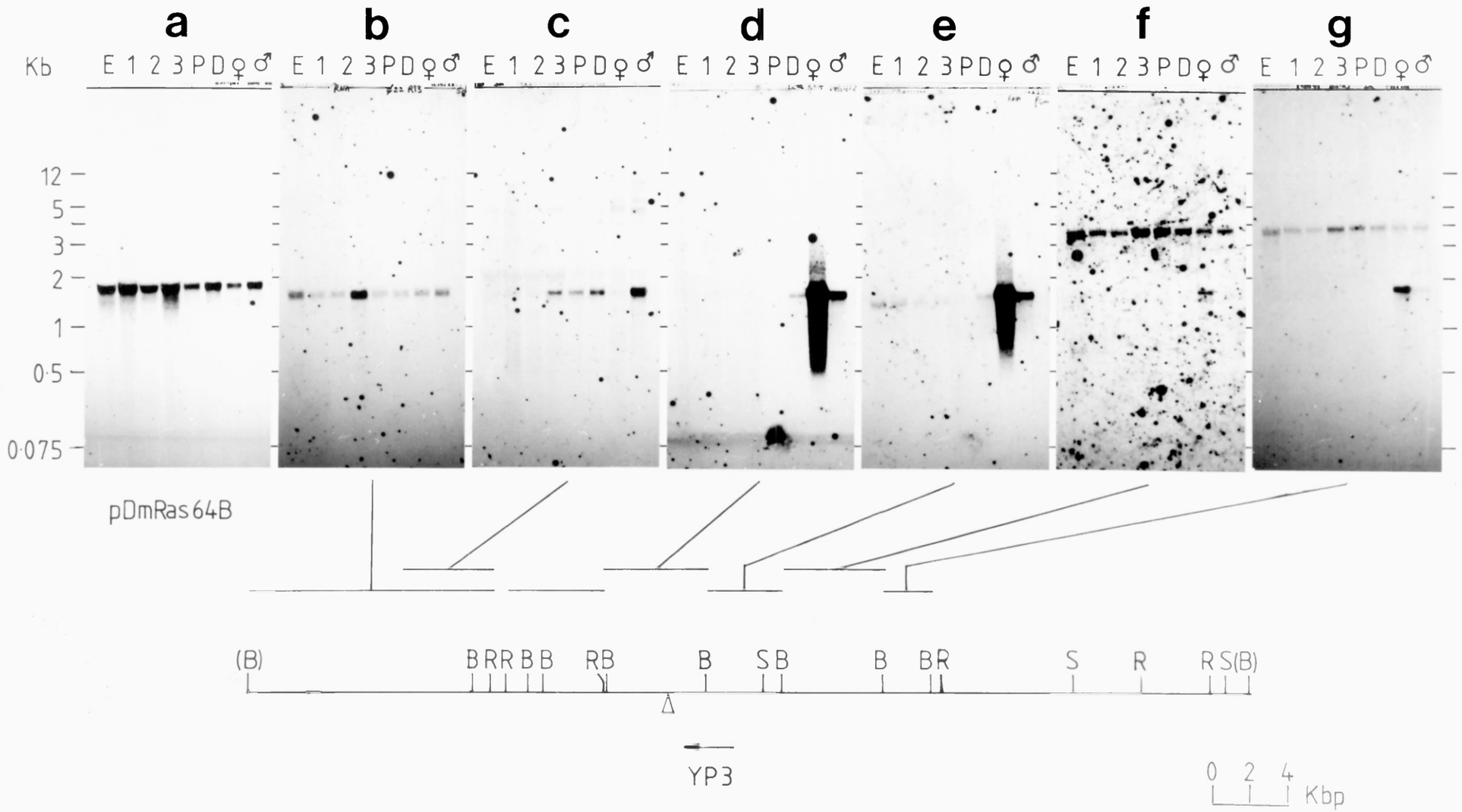
c) The 4.8kb insert of pCS2R-1 hybridised with a ≈1.5kb RNA present in late larvae, pupae and adult males but absent from adult females, embryos and early larvae. As larval and pupal RNA was isolated from animals of mixed sexes, the transcript detected in these samples might well represent the expression in males. Also shown below panel c, is the probe pCS2R-7, which hybridised to no transcripts on RNA blots.

d) The 5.5kb insert of pCS2R-8 hybridised with the 1.5-1.6kb *YP3* transcript in adult females. As transcription of *YP3* in males has not been previously reported, the transcript detected in the male lane might represent a low level of contamination of my preparation of male RNA with female RNA.

e) The 4.1kb *Bam*HI fragment of pSM2-41, B4.1, also hybridised with the *YP3* transcript in adult females. This probe also revealed the presence of another, ≈1.4kb transcript in embryos and early larvae

f) The 6kb *Bam*HI fragment of ϕCS15, B6, hybridised with 3.8-4kb message present as a constant proportion of polyadenylated RNA throughout development.

g) The 2.55kb *Bam*HI fragment of pSM2-41, B2.55, detects a transcript of identical size and profile of developmental expression as B6. This autoradiograph contains an artefactual band in the female track corresponding to pCS2R-8 probe irreversibly bound to *YP3* message. In all other cases, probes were readily removed by boiling the blot in wash buffer between probings. Removal of hybridised probes was monitored by autoradiography before reprobing (not shown).


a
b
c
d
e
f
g
Kb
E 1 2 3 P D ♀ ♂
12
5
3
2
1
0·5
0·075
pDmRas64B
(B) B R R B B R B B S B B B R S R R S (B)
YP3
0 2 4
Kbp

4.6 Use of clones of microdissected genomic DNA to initiate a molecular walk in 12B1,2

Microdissection of single polytene chromosome bands can provide sufficient DNA to construct a library of $10-10^3$ recombinant phage bearing inserts derived from the microdissected region (Scalenghe et al. 1981, Pirrotta et al. 1983). Robert Saunders performed the dissection of 12B1,2 and micro-library construction from Oregon R salivary gland chromosomes which I had prepared. These chromosomes were squashed after minimal fixation, 1min in 45% acetic acid, in order to limit acid depurination of the DNA. The dissected DNA was digested with the restriction enzyme *Eco*RI and ligated into the *Eco*RI site in the *cI* gene of the lambda insertion vector NM1149 (Murray 1983). Recombinants were selected by plating the *in vitro* packaged phage on *E. coli* NM514 *hfl*A cells (Section 2.8) on which they can form plaques. The number of total plaque forming phage was assayed using the hfl^+ strain Q358 (Section 2.8). The frequency of spontaneous clear plaques formed by vector DNA cleaved, re-ligated without insert DNA and packaged, was 0.25%. 72 clear plaques were obtained from the 12B1,2 dissections, these represent 1% of the total p.f.u. recovered. DNA was prepared from plate lysates of these phage, digested with *Eco*RI and run on agarose gels to determine the proportion containing inserts of *Drosophila* DNA. Eight of these 72 clones had inserts released by *Eco*RI digestion. These were purified from agarose gels and used to probe wild type and In(1)z^+64b9 chromosomes (Fig. 15b,d and Table 9). In(1)z^+64b9 breaks at 12B8,9 (Figs. 9,10 , Sorsa et al. 1973, Goldberg et al. 1982) and was used to check that the clones of microdissected DNA ("microclones") were indeed from 12B1,2, distal to the inversion breakpoint.

Under conditions of enzyme excess and with such impure DNA as is present during microcloning, *Eco*RI may cleave DNA with reduced sequence specificity to generate *Eco*RI* fragments, cleaved at AATT sequences. These could be ligated into *Eco*RI-cut vector but would not be released by *Eco*RI digestion of the recombinant DNA. To detect such *Eco*RI* fragments, I cut the phage DNA with *Hind*III and Bgl11 which release from the vector a 1.2kb fragment including the *Eco*RI cleavage site into which the *Drosophila* DNA was ligated. Thus insertions of *Eco*RI* fragments may be distinguished by the increase they cause in the molecular length of this *Hind*III, Bgl11 cut fragment. 15 of 36 clones tested in this way showed similarly-sized inserts of approximately 0.2kb. None of these small inserts could be used to generate a probe for use in hybridisation.

Table 9

Summary of *in situ* hybridisation of clones bearing inserts of microdissected DNA.

Before using clones of microdissected DNA for chromosome walking, I ascertained that these were indeed derived from the band, 12B1,2, which had been microdissected. To distinguish clearly between probes hybridising to 12B1-8 and 12B9-C I used the In(1)z^{+}64b9 chromosome which has a breakpoint at 12B8,9 (Section 4.3.2). Hybridisation of clone A34 to both chromosomes is shown in Fig. 15b,d. Experiments not performed are denoted "ND", probes which showed no hybridisation to polytene chromosomes are marked "-".

		Hybridisation site on Chromosomes from:	
Clone	Insert size (kb)	Wild type	In(1)z^{+}64b9
A2	5.5	-	-
A26	0.9	12B1,2	12B1,2
A32	9.0	-	12B1,2
A33	0.3	ND	ND
A34	2.5	12B1,2	12B1,2
B22	0.7/0.8 doublet	-	-
B24	0.2	12B1,2	12B1,2
B25	6.0	12B1,2	12B1,2

Fig. 15

In situ hybridisation of probes from the 12B-C walks.

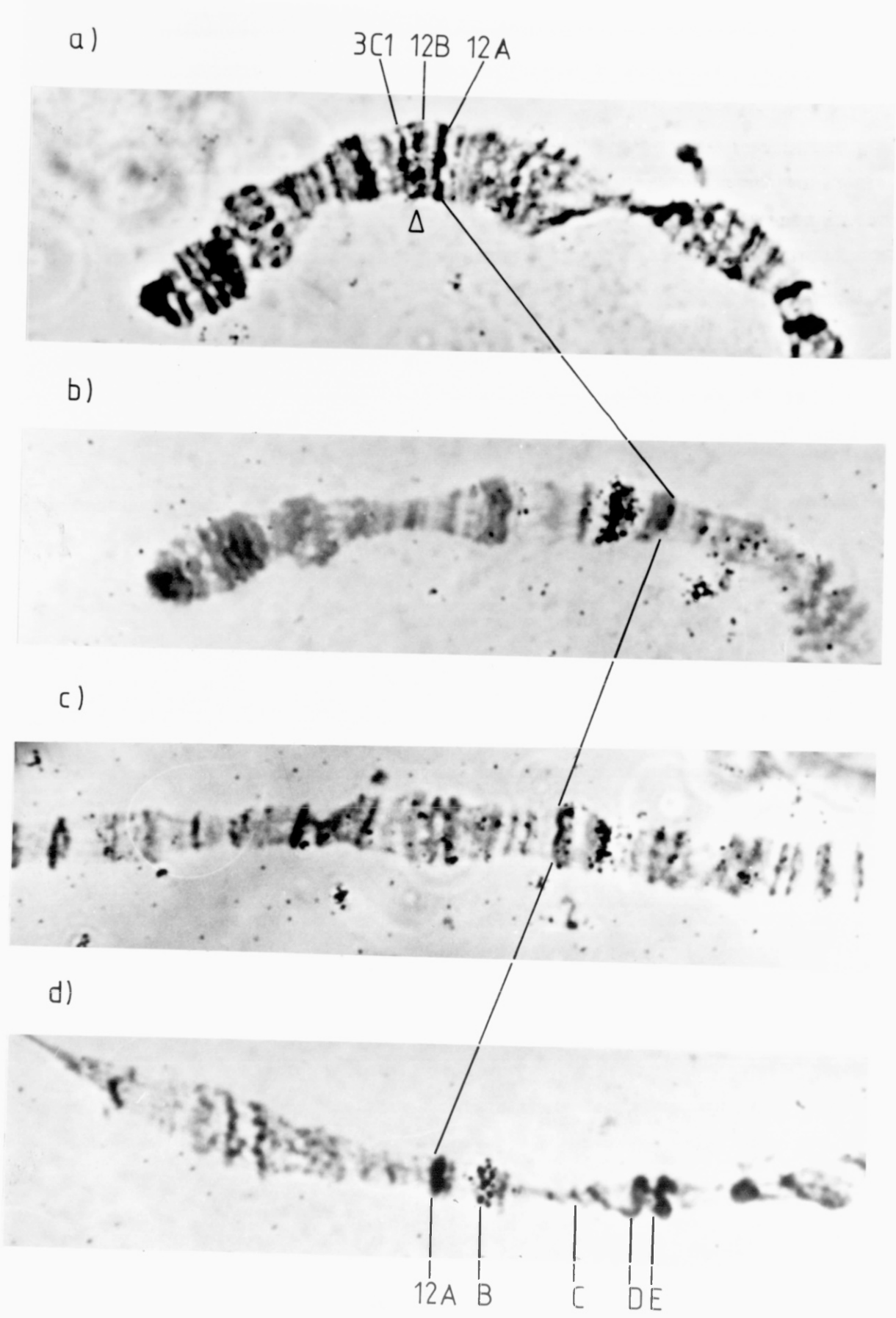
The relative positions of the distal end of the *YP3* walk and the clones of microdissected DNA were ascertained by *in situ* hybridisation to polytene chromosomes. Both of these probes hybridised to positions distal to the In(1)z^{+}64b9 inversion breakpoint at 12B8,9.

a) R2.3, a 2.3kb *Eco*RI fragment from the right hand end of the walk shown in Fig. 13b hybridised to 12B2-6 on the In(1)z^{+}64b9 chromosome.

b) Microdissected clone A34 hybridised to 12B1-2 on the In(1)z^{+}64b9 chromosome.

c) The same probe, R2.3, as a) above, hybridised to 12B2-6 on a wild type chromosome.

d) The same probe, A34, as b) above, hybridised to 12B1-2 on a wild type chromosome. Comparison of c and d shows that the two probes are separated by a region of chromosome band, perhaps 12B2-4.


a)
3C1
12B
12A
Δ
b)
c)
d)
12A
B
C
D
E

4.6.1 Phage clones

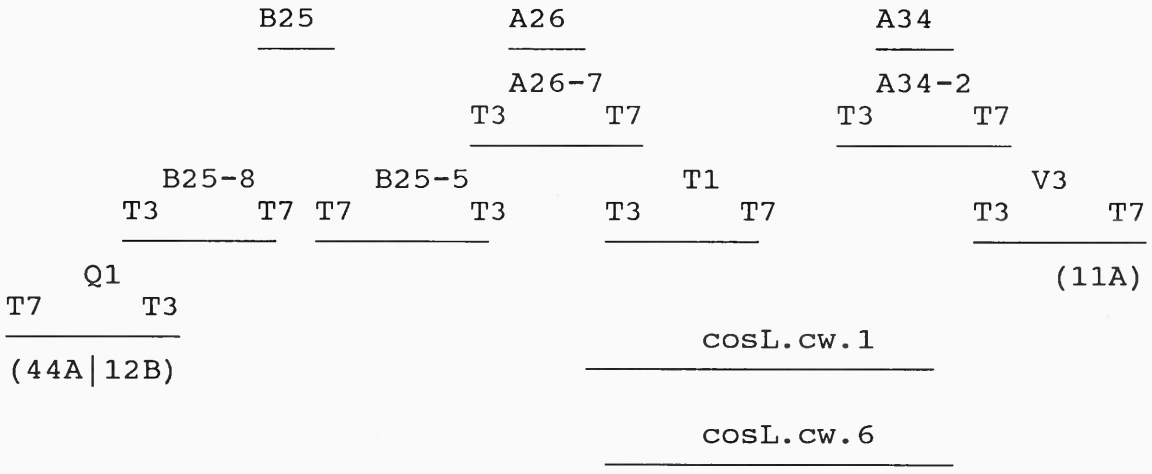
Phage clones were obtained from a lambda-DASH library of partially Sau3AI-digested Oregon R DNA constructed by Matthew Freeman (PhD thesis, University of London 1987). These clones were obtained by hybridisation-selection using as probes the inserts from microdissected clones A26, A34 and B25. Clones from this first round of screening were denoted by probe number and a suffix, (e.g A26-7). For the next round of screening, end-specific radiolabelled RNA probes were transcribed from the DASH clones and used to isolate further DASH clones from the library (Fig. 16). Clones from this second round of screening were denoted by single letters (e.g. Q1).

Two cosmids, pcosL.cw.1 and pcosL.cw.6, were kindly supplied by Santosh Krishnan and Erich Frei in the Department of Biology, Yale University. They had isolated these in the search for the *bendless* gene using the genomic insertion site, at 12B, of the marked P element p[*w*,*ry*$^{+}$E]2 (Levis et al. 1985). I made an attempt at this time to determine the orientation of the set of overlapping clones by *in situ* hybridisation (not shown). A 7.5kb *Eco*RI fragment of the T7 end of clone Q1 hybridised *in situ* to 12B but also to 44A . No other sites were seen. The most likely explanation for this result was that clone Q1 was an artefact resulting from the way the DASH walking library was constructed. The termini of the insert DNA were not dephosphorylated prior to ligation. Thus restriction fragments from different genomic loci could become ligated together. Such clones are selected by differential hybridisation with the end-specific probes of a clone isolated in the previous step but are of no use in extending the walk of clones colinear with the genomic locus at 12B. A 10.5kb *Sal*I fragment of the T7 end of clone V3 hybridised *in situ* to 11A. Thus within only 2 rounds of chromosome walking, both ends of the walk had encountered rearranged clones. Because of this, I was unable to determine the orientation of the clones I had isolated. Rather than continue with the bidirectional walking strategy I then began to walk distally from the most centromere-distal point of the walk initiated at YP3 to attempt to join this with the clones at 12B1,2.

Fig. 16

Ordering of clones isolated with microdissected DNA.

DNA purified from the clones on caesium chloride gradients was spotted onto multiple Hybond C Extra (Amersham) reinforced nitrocellulose filters and probed with the end-specific RNA probes from the following clones. The results of probing 12 clones with 14 end-specific probes are shown below. The relative orientation, and qualitative indication of regions of overlap are shown. This diagram does not indicate the size of the clones, nor the extent of overlap. Restriction fragments homologous to the T7 transcript of clone Q1 and the T7 transcript of clone V3 were used to probe polytene chromosomes. The cytological positions of these are recorded in brackets. It is clear that the walk has diverged from 12B1,2 at both the right and left hand ends. It has not been possible to orient these clones with respect to the centromere.



4.6.2 Attempt to connect the 12B1,2 and YP3 walks.

The rightmost restriction fragment of the YP3 walk is a 2.3kb *Eco*RI fragment of pSM2-41 termed R2.3 which was used as a probe to isolate a wild type genomic clone R2.3-1 from the lambda DASH library constructed by Matthew Freeman (Fig. 13b). The T3 transcript from this clone does not hybridise to any of the clones shown in Fig. 16 so the isolation of further clones will be needed to complete the walk from *YP3* to 12B1,2.

4.7 Future strategy to identify the *mus101* and *l(1)d.deg4* transcription units

Taking into account the published data on DNA content of chromosome bands (Spierer et al. 1983) I estimate that the region 12B1,2;8,9 uncovered by Df(1)w^{LCD} comprises approximately 200kb of genomic DNA, most of which is probably in the 12B1,2 bands. This should not present an unreasonable obstacle to isolation of the *mus101* and *l(1)d.deg4* genes given the data and materials described in this chapter. My future strategy will be as follows.

We are now in possession of carefully-constructed cosmid walking libraries in the pCosPer(*NotBamNot*) vector (gift of Dr J. Tamkun, Department of MCD Biology, University of Colorado, Boulder) and the Lorist 6 vector (Siden-Kiamos et al. 1990). I plan to connect the two regions of chromosome walk by isolating clones from these libraries. It is now possible using the polymerase chain reaction to synthesise specific probes from DNA microdissected from a single chromosome band (Saunders et al. 1989). These probes are more representative of the region dissected than clones obtained from a similar dissection. It should therefore be possible to obtain clones covering nearly all of the region uncovered by Df(1)w^{LCD} using the microamplified DNA to probe these cosmid libraries. The limits to the walk may be readily determined by *in situ* hybridisation to deficiency heterozygotes.

Probes derived from restriction fragments within the walk might be used to identify transcripts altered or missing from mutant larvae in RNA blots of any of the mutant alleles of *mus101* or *l(1)d.deg4*. Similarly, blots of genomic DNA could be used to identify restriction map polymorphisms associated with the mutations. I shall pursue both approaches. Identification of the *mus101* and *l(1)d.deg4* genes might be achieved by germline transformation with the pCosPer clones themselves, as this vector carries P element termini and the w^+ marker gene. 200kb of walk could be covered by 10-20 transformed lines, allowing for overlap of the clones. This would be a viable proposition, but RNA blotting or cDNA isolation would need to be carried out in parallel to ensure that the region contained no transcription units larger than the ≈30kb carried by a cosmid.

Small deletions for use in pinpointing the genes within the molecular walk could be selected by X-ray induced deletion of the p[*w*,*ry*$^+$E]2 insertion at 12B1,2 (Levis et al. 1985). X ray mutagenesis of Dp(1;Y)$y^+g^+na^+$ (Fig. 10) could be used to create

cytologically visible rearrangements associated with *mus101* and *l(1)d.deg4* mutations. Use of the duplication rather than a native X chromosome would simplify the recovery and analysis of these new breakpoints.

Recombination between *mus101*^{lcd} *d.deg4*$^{+}$ and *mus101*$^{+}$ *l(1)d.deg4* lethal chromosomes would selectively generate viable *mus101*$^{+}$ *d.deg4*$^{+}$ male flies. Because the two mutations were generated on different chromosomes, there will be restriction site polymorphisms between allelic loci on the two chromosomes. Thus, by probing genomic DNA digested with restriction enzymes having a 4bp recognition sequence with probes from the genomic walk, a characteristic DNA "fingerprint" would be obtained for regions of each parental chromosome throughout region 12B. It should therefore be possible to determine the segment of each recombinant chromosome derived from each of the *mus101*^{lcd} or *l(1)d.deg4* parents. In such a way the crossover points of each recombinant might be located, and the *mus101* and *l(1)d.deg4* genes oriented with respect to these crossover points.

Chapter 5: One of the protein phosphatase 1 isoenzymes in *Drosophila* is essential for mitosis

For the reasons stated in Section 1.3, protein phosphatases are likely to be required in the regulation of chromosome decondensation and other processes comprising the later stages of mitosis. Indeed mutations in type 1 protein phosphatase genes of fungal species have been identified which prevent sister chromatid disjunction and exit from mitosis. *bim* G^+ of *A. nidulans* (Doonan and Morris 1989) and *dis2*$^+$ of *S. pombe* (Ohkura et al. 1988 , Ohkura et al. 1989) both have a strikingly high level of sequence identity with the type 1 protein phosphatases of rabbit (Cohen 1988) and *Drosophila* (Dombrádi et al. 1989). Conditional mutations in both of these fungal genes have lethal effects late in mitosis but it seems that deletion of these genes is not lethal in either *Aspergillus* or in fission yeast due to the presence of functionally equivalent homologues (for review see Cyert and Thorner, 1989). I was interested to compare the roles of protein phosphatases in mitotic control in a multicellular organism with these findings by examining mutations in protein phosphatase genes of *Drosophila*.

5.1 Identification of *Drosophila* protein phosphatase genes.

Drosophila melanogaster has four PP1 cognates which we designate by their cytological location (Axton et al. 1990). A *Drosophila PP1 87B* cDNA, 1.2kb in length (formerly called "*Drosophila* PP-1α") was isolated from the adult head cDNA library of Kalderon and Rubin by Viktor Dombrádi using low stringency plaque hybridisation with a rabbit skeletal muscle cDNA encoding the catalytic subunit of protein phosphatase 1α. The nucleotide sequence of this clone was determined by him (Dombrádi et al. 1989). I showed that this *Drosophila* cDNA hybridises to four distinct sites on the salivary gland chromosomes (Fig. 17). The strongest of these is at 87B6-12, the transcription unit from which the cDNA is derived (see below). The remaining sites of hybridisation show less intense signals. These secondary sites, at 9C1-2, 13C1-2 and 96A2-5 are likely to share at least 70% nucleic acid identity with the probe given the hybridisation conditions used, according to the theoretical treatment of stringency of DNA hybridisation taken from Maniatis et al. (1982).

5.2 *ck19* mutations result in abnormal mitosis.

PP1 87B lies within a region of chromosome 3 that has been systematically studied by saturation mutagenesis (Gausz et al. 1981). The *PP1 87B* gene hybridises *in situ* to 87B6-12, within an interval defined by the proximal breakpoint of Df(3R)Kar^{3Q} and the distal breakpoint of Df(3R)E-079 (Figs. 18,19). Three of the recessive lethal complementation groups described by Gausz et al. (1981), *ck17-19*, are uncovered by both of these deficiencies. If *PP1 87B* is an essential gene, it should correspond to one

of these lethal complementation groups. I examined the stage of development at which these mutations caused lethality. I also repeated the tests for complementation of lethality on stocks of *ck17*^{e180}, *ck17*^{e280}, *ck18*^{e312}, *ck18*^{e290}, *ck19*^{e211}, *ck19*^{hs46} and *ck19*^{e078} (Table 10). *ck17*^{e180} complements the lethality of *ck17*^{e280} so my results show that there are in fact four lethal complementation groups in the region uncovered by the overlapping deficiencies Df(3R)Kar^{3Q} and Df(3R)E-079.

Mutant 3rd instar larvae of genotype *ck19*^{e211}/Df(3R)E-079 were superficially wild type in size and behaviour (they feed and crawl) but dissection revealed undeveloped imaginal discs, indicating failure of diploid cell proliferation. These larvae die either just before or just after pupariation. The phenotypic combination of late lethality, together with nonproliferation of imaginal cells, is common to many mutants exhibiting abnormal mitosis (Gatti and Baker 1989, and Section 1.1). I therefore examined mitosis in ganglion squashes prepared from larvae heterozygous for each of these chromosomes *in trans* with Df(3R)E-079. In this experiment abnormal mitoses were seen only in ganglia from members of the *ck19* complementation group *in trans* with the deficiency.

Fig. 17

The 1.2kb PP1α cDNA derived from the *PP1 87B* locus hybridises *in situ* to four chromosomal sites.

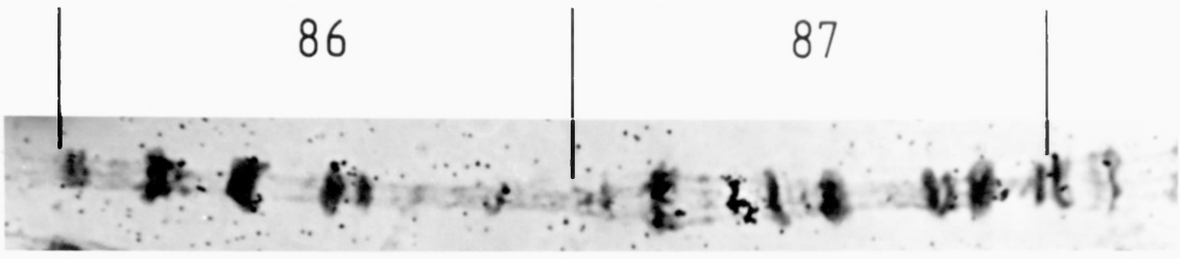
To localise the 1.2kb cDNA in salivary gland polytene chromosomes, this probe was hybridised *in situ*.

a) The main site of hybridisation, with the strongest signal, is the locus from which the cDNA was derived, at 87B6-12.

b) A weaker site of hybridisation, also on chromosome 3R revealed the presence of a related gene at 96A2-5.

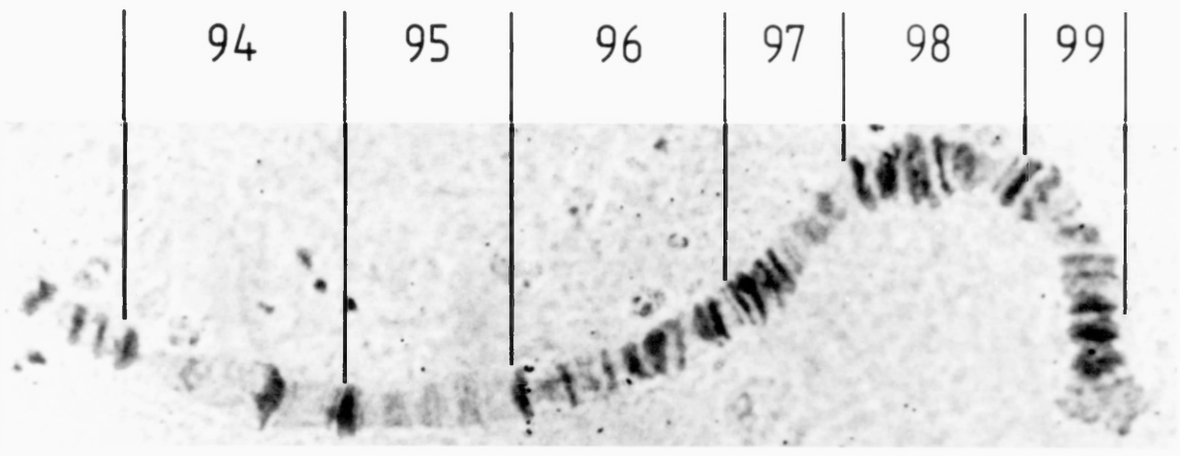
c) There were also 2 weak sites of hybridisation on the X chromosome at 9C1-2 and 13C1-2.

a)



10 µm

b)



c)

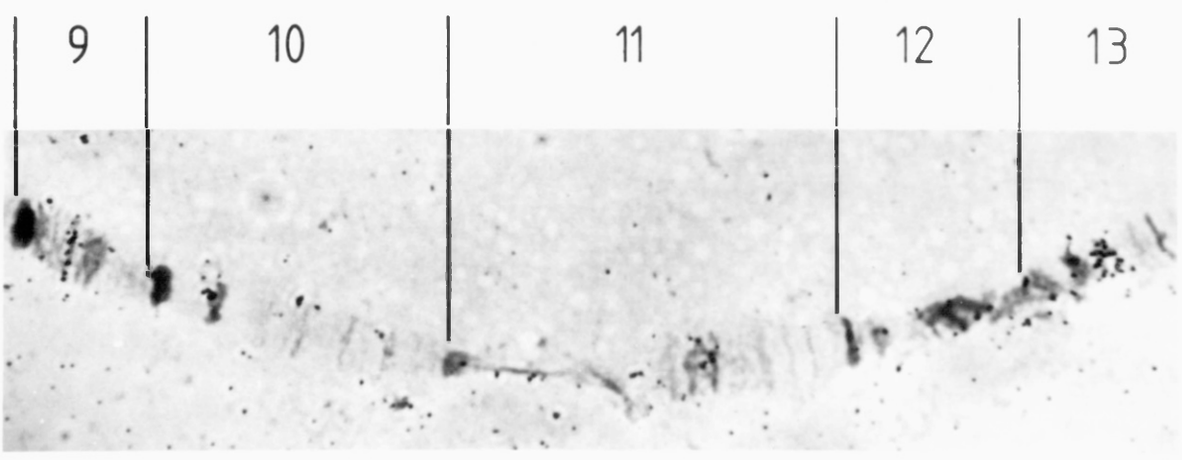


Fig. 18

The cytology of the 87B region of salivary gland chromosomes:

The position of *PP1 87B* determined by *in situ* hybridisation is indicated as an arrowed line above the conventional chromosome map drawn after Bridges (1941). Horizontal lines below the chromosome represent chromatin missing from deficiency chromosomes used in this study. Chromosome breakpoints were originally published by Gausz et al. (1981). Df(3R)Kar^{3J} does not uncover *PP1 87B* but is present on the *ck19*^{hs46} chromosome.

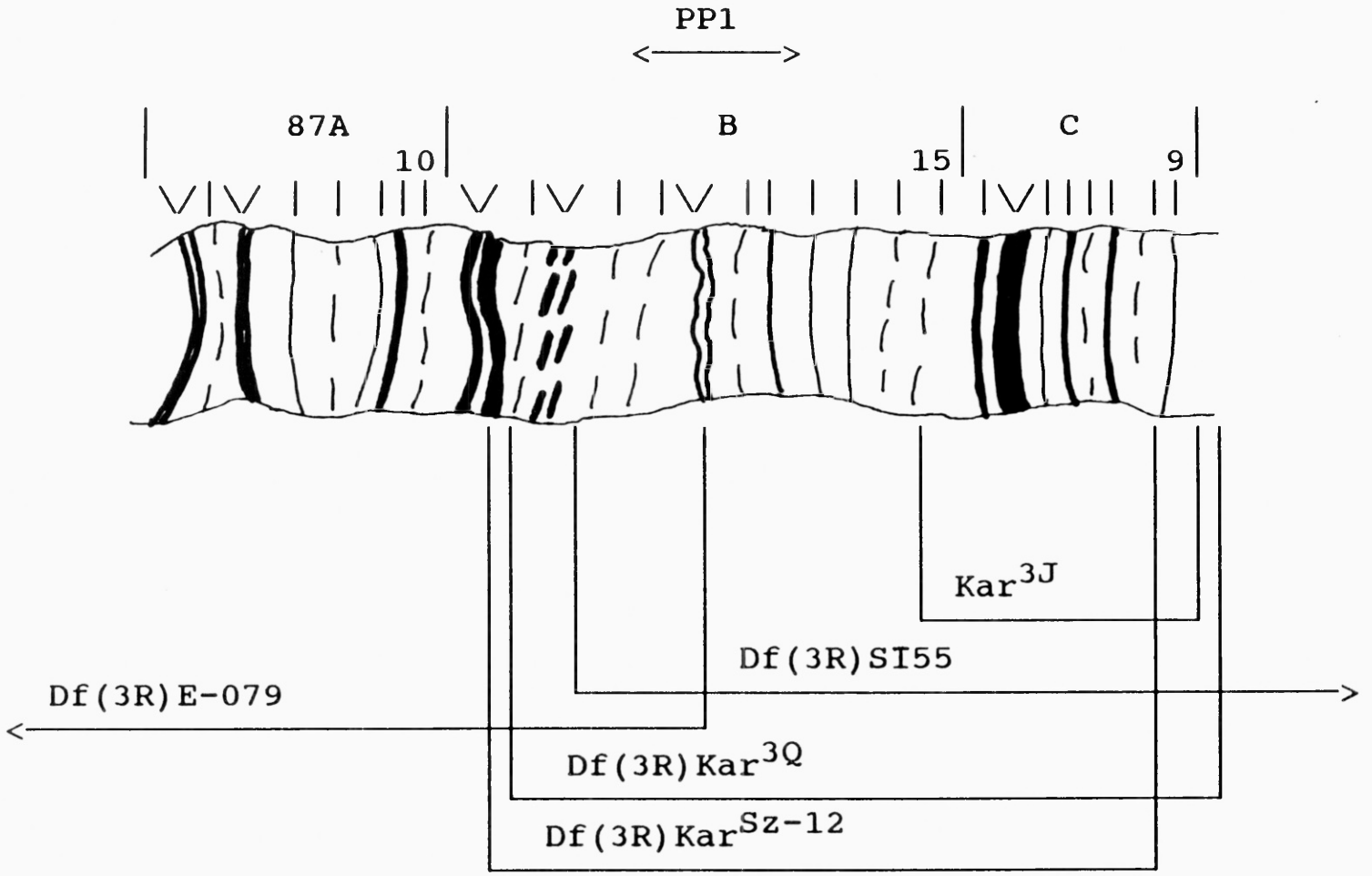

PP1
87A
10
B
15
C
9
Kar3J
Df(3R)SI55
Df(3R)E-079
Df(3R)Kar3Q
Df(3R)Kar Sz-12

Fig. 19

Deficiencies which uncover the *PP1 87B* locus.

Heterozygous Df(3R)/+ salivary gland polytene chromosomes were examined to confirm the breakpoints of the deficiencies used in mapping the *l(3)ck* series of lethal mutations. The extent of each of these deficiencies is shown schematically in Fig. 18.

a) *In situ* hybridisation of the 1.2kb PP1α cDNA to 87B6-12.

b) Df(3R)E-079/+

c) Df(3R)Kar^{Sz-12}/+

d) Df(3R)Kar^{3Q}/+

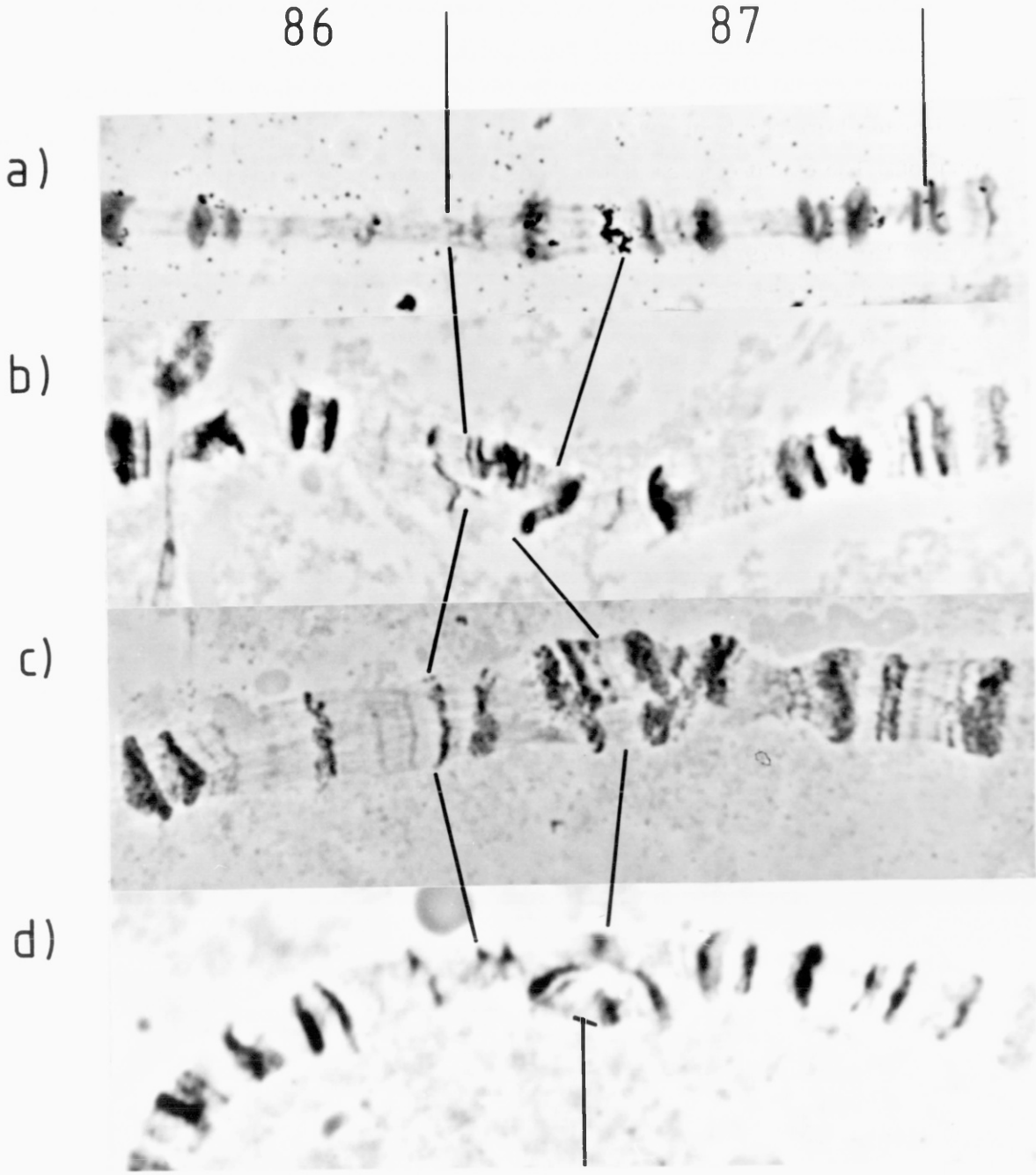
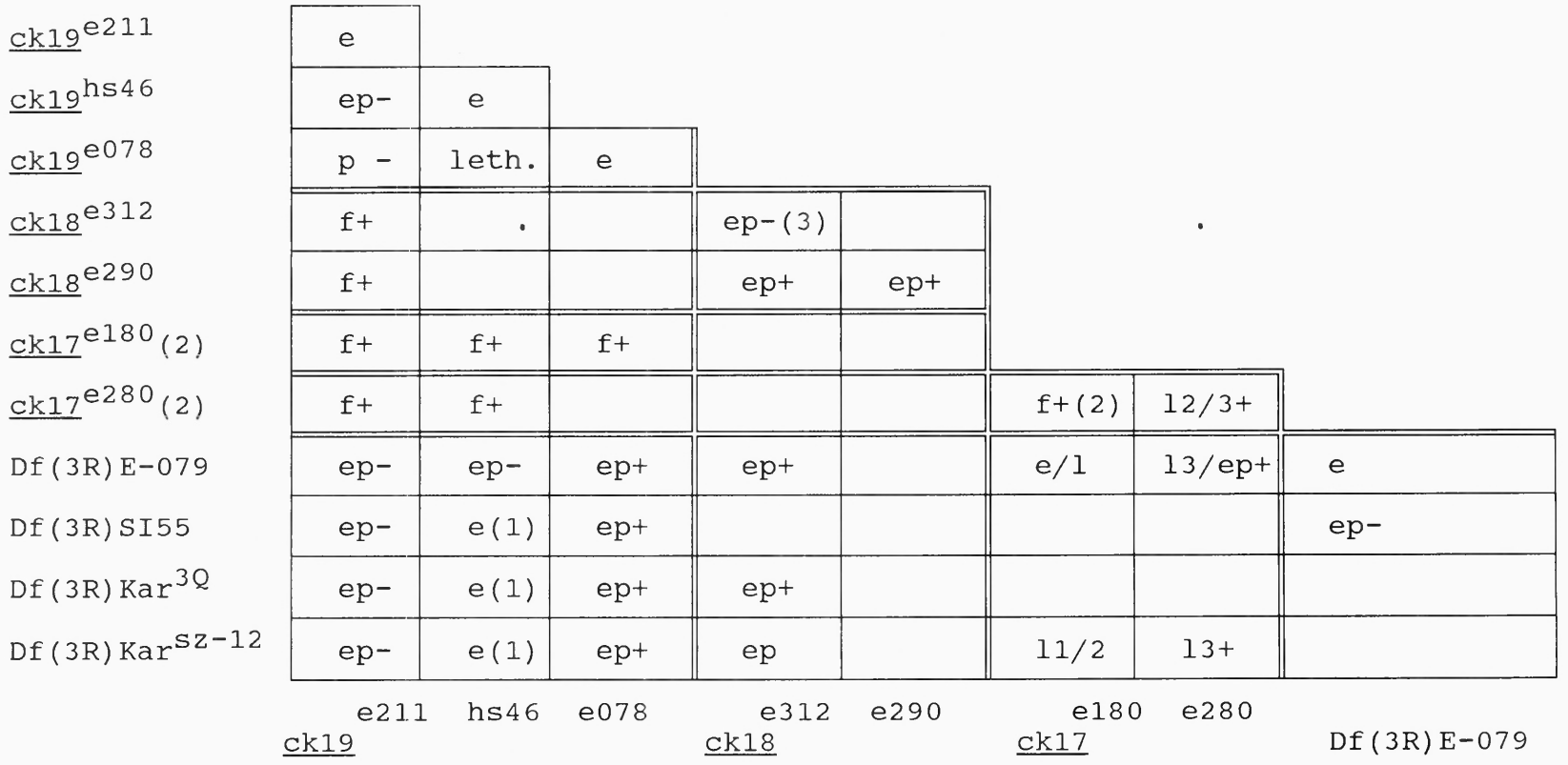

86
87
a)
b)
c)
d)
B6-12
10 µm

Table 10

Complementation analysis of lethal mutations and deficiencies in 87B.

This table records the lethality and associated mutant phenotypes of heterozygous combinations of *l(3R)ck* mutations and deficiencies. The stages at which the majority of the mutants of the indicated genotype died are lettered: e=embryo, l=larva, l1/2=first and second instar larvae, l3=third instar larva, ep=early pupa, f=viable and fertile. The effect on diploid cell proliferation of each of these mutant combinations was also tested by examining the development of the imaginal discs of third instar larvae where appropriate. For all combinations which gave third instar larvae, mitosis in ganglia was analysed in squash preparations. (-) denotes rudimentary imaginal disc development and aberrant mitosis. (+) indicates that imaginal discs and mitosis were indistinguishable from those of wild type. Several additional points must be noted. (1) The embryonic lethality of $ck19^{hs46}$ *in trans* with deficiencies other than Df(3R)E-079 is due to the presence of $Df(3R)Kar^{3J}$ on the $ck19^{hs46}$ chromosome (Fig. 18). (2) My complementation analysis disagrees with that of Gausz et al. (1981) who concluded that $ck17^{e180}$ failed to complement the lethality of $ck17^{e280}$, and that these mutations were therefore likely to be allelic. I have found, in contrast, that these mutations complement one another fully. (3) $ck18^{e312}$ when homozygous exhibits a strongly abnormal mitotic phenotype with polyploid cells and hypercondensed chromosomes. However, this recessive phenotype is not uncovered by any of the deficiencies tested, nor revealed *in trans* with $ck18^{e290}$ or any of the other *l(3)ck* mutations tested. Either expression of the $ck18^{e312}$ mutant mitotic phenotype depends on paired $ck18^{e312}$ chromosomes, or, more likely, the $ck18^{e312}$ chromosome bears a mitotic mutation elsewhere on the chromosome in addition to the *ck18* lethal.


ck19e211
ck19hs46
ck19e078
ck18e312
ck18e290
ck17e180(2)
ck17e280(2)
Df(3R)E-079
Df(3R)SI55
Df(3R)Kar3Q
Df(3R)Karsz-12
e
ep-
e
p -
leth.
e
f+
.
ep-(3)
f+
ep+
ep+
f+
f+
f+
f+
f+
f+(2)
l2/3+
ep-
ep-
ep+
ep+
e/l
l3/ep+
e
ep-
e(1)
ep+
ep-
e(1)
ep+
ep+
ep-
e(1)
ep+
ep
l1/2
l3+
e211
hs46
e078
e312
e290
e180
e280
ck19
ck18
ck17
Df(3R)E-079

5.2.1 Chromosome condensation and chromatid separation.

I found ganglia from mutant larvae of genotype $ck19^{e211}$/Df(3R)E-079 to be smaller than those of wild type larvae of equivalent age, and to have significantly fewer cells per microscope field in squash preparations (mean 77% of wild type). I conclude from this that cell division is impaired in mutant ganglia as well as in imaginal discs. Wild type neuroblasts have by the crawling stage (106-112h post-hatching at 25°C,(Truman and Bate 1988)) produced more of their small progeny than their mutant counterparts. Mitoses were scored per microscope field and also as a proportion of the total number of nuclei in the preparation. Despite the difference in cell density between wild type and mutant, the following conclusions drawn from observations of the number of mitoses per field were not qualitatively different to those obtained by counting all cells.

The frequency of normal metaphase and anaphase mitotic figures was significantly reduced in squashes of mutant $ck19^{e211}$/Df(3R)E-079 ganglia (51% of wild type on a per field basis, 66% of wild type on a per cell basis). The ratio of metaphases to anaphases among these normal cells is about 3 in wild type, and approximately 4 in the mutant (Table 11a). This difference is not however statistically significant. A chi-squared test of homogeneity performed on the numbers of normal metaphases and anaphases in mutant and wild type found these to be consistent with the null hypothesis of no difference in ratio between mutant and wild type at the 99% confidence level. Therefore the cells of this "normal" subpopulation in mutant ganglia are undergoing mitosis without impediment to the timing of chromatid separation. The proportion of all cells in mitosis was approximately twofold higher in the mutant ganglia (190% of wild type mitoses per field, 240% cell/cell). This significant increase in mitotic index was due to mutant cells showing various abnormalities (Table 11a,b), including extremely overcondensed chromosomes (Fig. 20e-h), extra chromosomes (hyperploidy - Fig 20b,c); or both abnormal features (Fig. 20d,i). I infer that these aberrant cells are delayed in their progress through mitosis for lack of *PP1 87B* function.

The presence of aneuploidy and polyploidy (4N,8N) suggests that the mutant is defective in chromosome segregation or cytokinesis. Overcondensed sister chromatids are seen which have disjoined but show no sign of anaphase orientation (Fig.20e). In some mitoses, anaphase separation has occurred but the chromatids in one of the resulting groups are overcondensed (Fig. 20f). Many cells have overcondensed chromosomes which appear to be chromatids in anaphase-like groupings (Fig. 20g). Commonly seen figures of the type shown in Fig. 20h may result from successful telophase decondensation of only one polar group. However, given the extreme degree of chromatin condensation this hypothesis must be considered with caution. Mutant

cells arrested at metaphase in an equivalent state of pycnosis would be unlikely to display well-defined discrete chromatids.

Mitosis in *ck19*^{hs46}/Df(3R)E-079 neuroblasts did not differ significantly from *ck19*^{e211}/Df(3R)E-079 (data not presented). In contrast, less than 4% of mitotic figures in *ck19*^{e078}/Df(3R)E-079 were found to be abnormal, most being cells with overcondensed chromosomes without hyperploidy (Table 11b). Imaginal disc development was nearly wild-type. The mitotic phenotype of *ck19*^{e078}/Df(3R)E-079 is not temperature sensitive, larvae kept at 29°C for 12h prior to squashing showed no greater frequency of mutant figures than those at 18°C (data not presented).

Table 11

Frequencies of mitotic abnormalities in mutant, rescued and wild type larvae:

These data were recorded from different sets of acetic orcein squashes of ganglia from third instar larvae of the genotypes indicated. Examples of different types of abnormal mitotic figures are illustrated in Fig. 20.

a) Frequencies of mitotic figures in neuroblast squash preparations expressed as a percentage of total mitotic figures scored.

b) Frequencies of mitoses per microscope field (facing page). The numbers quoted in the upper part of the table are mean numbers of cells per field (the standard deviation is shown in brackets). Means which differ significantly from wild type (Student's t test at 99% level of confidence) are shown in **bold** type. For a discussion of rescue of mitosis by P element mediated germline transformation, see Section 5.5.

---

a)

	Wild type	$\underline{ck19}^{e211}$/ Df(3R)E-079
Wild type metaphases	74.6	24.2
Wild type anaphases	25.4	5.8
Overcondensed	0	52.5
Hyperploid	0	2.8
Overcondensed hyperploid	0	14.7
Total figures	861	1241
Total animals	10	10

b)

Per field:	Wild type	ck19^{e211}/ Df(3R)E-079	P[w^{+}PP1^{+}]16; ck19^{e211}/ Df(3R)E-079	ck19^{e078}/ Df(3R)E-079
Wild type metaphases	2.05 (1.44)	**1.10** (1.29)	2.34 (1.44)	2.22 (1.77)
Wild type anaphases	0.60 (0.83)	**0.25** (0.53)	0.77 (0.96)	0.83 (0.97)
Wild type meta./wild type ana.	3.42	4.40	3.04	2.67
Aberrant mitoses	0	**3.66** (3.28)	0	**0.11** (0.35)
Mitoses	2.65 (1.75)	**5.01** (3.65)	3.10 (1.70)	3.17 (2.16)
Cells	146.00 (43.28)	**112.00** (38.15)	150.90 (34.43)	150.36 (46.47)
Total fields	78	87	121	96
Animals	7	4	5	4

Fig. 20

Acetic orcein-stained ganglion squashes illustrating the range of mutant mitotic figures seen in *ck19* mutants.

Squashes were carried out as described in Section 2.3.1 without colchicine or hypotonic treatment.

a) This panel shows chromosomes from a wild type female larva whereas panels b-i are all from $ck19^{e211}$/Df(3R)E-079 mutant larvae. The panels illustrate:

b) A hyperploid XY cell with one extra metacentric chromosome but normal condensation.

c) A 4N polyploid with normal condensation, from XY larva.

d) An overcondensed hyperploid nucleus.

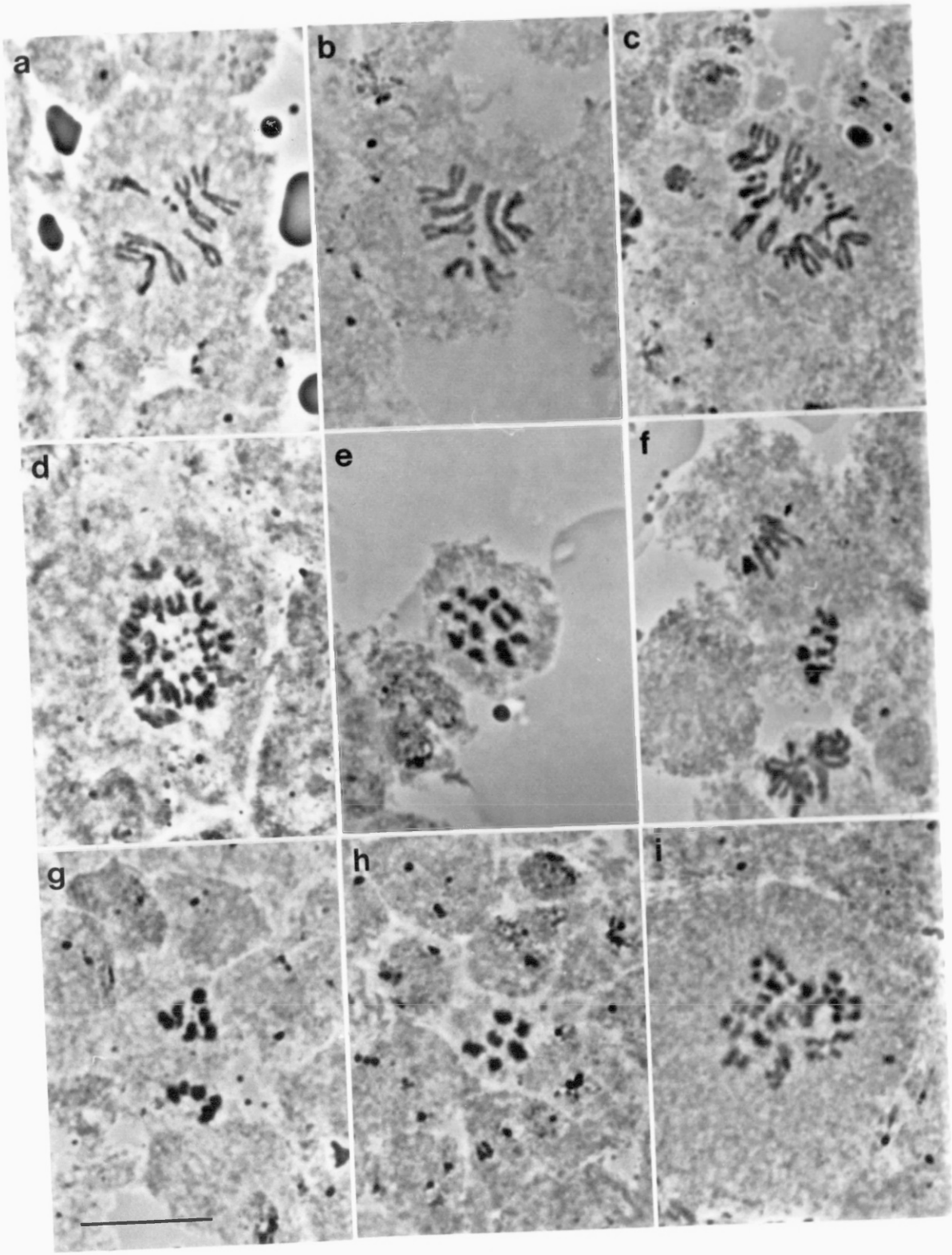
e) Strongly overcondensed, disjoined sister chromatids which do not show any anaphase orientation.

f) A late anaphase figure in which the lower chromatid group is overcondensed.

g,h) Strongly overcondensed chromosomes of anaphase-like figures, (see text).

i) Overcondensed hyperploid cell with chromatin of degenerate appearance.

The bar is 10μm.


a
b
c
d
e
f
g
h
i

5.2.2 The effect of *ck19* mutations on mitotic spindles.

While squashed preparations provide information about the chromosome cycle, the mitotic spindles are destroyed by fixation in acid, and even with alternative fixation, squashing cells would distort the spindle structure. To overcome these problems I examined mitotic neuroblasts in whole, formaldehyde-fixed ganglia using confocal indirect immunofluorescence microscopy. Serial optical sections of cells stained to reveal DNA and microtubules were superimposed in order to examine chromosome and spindle structure in three dimensions. Superficial giant neuroblasts of the medial region of the brain hemispheres of mid-third instar larvae are shown in Fig. 21. Metaphase and anaphase spindles in the wild type have the characteristic ellipsoidal shape shown in panels a,b. An optical section of the metaphase plate of a wild type cell is shown in panel e. The majority of 36 $ck19^{e211}$/Df(3R)E-079 mutant spindles in the brain were of the type shown in Fig. 21d. These spindles show unusually little space between almost parallel bundles of spindle microtubules. Overcondensed metaphase chromosomes can be seen associated with such spindles of "collapsed" appearance. Strongly overcondensed chromatids in anaphase groups are found in whole neuroblasts (Fig. 21e) as well as in squashes (see above). The microtubule-free "hole" about the centrosome which is typical of 50 wild type giant neuroblast spindles examined can be seen particularly well in Fig. 21a. Such a centrosomal arrangement of microtubules was not seen in most mutant spindles.

The ventral lobe of late (crawling) third instar $ck19^{e211}$/Df(3R)E079 larvae is slim and poorly developed in comparison with wild type. In contrast to the brain hemispheres of earlier larvae, superficial giant neuroblasts at this stage and position were found to contain only overcondensed chromosomes. Of these cells, 34% were hyperploid, with no associated mitotic microtubules (Table 12). In 67 giant neuroblasts in ventral ganglia from 18 wild type crawling third instar larvae processed in an identical manner, condensed chromosomes in prophase, metaphase or anaphase configurations were invariably surrounded by a mitotic spindle. I suspect that hyperploid mutant cells without spindles are a terminal phenotype. In late third instar larvae languishing for several days without pupating, squashes reveal an excess of overcondensed hyperploid nuclei of degenerate appearance (Fig. 20i)) which I believe to be the same terminal cells. Multipolar hyperploid cells (Fig. 21f, Table 12) are those in which centrosome replication has continued in the absence of cell division.

Fig. 21

Mitotic spindles in wild type and *PP1 87B*-deficient neuroblasts:

Ganglia were prepared from third instar larvae for confocal indirect immunofluorescence microscopy as described in Section 2.3.6. Tubulin in this figure is stained blue, DNA red. Unless otherwise stated, the whole mitotic apparatus is shown, reconstructed from several 1μm confocal optical sections.

Panels a-c are from wild type larvae, showing:

a) A wild type metaphase.

b) A wild type anaphase.

c) A single optical section of a wild type metaphase plate with the spindle poles above and below the plane of focus.

Panels d-f are from $ck19^{e211}$/Df(3R)E-079 mutant neuroblasts and show:

d) A mutant metaphase, 18 of 25 mutant metaphases showed this unusual "collapsed" spindle morphology.

e) A mutant anaphase with overcondensed chromatids.

f) A single optical section of a cell with a tetrapolar spindle, one pole of which is above the plane of focus.

The bar is 5μm.

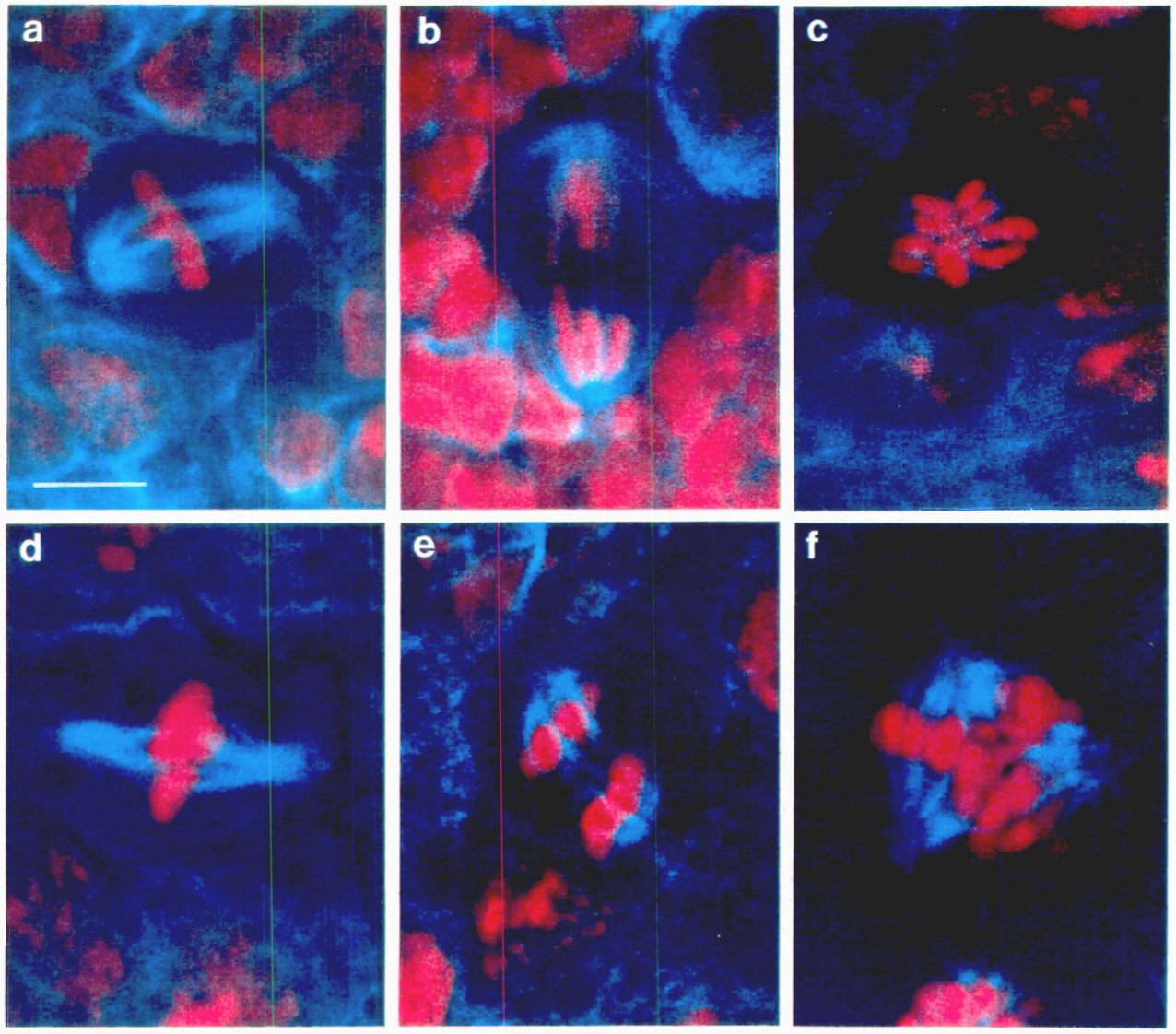

a
b
c
d
e
f

Table 12

Heterogeneity of *ck19*^{e211}/Df(3R)E-079 spindles associated with condensed chromosomes.

Data are based on confocal optical sectioning of 65 giant neuroblasts from whole mounts of the ventral thoracic region of the ventral ganglia of 18 late third instar larvae. The table records the state of chromosome condensation and mitotic spindle organisation these cells. No normally condensed chromosomes were seen in this experiment.

	Spindle:			
Chromosomes:	Bipolar	Multipolar	Disorganised	Absent
Overcondensed	7	0	0	5
Overcondensed and hyperploid	10	11	10	22

5.3 Mutations in *PP1 87B* correspond to lethal complementation group *ck19*.

I examined stocks bearing lethal mutations in complementation groups *ck17-19* for rearrangements of chromosomal DNA in the vicinity of the PP1 locus. Blots of genomic DNA were prepared from heterozygous flies in which a wild type allele of the lethal mutation is supplied by a TM3 or TM6B balancer chromosome. These were probed with 5' and 3' noncoding fragments of the *PP1 87B* gene. These probes, which specifically hybridise to the *PP1 87B* locus in genomic DNA blots, were prepared from the 1.2kb cDNA (Fig. 22, and Dombrádi et al. 1989). The 5' probe comprises nucleotides 1-172, representing 129bp of 5' non-coding region and 15 codons of the N terminal end of the PP1 open reading frame. The 3' probe is entirely non-coding sequence, nucleotides 1047-1208. I subcloned the 5' probe, an *Eco*RI-Xho1 fragment, and the 3' probe, an *Eco*RI-Pst1 fragment into Bluescribe^{+} for further use.

ck19^{e078}, *e180*, *e280*, *ck18*^{e312} and the TM3 and TM6B balancer chromosomes all showed the same *Eco*RI and *Bam*HI fragments as the Oregon R wild type with the *PP1 87B* probes (Fig. 23). *e180* and *e280* were both tested because my own complementation analysis of these "*ck17* alleles" indicated that the *e180/e280* trans-heterozygotes are viable and fertile (Table 10). Restriction fragments differing from wild type were found associated with lethal chromosomes *e211* and *hs46* of the *ck19* lethal complementation group (Fig. 24). *Eco*RI and *Bam*HI fragments from the *ck19*^{hs46} chromosome are not homologous to the 5' probe used, and the 3' probe detects bands generated by both enzymes which are some 500bp smaller than their wild type alleles. I therefore concluded that the *ck19*^{hs46} chromosome is deficient for approximately 500bp of the 5' end of the *PP1 87B* gene including the whole 170bp homologous to the 5' noncoding probe (Fig. 24). Thus this allele lacks the startpoint of translation and might be expected to be a null.

To investigate the DNA rearrangement associated with the *ck19*^{e211} allele further, I prepared a library of *Bam*HI-cut genomic DNA from *ck19*^{e211}/TM3 flies in the lambda vector EMBL4 (Frischauf et al. 1983). I obtained clones of an 11kb *Bam*HI fragment from the *ck19*^{e211} mutant and 6.5kb from the balancer chromosome. I compared the *ck19*^{e211} allele to the wild type gene by restriction mapping with enzymes *Sal*I, *Eco*RI and *Bam*HI and Southern blotting with the *PP1 87B* cDNAs and the 5' and 3' probes. While the *ck19*^{e211} restriction map is the same as the wild type from the *Sal*I site in the coding region through to the *Bam*HI site at the 3' end of the p*w*813 clone, the restriction map of the mutant diverges from wild type 5' to the 5' probe (Fig 22). This result strongly suggested that there was a DNA rearrangement at the 5' end of the *PP1 87B* gene. Dr Cohen and co-workers sequenced the *PP1 87B* gene from my genomic clone of *ck19*^{e211} and showed that the mutant sequence is the same as wild type in the coding and 3' regions but diverges 165 nucleotides 5' to the start of the *PP1* ORF thus removing sequences 5' to this point which are probably required for

PP1 87B transcription. One possible *cis*-acting regulatory element missing in the mutant is the sequence TATAAAA 290bp 5' to the initial ATG of the ORF (Dombrádi et al. 1990). From this analysis it might be expected that the DNA rearrangements found in the $ck19^{hs46}$ and $ck19^{e211}$ mutant alleles would interfere with transcription from the *PP1 87B* locus.

Fig. 22

Molecular organisation of the region flanking *PP1 87B*:

The 6.5kb wild type genomic DNA fragment carried on p*w*813 (upper line) is sufficient to rescue *PP1 87B* mutants. The insertion of the AM oligonucleotide to generate the disrupted control construct pAM12 results in the replacement of the *Nru*I site (N) by an *Xba*I site (X). The structure of the transcription unit was deduced by comparison of *PP1 87B* cDNAs with the cloned genomic locus by restriction analysis and Southern blotting. Regions of cDNA homologous to PP1 open reading frame are indicated by double horizontal lines. RNA blots probed with clones of the genomic locus reveal the presence of an adjacent transcription unit producing a 2.0kb RNA with no homology to *PP1 87B*. *PP1 87B*-specific probes corresponding to the 5' end of the gene and a 3' untranslated region are indicated. DNA present in three *ck19* mutant alleles is represented by the three solid lines in the lower part of the diagram. Other restriction enzyme cleavage sites are: *Bam*HI (B), *Eco*RI (R), *Sal*I (S).

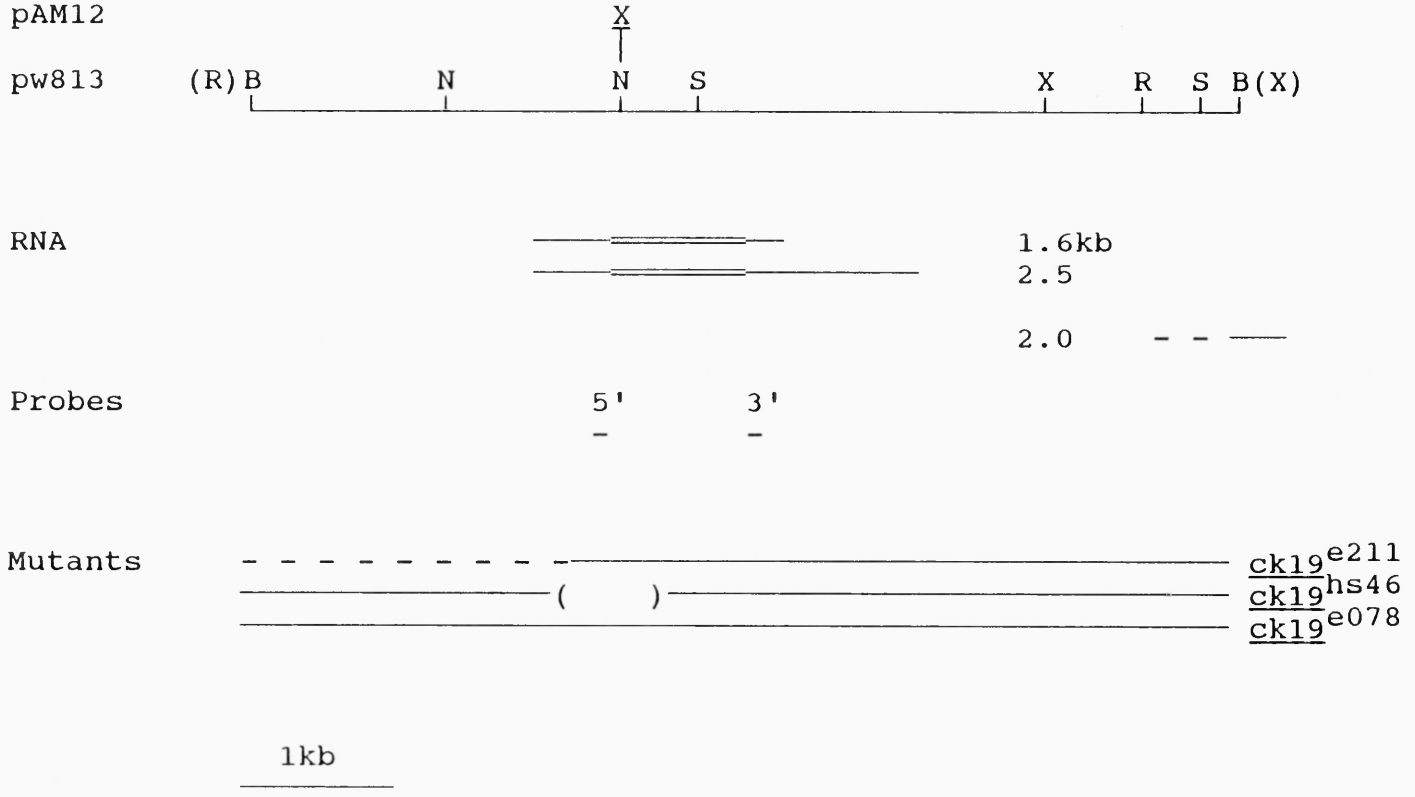

pAM12
X
pw813
(R) B
N
N
S
X
R
S
B(X)
RNA
1.6kb
2.5
2.0
Probes
5'
3'
Mutants
ck19e211
ck19hs46
ck19e078
1kb

Fig. 23

Restriction site polymorphisms specific for the *ck19*^{e211} chromosome suggest rearrangement of the *PP1 87B* gene in this mutant.

This experiment was carried out in order to screen lethal mutations in the 87B chromosomal region for rearrangements affecting the *PP1 87B* locus. Genomic DNA from the four lethal stocks indicated, heterozygous for the TM3 balancer chromosome, was digested with *Bam*HI (B), *Eco*RI (R) or a combination of these enzymes (RB). A Southern blot of this DNA was probed with the *PP1 87B* 3' specific probe. All mutations were induced on Oregon R wild type chromosomes. TM3 and the mutant chromosomes, with the exception of *ck19*^{e211} show the wild type pattern of bands. Note that *PP1 87B* falls within Df(3R)E-079 as judged by the intensity of the hybridisation signal.

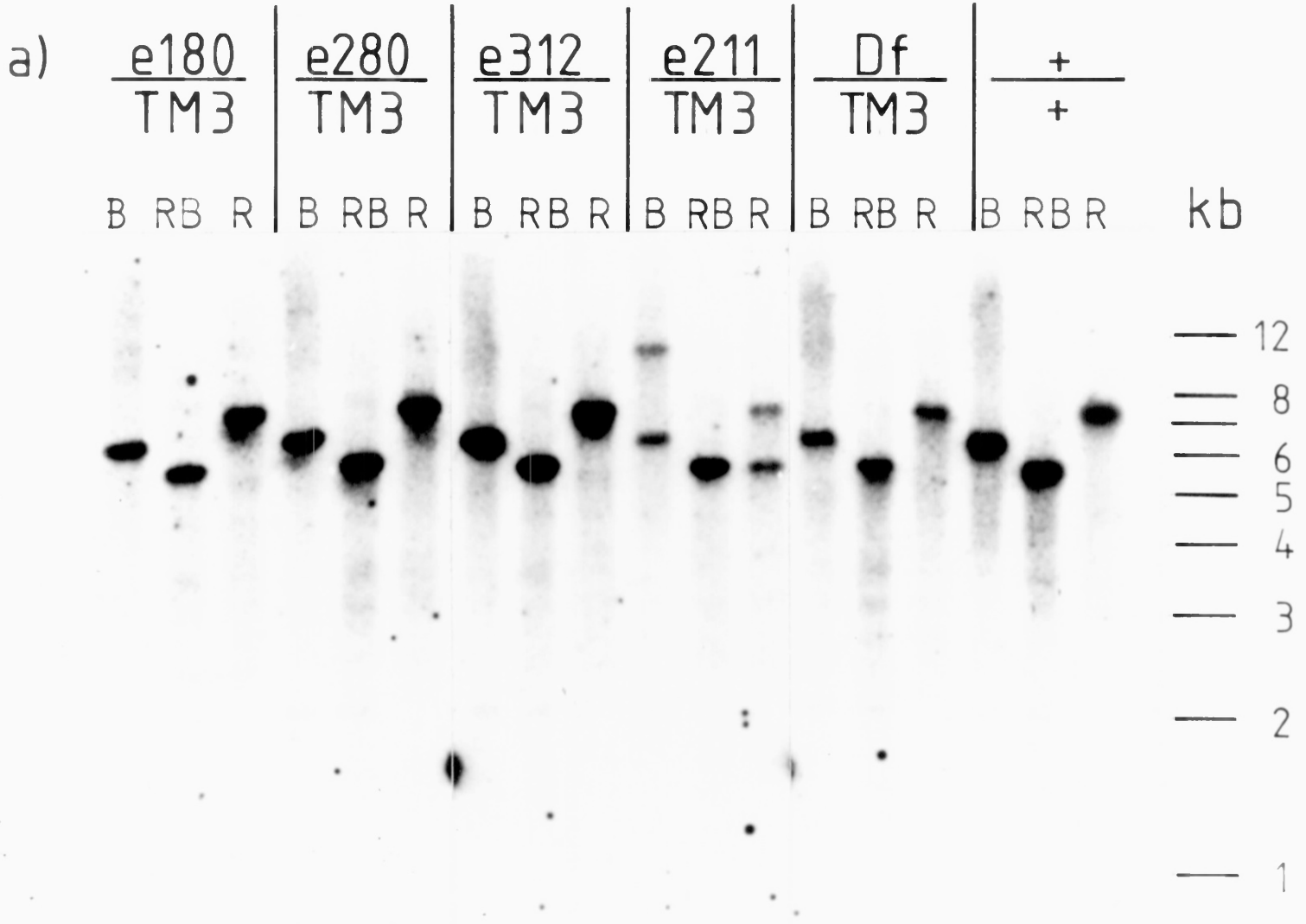

a)
e180/TM3
e280/TM3
e312/TM3
e211/TM3
Df/TM3
+/+
B RB R B RB R B RB R B RB R B RB R B RB R
kb
12
8
6
5
4
3
2
1

Fig. 24

Blots of genomic DNA from heterozygous flies show DNA rearrangements in the *PP1 87B* gene of *ck19* mutants:

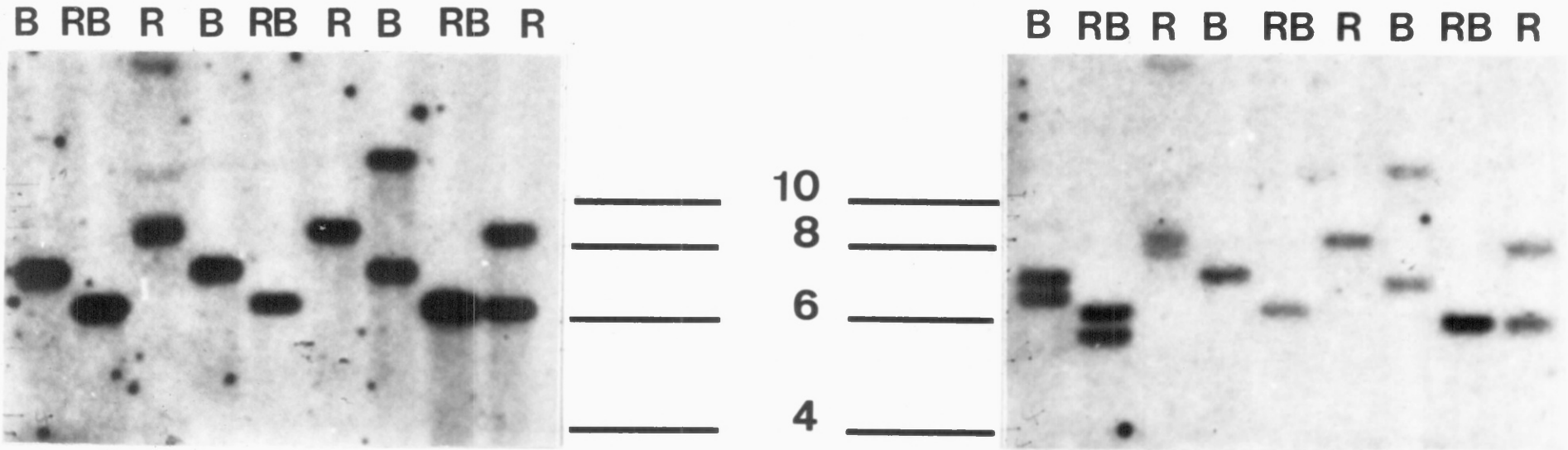
DNA from the indicated fly stocks was digested with *Bam*HI (B), *Eco*RI (R), or a combination of the two enzymes (RB). Molecular size is indicated in kilobase pairs (kb). Southern blots of this DNA were hybridised to ^{32}P-labelled 5' and 3' *PP1 87B* probes shown in Fig. 22. The TM6B balancer and wild type (+) chromosomes yield an identical pattern of bands. In the *ck19*^{hs46}/TM6B lanes note that the *PP1 87B* 3' probe hybridises to restriction fragments some 500bp shorter than wild type. These are derived from the mutant chromosome and represent a small deficiency in the region homologous to the 5' probe (see Fig. 22).

5' PROBE

3' PROBE

$$\frac{hs46}{TM6B} \quad \frac{+}{+} \quad \frac{e211}{TM6B}$$

$$\frac{hs46}{TM6B} \quad \frac{+}{+} \quad \frac{e211}{TM6B}$$



5.4 Transcription of *PP1 87B*

RNA blots were probed with *PP1 87B* probes and subsequently with pDmras64B (Mozer et al. 1985) as a control for loading (Fig. 25). The 1.2kb *PP1 87B* cDNA, 5' and 3' probes all recognise two abundant transcripts of approximately 1.6 and 2.5kb under stringent hybridisation conditions demonstrating that both mRNAs are products of the same gene. *PP1 87B* transcripts are approximately the same proportion of total poly (A)-rich RNA in the embryonic, larval, pupal and adult stages. For the messages to exhibit an uniform developmental profile it is likely that the *PP1 87B* messages are to be found in most tissues because different developmental stages vary enormously in the relative proportions of the tissues present.

5.4.1 A *ck19* allele which abolishes transcription from *PP1 87B.*

In order to determine whether mutations in *ck19* affected *PP1 87B* expression I prepared RNA blots of poly A^+ RNA from third instar larvae of $ck19^{e078}$ $ck19^{e211}$ and $ck19^{hs46}$ all hemizygous with Df(3R)E-079 (Fig. 26). $ck19^{e078}$ mutant larvae were found to contain both the 1.6kb and 2.5kb *PP1 87B* transcripts at wild type levels. $ck19^{e211}$ produced undetectable amounts of *PP1 87B* RNA. $ck19^{hs46}$ does not produce the 1.6kb and 2.5kb transcripts, but instead, two smaller, less abundant transcripts were observed. Transcripts of a similar size, approximately 2.0 and 1.3kb, are also present at low levels in wild type RNA perhaps reflecting the activity of an alternative promoter or start of transcription. Thus there are two explanations of the $ck19^{hs46}$ transcription pattern. Firstly, the $ck19^{hs46}$ RNAs may be the wild type minor transcripts, overexpressed in the absence of the 1.6kb and 2.5kb species. Alternatively, the $ck19^{hs46}$ RNAs may be truncated and poorly expressed versions of the 1.6kb and 2.5kb transcripts which coincide in size with wild type minor RNAs.

Fig. 25

Transcription of *PP1 87B* during development in wild type *Drosophila*

a) Polyadenylated RNA was isolated from the following developmental stages: E=0-12h embryos, 1, 2, 3=1st, 2nd, 3rd larval instars, P1=early pupae, P2=late (dark) pupae, F=female adults, M=male adults. This blot was probed with the *PP1 87B* 3' probe and showed the same result when reprobed with the 5' probe (not shown). Abundant transcripts of about 2.5 and 1.6kb were found to be expressed as an approximately constant proportion of total polyadenylated RNA throughout development. Two much less abundant transcripts of about 2.0 and 1.3kb also show a similar pattern of expression.

b) The same blot reprobed with pDmras64B (Mozer et al. 1985) as a control for loading.

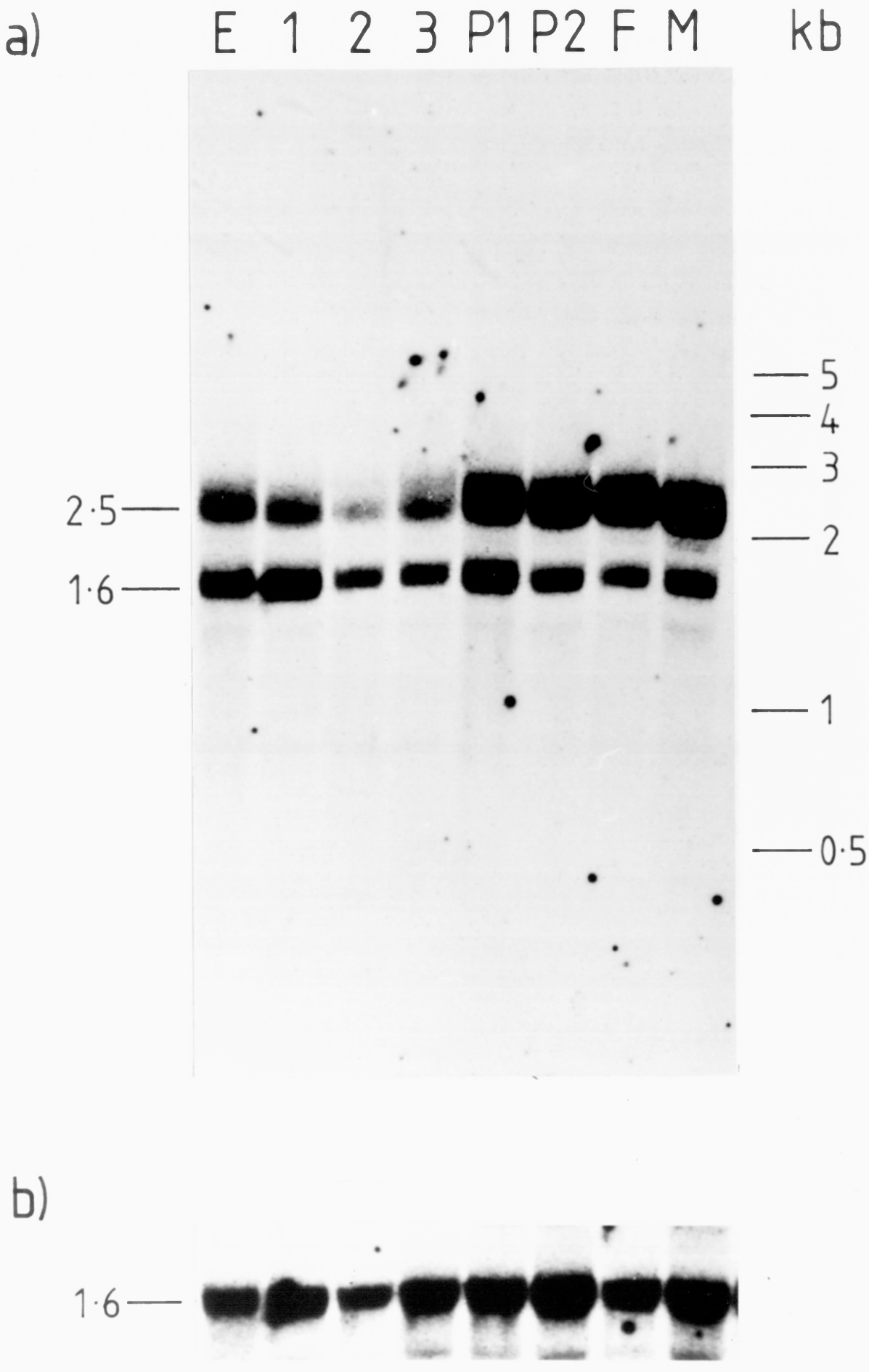

a)
E 1 2 3 P1 P2 F M kb
2·5
1·6
5
4
3
2
1
0·5
b)
1·6

Fig. 26

Transcripts of mutant and wild type alleles of *PP1 87B*:

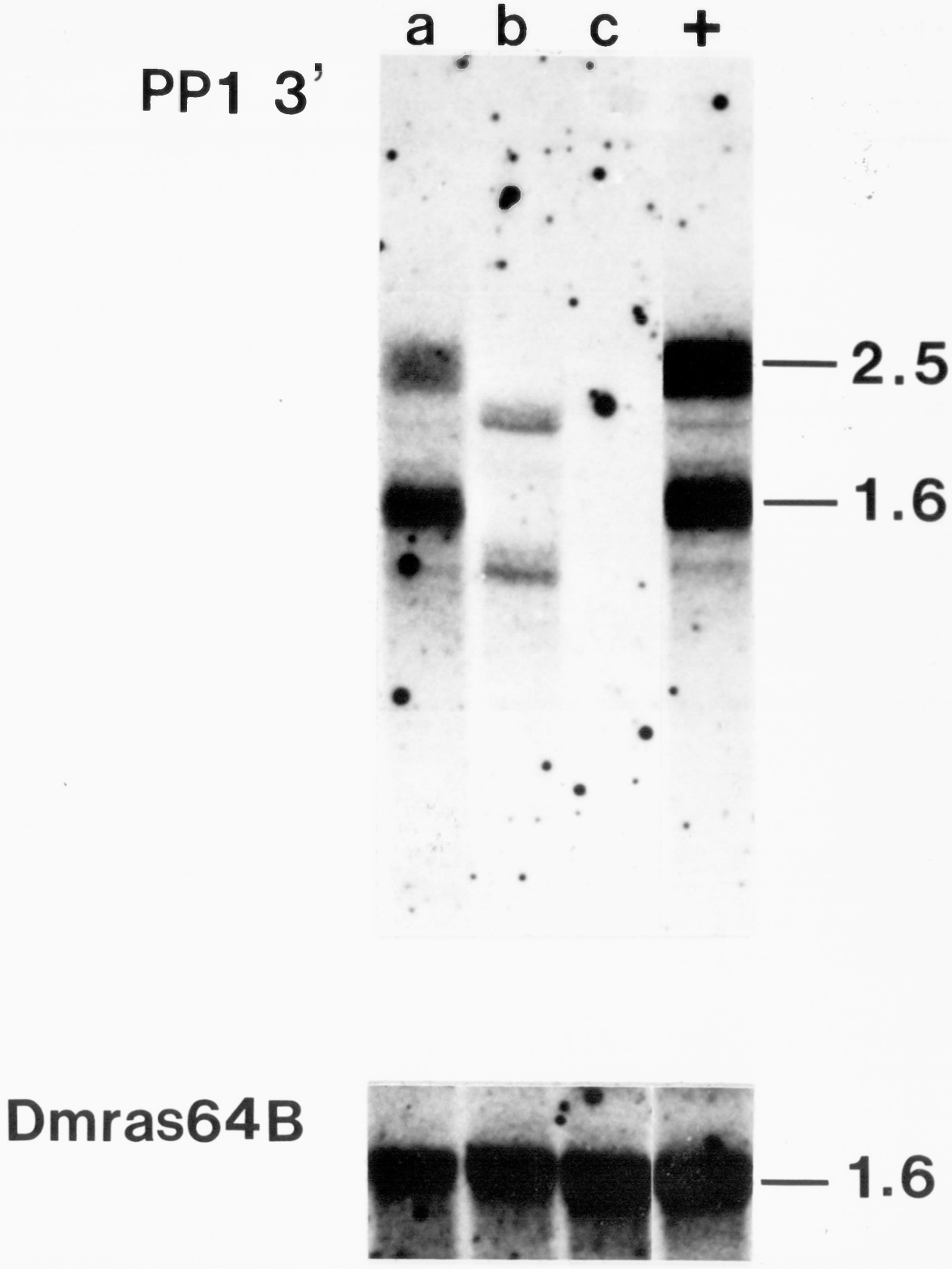
An RNA blot of polyadenylated RNA from 3rd instar larvae was first hybridised with *PP1 87*B 3' probe as previously described in Fig. 25. After autoradiography, the blot was stripped by boiling in wash buffer and reprobed with pDmras64B (Mozer et al. 1985) as a control for loading. The RNA was prepared from larvae heterozygous for Df(3R)E-079 and the following third chromosomes:

a) $ck19^{e078}$. The wild type transcripts are present at normal or slightly reduced levels.

b) $ck19^{hs46}$. Transcripts in this mutant are altered in both size and abundance.

c) $ck19^{e211}$. This mutation abolishes zygotic expression of *PP1 87B* in these larvae.

d) Wild type.


a b c +
PP1 3'
2.5
1.6
Dmras64B
1.6

5.4.2 Isolation of further *PP1 87B* cDNA clones

I used the 1.2kb cDNA derived from *PP1 87B* to isolate, by colony hybridisation, cDNA clones from a 0-4h embryonic library in the plasmid vector pNB-40 (kind gift of N. Brown: Brown and Kafatos (1988)). This vector bears the cDNA insert in a defined orientation to enable *in vitro* transcription. This orientation also facilitates comparison of homologous clones after restriction enzyme cleavage. *PP1 87B* clones were identified in Southern blots using the 5' 172bp probe described above. *PP1*-positive cDNAs are abundant and comprised about 0.2% of this library (177 in 10^4 total clones examined). The sizes and hybridisation patterns of some of these clones are described in Table 13.

I could recognise two classes of insert after digestion with *Eco*RI and *Hind*III to release the cDNA from the pNB-40 vector, those without recognition sites for the enzyme, and a second group which contained an internal *Eco*RI site. The first group gave a strong signal on hybridisation with the *PP1 87B* 5' probe and were thus identified as *PP1 87B*. The 1.0kb fragment of clone 5.11 did not hybridise with either of the *PP1 87B* probes used. I used this fragment to probe the other cDNAs and found it to be specific for the second class. The 1.0kb 3' *Eco*RI fragment of clone 5.11 hybridised *in situ* to a single site at 96A2-5 (not shown), thus identifying the second class of embryonic cDNAs as representing transcripts of *PP1 96A*.

My restriction mapping and Southern blotting of the cDNAs 5.19, 5.8, and genomic clone p*w*813 indicated that the cDNAs were co-linear with the genomic clone over the *PP1* coding region (not shown). DNA sequencing of these cDNAs by members of Dr Cohen's laboratory confirmed that the *PP1* open reading frames of 5.19, 5.8 and genomic clone p*w*813 were identical to that of the published 1.2kb cDNA. The genomic locus contains no introns. cDNA 5.8 is polyadenylated and similar at its 3' end to the 1.2kb cDNA. 5.19 differs in length from 5.8 at the 3' but not at the 5' end suggesting the hypothesis that these two clones may be derived from the 2.5kb and 1.6kb RNAs respectively.

This hypothesis predicts that a 3' fragment of 5.19 with no sequence identity to 5.8 should hybridise specifically to the 2.5kb transcript in RNA blots. This experiment remains to be done. The use of such a probe for *in situ* hybridisation to tissue sections might reveal subtleties of expression of the *PP1 87B* gene. However, the proteins that would be produced by the 2.5 and 1.6 messages would be identical and the relative abundance of the two transcripts is constant throughout development so it is hard to see how *a priori* such differential expression of *PP1 87B* could have a physiological function in the organism. The two major *PP1 87B* transcripts may differ in splicing of the 3' noncoding region. A more likely explanation is that the 1.6 and 2.5kb RNAs result from differential polyadenylation of *PP1 87B* transcripts at alternative sites 3' to the coding region.

Table 13

Summary of cDNA clones isolated from the 0-4h embryonic library using the 1.2kb *PP1 87B* clone

Clones were isolated by colony hybridisation and characterised by Southern blotting with the probes indicated below. The sizes of the inserts released by restriction digestion with *Eco*RI and *Hind*III are shown, as well as a qualitative indication of the intensity of hybridisation of the different probes. An intense hybridisation signal is denoted "++", weak hybridisation "+", and no signal "-". The lower class of cDNA contain an internal *Eco*RI site and were derived from the *PP1 96A* gene. Those without an internal *Eco*RI site were derived from *PP1 87B*.

Clone cDNA	Insert size (kb)	PP1 87B probes		cDNA5.11 probes	
		5' probe	1.2kb	1.4kb (5')	1kb (3')
87B					
5.6	1.5	++	++	++	-
5.8	1.6	++	++	++	-
5.10	1.4	++	++	+	-
5.19	2.3	++	++	ND	ND
5.22	1.55	++	++	ND	ND
5.35	2.2	++	++	ND	ND
96A					
3.1	1.4	+	+	++	-
	0.5	-	-	-	++
5.11	1.4	+	+	++	-
	1.0	-	-	-	++
5.13	1.4	+	+	++	-
	0.5	-	-	-	++

5.5 Germline transformation with *PP1 87B* rescues *ck19* mutants.

The co-localisation of a lethal mutation with a DNA lesion which abolishes transcription of the *PP1* gene is not in itself sufficient evidence that *PP1 87B* is required for viability. Hence I tested the wild type *PP1 87B* transcription unit for its ability to complement the lethality of *ck19* mutants by introducing a 6.5kb *Bam*HI fragment (Fig. 22) into the germline by P element mediated transformation (Rubin and Spradling 1982). I isolated wild type clones using the *PP1 87B* 3' probe. These clones, in the CosPer cosmid vector, were from a library provided by John Tamkun (Dept. of MCD Biology, Univ. of Colorado, Boulder, USA). From one of these, pcos87Ba, a 6.5kb *Bam*HI fragment containing the *PP1 87B* coding region was subcloned into the P*w*8 vector (Klemenz et al. 1987) to generate the wild type transformation construct p*w*813 (Fig. 22).

As it remained possible that rescue could result from another gene carried on the *Bam*HI fragment I inserted a linker containing translational termination codons in all 3 reading frames into the open reading frame of the [$PP1^{+}$] gene in the p*w*813 construct. Using the sequence of the 1.2kb cDNA (Dombrádi et al. 1989) as a guide, I decided to introduce this oligonucleotide at an *Nru*I site toward the 5' end of the ORF so that this insertion would stop the *PP1* ORF after the first 8 amino acid residues. This generated a synthetic mutant allele [$PP1^{AM}$] in the control construct pAM12. To achieve this, p*w*813 was partially cleaved with *Nru*I, which cuts once within the sequence of the *PP1 87B* 1.2kb cDNA, but seven times in the whole construct. Full length linear plasmid was purified by gel electrophoresis and ligated to the dephosphorylated palindromic duplex oligonucleotide Amber MURFI (AM) (5' CTAG TC TAGA CTAG 3') as described by Perlman and Halvorson (1986). This oligonucleotide was kindly synthesised by Dr Conrad Lichtenstein. The ligated DNA was used to transform *E. coli* XL-1 (Stratagene). Plasmids were screened for replacement of the original *Nru*I site at the 5' end of the *PP1 87B* ORF by the *Xba*I site borne on the oligonucleotide. Plasmids were digested with *Xba*I or *Nru*I, Southern blotted in duplicate and probed with the 5' and 3' probes described above. As may be seen from Fig. 22, the two probes recognise the same single fragment of wild type construct p*w*813 after digestion with *Xba*I but are separated by an *Nru*I site. Only insertion of the AM oligonucleotide results in an *Nru*I fragment recognised by both probes and separate *Xba*I fragments each recognised by one probe. The pattern of fragments on cleavage with both enzymes is of course identical for wild type and disrupted constructs. DNA sequencing of the insertion site in the pAM12 construct carried out by members of Dr Cohen's laboratory confirmed that a single linker had been correctly inserted.

Approximately 25% of embryos survived injection, 30% of these emerged as adults, 75% were fertile of which 5% (0.3% of injected embryos) gave transformed progeny bearing the w^{+} eye color marker. 9 independent transformed lines were

isolated containing the wild type *PP1 87B* construct p*w*813, and 6 lines with the disrupted construct pAM12. G_0 (injected) flies and their transformed G_1 progeny were crossed to w^{1118} G_2 and G_3 flies were crossed *inter se* to generate lines homozygous for the insertions. *In situ* hybridisation with vector probe was used to determine insertion sites. Lines with a single euchromatic insertion site were chosen for use in rescue experiments. To control for any effect of chromosomal position on expression of the *PP1* gene, independently-derived transformants were employed (Spradling and Rubin 1983).

Table 14

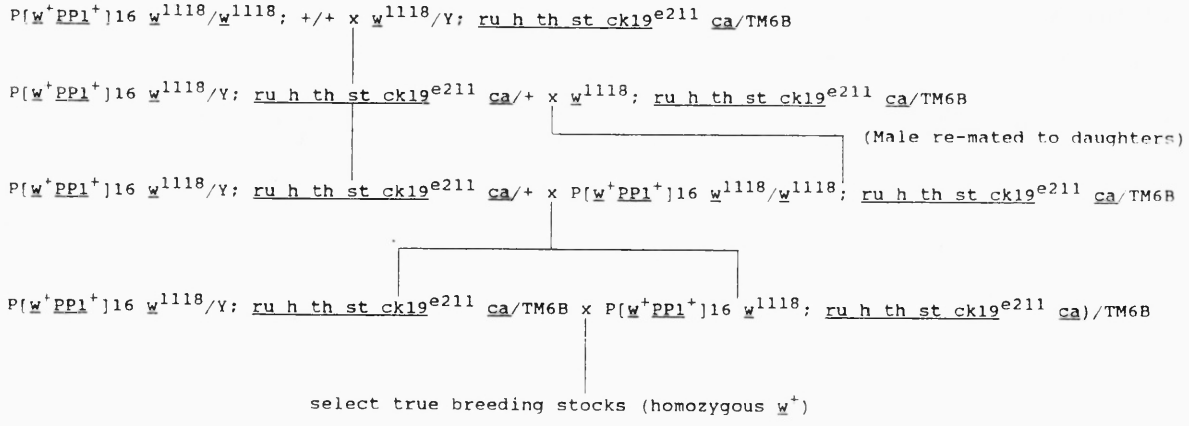
Transformed lines used to demonstrate rescue of *ck19* mutants by P elements bearing the wild type *PP1 87B* gene.

Independent insertions of the wild type (p*w*813) and disrupted (pAM12) constructs were obtained and the insertion site in each stock localised by *in situ* hybridisation (not shown). The chromosomal positions of the constructs in stocks bearing single P element insertions are shown below.

Stock	Insertion
p_w813	
P[w^+ *PP1* $87B^+$]6	20A
P[w^+ *PP1* $87B^+$]15	60B
P[w^+ *PP1* $87B^+$]16	5E4-7
P[w^+ *PP1* $87B^+$]17	70A
pAM12	
P[w^+ *PP1* $87B^{AM}$]B1	10C9-D1
P[w^+ *PP1* $87B^{AM}$]E1	47C3-7
P[w^+ *PP1* $87B^{AM}$]F3	36B

P[w^+ *PP1 87B*$^+$]6 was not used in complementation experiments because this insertion, though viable, is associated with an X-linked roughened eye phenotype. Such a phenotype could result from the element's insertion in a gene which, when thus mutated gives roughened eyes. Another possibility is that the *PP1 87B* gene on the P element is irregularly expressed due to the position of its insertion site and thus causes the roughened eye phenotype through cell death or differential growth. This insertion is near the heterochromatin of the X chromosome, so irregular expression might result from position-effect variegation as has been discussed in Section 1.5. Homozygosity for any of the other insertions does not cause any visible abnormality, or sterility, so one or two extra copies of the *PP1 87B* gene do not appear to be deleterious.

Appropriate stocks were constructed to generate mutant animals with and without the transposon that carries the additional wild type *PP1 87B* transcription unit. The crosses used to put the P[w^+PP1^+]16 insertion and the *ck19*^{e211} mutation together in the same stock are shown below. "*rf2*" is the trivial name of the multiply marked *ck19*^{e211} recombinant used in the rescue experiments.



Males bearing X-linked P elements and heterozygous for the *PP1* mutation and third chromosome balancer, TM6B, were crossed to balanced females heterozygous for the deficiency Df(3R)E-079 (Table 1a). These single pair crosses generated female progeny with, and male progeny lacking the P element construct. This could be confirmed in adults using the w^+ eye color marker carried by the P*w*8 vector (Klemenz et al. 1987). Table 1b shows that while mutant males generated in this way do not live to adulthood, their mutant sisters bearing the [$PP1^+$] construct do survive. In addition, these rescued females are fully fertile.

Table 15b shows that unlike the [$PP1^+$] construct, [$PP1^{AM}$] fails to rescue $ck19^{e211}$/Df(3R)E-079 mutants. In a similar series of experiments, $ck19^{hs46}$ and $ck19^{e078}$ were also rescued by P element insertions of [$PP1^+$] but not by [$PP1^{AM}$]. Since $ck19^{e211}$, $ck19^{hs46}$ and P[w^+, *PP1* $87B^{AM}$]B1 fail to complement the lethality of $ck19^{e078}$/Df(3R)E-079 larvae, which are, however rescued by P[w^+ *PP1* $87B^+$]16 (not shown) I conclude that $ck19^{e078}$ is a hypomorphic mutant of *PP1 87B* with residual function nearly sufficient for normal mitosis.

I analysed neuroblast squashes from larvae generated by the rescue experiment described in Table 15a to ensure that the stocks used in that experiment exhibited the lethal mitotic phenotype. The results of this experiment summarised in Table 3 demonstrate that the presence of the P element construct bearing the wild type *PP1* gene restores not only the adult viability of the $ck19^{e211}$ mutant (Table 15b), but also restores imaginal disc cell proliferation and normal mitosis in neuroblasts (Table 16). The rescue of mitosis is shown quantitatively in Table 11b. The disrupted construct $PP1^{AM}$ cannot effect rescue.

I am led to conclude that the *ck19* complementation group is equivalent to the *PP1 87B* gene, and that this gene is essential for viability.

Table 15

Rescue of lethal *ck19* alleles by a functional *PP1 87B* gene:

a) Inheritance of P element bearing PP1 87B and the eye color marker w^+.

The male parent carries the P element on the X chromosome, and is heterozygous for $ck19^{e211}$ and the TM6B balancer chromosome. The female parent is heterozygous for TM6B and a deficiency for *PP1 87B*, Df(3R)E-079. The phenotypes of the progeny are shown in the table.

b) Numbers of progeny in each phenotypic class generated by crosses like that shown in a).

In one set of crosses the parental male X chromosome carries the wild type gene designated P[*PP1 87B*$^+$]16. In the second set of crosses, the parental male X bears the disrupted gene P[*PP1 87B*^{AM}]B1. Numbers of progeny expected if [*PP1*$^+$] but not [*PP1*^{AM}], rescues the $ck19^{e211}$ mutation are in brackets.

a)

Paternal gamete	Maternal gamete: w^{1118} Df(3R)E-079	Maternal gamete: w^{1118} TM6B Tb
Y;ck19^{e211}	$\sigma^{>}$ w	$\sigma^{>}$ w;Tb
Y;TM6B	$\sigma^{>}$ w;Tb	embryo dies
w^{1118} P[w^{+}];ck19^{e211}	ς_{+} w^{+}	ς_{+} w^{+};Tb
w^{1118} P[w^{+}];TM6B	ς_{+} w^{+};Tb	embryo dies

b)

Progeny phenotype	Paternal X insertion: P[w^{+}PP1^{+}]16	Paternal X insertion: P[w^{+}PP1^{AM}]B1
$\sigma^{>}$ w	0 (0)	0 (0)
$\sigma^{>}$ w Tb	175 (178.4)	245 (234.5)
ς_{+} w^{+}	109 (89.2)	0 (0)
ς_{+} w^{+} Tb	162 (178.4)	224 (234.5)

Table 16

Rescue of imaginal disc proliferation and mitotic phenotype of ck19 mutant alleles by a functional *PP1 87B* gene:

Sibling larvae derived from crosses shown in Table 15a were examined for failure of imaginal disc growth and for abnormal mitosis. Df(3R)E-079 and a previously unpublished deficiency Df(3R)SI55 that uncovers *l(3)ck19* (kindly supplied by János Gausz (Fig. 18)) were used to uncover the lethal mitotic phenotype associated with the $ck19^{e211}$ mutation. Ganglia from 10 larvae of each genotype were analysed in squash preparations for presence of overcondensed or hyperploid mitoses of the types shown in Fig. 20. (+) denotes a result indistinguishable from wild type and (-) denotes mutant.

	Sex	Imaginal disc growth	Neuroblast mitosis
$\underline{w}^{1118}$ P[$\underline{w}^{+}$$\underline{PP1}^{+}$]16/ $\underline{w}^{1118}$; $\underline{ck19}^{e211}$/Df(3R)E-079	female	+	+
$\underline{w}^{1118}$/ Y; $\underline{ck19}^{e211}$/ Df(3R)E-079	male	-	-
$\underline{w}^{1118}$ P[$\underline{w}^{+}$$\underline{PP1}^{+}$]16/ $\underline{w}^{1118}$; $\underline{ck19}^{e211}$/ Df(3R)SI55	female	+	+
$\underline{w}^{1118}$/ Y; $\underline{ck19}^{e211}$/ Df(3R)SI55	male	-	-
$\underline{w}^{1118}$ P[$\underline{w}^{+}$$\underline{PP1}^{AM}$]B1/ $\underline{w}^{1118}$; $\underline{ck19}^{e211}$/Df(3R)E-079	female	-	-
$\underline{w}^{1118}$/ Y; $\underline{ck19}^{e211}$/ Df(3R)E-079	male	-	-
$\underline{w}^{1118}$ P[$\underline{w}^{+}$$\underline{PP1}^{AM}$]B1/ $\underline{w}^{1118}$; $\underline{ck19}^{e211}$/Df(3R)SI55	female	-	-
$\underline{w}^{1118}$/ Y; $\underline{ck19}^{e211}$/ Df(3R)SI55	male	-	-

5.6 Conclusions

Drosophila has four highly conserved loci with homology to the coding sequence of the catalytic subunit of type 1 protein-serine-threonine phosphatases (PP1s). I have identified mutations in one of these genes, at 87B on chromosome 3. One of the mutants, $ck19^{e211}$, shows no detectable transcription of the *PP1 87B* gene. Another mutant allele, $ck19^{hs46}$, produces RNAs which differ from wild type in size and abundance. Mutants die at the larval-pupal boundary with little or no imaginal cell proliferation. Neuroblasts are delayed in progress through mitosis and show defective spindle organisation, abnormal sister chromatid segregation, hyperploidy and an excessive degree of chromosome condensation. In contrast to *A. nidulans* and *S. pombe*, the homologous *Drosophila* PP1 genes do not provide sufficient activity to complement the loss of *PP1 87B*.

Germline transformation of mutant flies with the wild type *PP1 87B* gene results in rescue of normal mitosis, viability and fertility. These results suggest that PP1 activity is required for mitotic progression and that the other loci cannot supply sufficient activity to complement loss of expression of the *PP1 87B* gene. Alternatively, the *PP1 87B* product may have a distinct specialised function in mitosis.

Chapter 6: Genetic analysis of chromatin structure and its regulation during the mitotic cell cycle.

The aim of my research has been to screen, by direct cytological analysis, mutations causing lethality at the larval-pupal boundary. By this method I have attempted to identify and characterise genes whose products are essential in the structure of the mitotic chromosome or in the regulation of the processes of chromosome condensation and decondensation.

6.1 Strategies for isolation of genes essential for mitosis in *Drosophila*

Successful analysis of the genes encoding functions which control the transition from interphase (G2) to mitosis has been achieved in fungi by making use of cell division cycle (cdc) mutants which block the cells in interphase. This appproach has as yet yielded little information about the nuclear response to these mitosis-inducing signals. In contrast, cytological screening of conditionally lethal mutations in *Schizosaccharomyces pombe* and *Aspergillus nidulans* has met with more success leading to the identification of genes whose products are required in maintenance of nuclear structure (*crm1*$^+$) and several whose products are essential for decatenation or disjunction of sister chromatids (reviewed by Yanagida 1990). This last class includes topoisomerase II, and *nuc2*$^+$, a nuclear protein capable of binding AT-rich DNA and which has been proposed to associate mutually forming part of the chromosome scaffold (Hirano et al. 1990).

Our aim is to be able to extend this analysis to multicellular organisms, not only to identify evolutionarily conserved molecular structures, but to provide a bridge linking the wealth of classical cytology with our growing molecular understanding of chromosome composition and its cell-cycle regulation. This approach is feasible in *Drosophila*, which is most suitable for advanced genetics and has few, relatively large chromosomes. The major obstacle to this work is a technical consequence of the multicellular nature of *Drosophila*, namely, genes cannot be readily isolated by direct complementation of *Drosophila* mutants with libraries of wild type genes. However, some success has already been achieved by interspecific complementation of *S. pombe* mutants with *Drosophila* cDNAs (L. Alphey, pers. comm.). I believe cytological screening of collections of *Drosophila* mutants to be worth the effort because the chromosomes are large enough for us to be sure that there is a real and complex cycle of condensation and decondensation of chromatin. The small size of most fungal chromosomes also precludes analysis of mutations with region-specific effects on mitotic chromosomes. *Drosophila* chromosomes ought therefore to be a relatively more sensitive material with which to identify determinants of condensed chromatin structure by analysis of mutations which disrupt this structure.

In view of the relatively large number of nonhistone proteins found in preparations of mitotic chromosomes, one would predict a large class of mutations that produce irregular mitotic chromosome condensation and result in chromosome breakage. These mutations would be those affecting proteins which are themselves integral to the structure of chromatin. These could be proteins which bind DNA, nucleosomes, or which are components of the higher order structure of the mitotic chromosome. Some such mutations are already known in *Drosophila* but few have been extensively characterised (Gatti 1979, Gatti and Baker 1989).

6.2 Determinants of higher order mitotic chromosome structure

Are higher order structures in the metaphase chromosome (Belmont et al. 1987) entirely the result of reorganisation at a lower level? Mechanisms already proposed to explain mitotic chromatin organisation depend essentially on "loop-fastening" proteins to organise DNA. Firstly cooperative binding of histone H1 is required to aggregate turns of histone-bound DNA into 30nm chromatin fibre (Thoma et al. 1979). Secondly, the bases of 10^3-10^4bp radial loops are bound by topoisomerase II and other nonhistone proteins in the models of Laemmli and co-workers (see Introduction). Either the processes of coiling and packaging of the 30nm chromatin fibre and coming together of aggregates of radial loop domain-binding proteins are indeed sufficient to form the entire structure of the metaphase chromosome, or there exist higher levels of organisation intrinsic to the chromosome which define a still further level of packaging complexity. By analogy with the conservation of anaphase (Rabl) orientation of chromosomes in the interphase nucleus I suggest that we might find this organisation, if such exists, in a rearranged form in the interphase nucleus. Certainly very large chromosomal domains which have the potential to be topologically independent are known. These are the $\approx 10^6$bp units between attachment sites linking chromatin to the nuclear lamina (Hochstrasser et al. 1986). Such units are 1-2 orders of magnitude larger than the scaffold- (or matrix-) bound loops (Lewis and Laemmli 1982). It has been proposed that laminar attachment sites maintain the untangled orientation of the chromosomes in the nucleus (Section 1.2). It is not known whether these sites are simply released from the nuclear lamina at prophase and are without function in the mitotic chromosome or whether they can contribute to chromosome packing by mutual association. It has certainly been noted that these laminar attachment sites are buried within the metaphase chromosome structure because nuclear envelope re-formation depends on chromosome decondensation (Newport and Spann 1987).

6.3 *l(3)snap* and *mus101*

l(3)snap and *mus101* mutations disrupt the integrity of condensed mitotic chromosomes. In the case of both mutations, extended or insufficiently packaged regions of chromatin remain at metaphase despite apparently normal condensation of a

large proportion of the chromosomes. The *l(3)snap* defect shows no apparent specificity for particular chromosomal regions but *mus101* mutations preferentially affect the pericentric and Y chromosome heterochromatin. The possible causes of the "stretched" or unpackaged regions could be any of several. Mutant proteins could produce these phenotypes by acting, or failing to act in any of the processes of DNA replication, repair, and chromatin condensation.

Failure of chromatid replication in localised regions could lead to linked structures comprising sister (G2) chromatids which were still single (G1-like) in parts. Such chromosomes would be abnormally packaged at mitosis. If sister chromatids coil in helices of opposite handedness (Boy de la Tour and Laemmli 1989), which way would a shared single chromatid segment coil? Additionally, chromatin proteins may be added or modified during replication which mark DNA as having been replicated. Others might be incorporated which determine the response of chromatin to condensing factors. Without completion of replication, such permissive modifications might not occur.

More likely to cause failure of chromatin condensation would be the persistence into M phase of DNA lesions such as single strand breaks or gaps resulting from regional imperfections in DNA replication. Such lesions might prevent binding of chromosomal proteins, both directly and by failure to transmit supercoiling or torsional forces which may be necessary for protein binding and higher order chromosomal packaging. Sections containing DNA lesions, or abnormally or sparsely bound chromatin proteins will be particularly susceptible to chromosome breakage. Similarly, mutations in repair enzymes which correct DNA lesions persisting from replication would permit incorrect packaging and abnormal mitotic condensation. Also, repair functions are present in mitotic cells and may be required during chromosome condensation and mitosis itself to deal with spontaneous, mechanical and enzymic damage to DNA during these processes.

While basic components of the cell cycle oscillator and many chromosomal proteins (e.g. the nucleosomal histones) are conserved, it is clear that the chromosomes of different species differ widely in the size and structural complexity of mitotic chromosomes. Is this complexity matched by a corresponding variability in structural chromatin proteins? Or does an evolutionarily invariant mitotic chromosome structure arise from number of conserved proteins which act entirely independently of DNA sequence? If chromosome organisation is entirely without regard to DNA sequence, nonfunctional "garbage" DNA would have been able to accumulate without evolutionary selective pressure. However, much noncoding DNA, especially satellite DNA, is relatively homogeneous in sequence. Chromatin proteins with specific binding affinities for such sequences have the potential to constrain the sequence divergence of their binding sites which would otherwise result from accumulation of mutations. Such

proteins might also exert an selective influence on the spread of such sequences through the genome.

In spite of the prevalence of apparently useless "garbage" DNA we should also consider the observation that larger genomes reflect, in part, the greater number of genes in multicellular organisms with specialised cells or tissues. The demands of maintaining different programs of differential gene activity from the same set of chromosomes in a variety of tissues may require the chromosomes of multicellular organisms to be correspondingly more complex in composition. There is wide variety in size and morphology of *Drosophila* diploid chromosomes in different tissues. The chromosomes of the eye disc are less than 1μM long compared to 5-10μM in giant neuroblasts This corresponds to up to a thousandfold difference in chromosome volume. These differences between chromosomes in mitotic tissues may simply be quantitative, that is, the degree of compaction of mitotic chromosomes may be governed by the amount of chromosomal protein incorporated or the amount of time available before mitosis for chromosome condensation. In turn, these factors may be controlled by the volume or space available in the mitotic cell.

However, particular cell types might carry tissue-specific distributions of chromatin proteins inherited as part of tissue determination, a speculative idea entertained by Brown (1984). If so, these proteins would have to remain associated with chromatin through mitosis and might therefore have a structural role. Thus chromosomes in different tissues would be qualitatively different in composition and respond with differing sensitivities to mutations in the genes encoding tissue-specific chromatin proteins. This hypothesis is particularly interesting given the strong mutant effect *l(3)snap* mutations have on mitotic chromosome condensation in neuroblasts and ganglion mother cells without apparently affecting diploid cell division in other tissues.

Alternatively, it now seems likely that phosphorylation of replication and transcription factors by $p34^{cdc2}$ provides a way in which these are removed from chromatin, permitting mitotic packaging to occur (Nurse 1990). Mutant proteins which could bind in interphase but which did not respond to this signal could well interfere with proper condensation in a tissue-specific manner.

6.3.1 Is *mus(1)101* required for DNA replication or for mitotic chromatin packaging?

The Y chromosome is most sensitive to the loss of *mus101* function after temperature shift of ganglia bearing the conditional allele $mus101^{ts1}$. Although no data are supplied, Gatti et al. (1983) concluded, from studies with $mus101^{ts1}$ and T(2,3)bw^{vDE4}, that the $mus101^{ts1}$ mutation preferentially affects the condensation of the heterochromatin of the second chromosome. This preferential decondensation differs from that seen with H33258 treatment of living ganglia where the pericentric heterochromatin of chromosome 2 is not affected (Pimpinelli et al. 1975). Nor is this

heterochromatin brightly stained by this dye which selectively binds AT-rich DNA (Holmquist 1975) Interestingly, the only major satellite sequence consisting of pure AT is the $(AATAT)_n$ sequence which despite constituting 3.1% of the entire genome of *Drosophila melanogaster* (Lohe and Roberts 1988) is apparently absent from the centric heterochromatin of chromosome 2 (Lohe, pers. comm. (in) Ashburner 1989).

It is conceivable that H33258 prevents mitotic condensation of this AT-rich DNA by blocking binding of a specific set of proteins such as the D1 protein, known to bind specifically to the $(AATAT)_n$ satellite (Levinger 1985). Were the product of the *mus101*$^+$ gene a satellite DNA-binding protein with a different sequence specificity, we should expect the regions decondensed due to *mus101* malfunction to be rich in the sequences normally bound. Several sequences do in fact show the right distribution, including the most abundant satellite $(AAGAG)_n$ which is 5.6% of the genomic DNA and a major component of the satellite DNA of the Y and chromosome 2.

6.3.2 DNA damage and the euchromatin-heterochromatin boundary

There is a large body of published work to support the view that DNA replication is error-prone, and the lesions introduced both by replication, and by spontaneous or mutagen-induced alteration of DNA structure are repaired by defined enzymic pathways. Whether the biochemical and mechanical processes of chromosome condensation and mitosis produce DNA damage which is repaired by particular enzymes in normal cells has not been investigated. However, it may be worthwhile to consider this possibility as well as the more obvious observation that regions at the junction of domains of differentially condensed chromatin may well be subject to particular topological stresses.

The nature of the DNA occupying the boundary between euchromatin and heterochromatin is of particular interest. It is presently unclear how this region is regulated with respect to chromatin condensation. The boundary does appear to be particularly vulnerable in mitotic cells to damage by mutagens (Gatti et al. 1974, 1975, 1979, Pimpinelli et al. 1976.). Sister chromatid exchange (SCE) occurs most commonly at heterochromatin-euchromatin boundaries in cells treated with bromodeoxyuridine (BrdU). This is also true of mammalian cells (e.g. Kato 1979). The susceptibility of the boundary to damage is genetically determined, as has been shown by Gatti (1979). 80% of the heterochromatic breaks which occur spontaneously in mitotic neuroblasts of mutagen-sensitive mutants were found near the junction with the euchromatin. Of these, *mus(1)109* causes heterochromatic breakage, mostly near the junction, but rarely exhibits breaks in the euchromatin. This observation supports the hypothesis that the *mus(1)109*$^+$ gene functions to maintain the integrity of this region.

Sperm nuclei, despite different chromatin structure may also show a similar uneven distribution of damage. The distribution of translocation breakpoints induced by X-rays is commensurate with the relative lengths of heterochromatin and

euchromatin seen at mitotic prometaphase. Paradoxically, duplications and deficiencies of heterochromatin do not appear to influence the breakage frequency (reviewed by Ashburner 1989). This is open to two obvious interpretations, either there exist susceptible sites in the centromeric region of the X proximal to the region duplicated or deleted in these experiments, or any euchromatic-heterochromatic boundary is a hypersensitive target regardless of its particular sequence.

The significance of the under-representation of breakpoints within the heterochromatic regions themselves may be interpreted in at least three ways: It seems unlikely that damage caused by ionising radiation could be somehow "transferred" to the euchromatic boundary (Muller 1945). Heterochromatin may simply be less susceptible to damage by ionising radiation due to its sequence or condensation state. For example, an abundance of cysteine sulphydryl groups or other reducing agents decreases the sensitivity of chromatin to ionising radiation (discussed by Muller 1954). Alternatively, breaks within repeated DNA could generate "adhesive" ends which might facilitate repair by promoting association of homologous DNA. This last hypothesis is based on an idea proposed by Zhimulev et al. to explain the ectopic pairing of "weak points" in salivary gland polytene chromosomes formed by intercalary heterochromatin. However, the proportion of breaks induced by X-irradiation etc. observed in mitotic cells may be under-recorded owing to the persistence of sister chromatid pairing in the centromeric heterochromatin during metaphase and c-metaphase.

It would probably be unwise to assume that DNA repair is deficient in the centromeric heterochromatin or that mitotic chromosome condensation is incompatible with access of repair enzymes. Observations of DNA polymerase access made using *in situ* nick translation (e.g. Sperling et al. 1985) do not necessarily provide useful information about repair access because DNA-manipulating enzymes may also be structural components of chromatin itself. Indeed, a challenge to the view that condensed chromatin is inaccessible to repair comes from the discovery of DNA repair which functions in mammalian cells artificially arrested at metaphase (Mullinger and Johnson 1985). If DNA repair enzymes can operate on metaphase chromosomes, heteropycnotic interphase chromatin should present no obstacle to repair. Indeed, some form of DNA repair may even be preferentially active in heterochromatic regions.

These studies are also of interest with respect to the possible role of *mus101* in maintaining the integrity of the mitotic chromosome. When repair synthesis is inhibited with drugs such as hydroxyurea, chromosomes damaged by U.V. light decondense. Frequently the centromeric and telomeric heterochromatin are the first regions to be affected. If this may be taken to mean that heterochromatin is particularly susceptible to loss of repair activity, and if DNA repair enzymes are required throughout mitosis in non-mutagenised cells (for which there is no evidence) then the *mus101* repair defect might indeed cause specific decondensation of heterochromatin.

6.3.3 A checkpoint links mitotic entry to DNA replication and repair.

In yeast and mammalian cells there exists a mechanism ensuring the obligatory coupling of the completion of DNA synthesis to mitotic initiation (reviewed by Hartwell and Weinert 1989). It is thought that, in embryonic cleavage divisions, the pathway, or S-M checkpoint linking mitotic entry to the completion of replication is absent, or that the system detecting un-replicated DNA is significantly less sensitive than it is in somatic cells (Raff and Glover 1988, Dasso and Newport 1990). Enoch and Nurse (1990) demonstrated that the level of *cdc25*$^{+}$ product is a key determinant of this checkpoint. When levels are high (e.g. when the *cdc25*$^{+}$ message is overexpressed) in *S. pombe*, blocking DNA replication with hydroxyurea no longer prevents mitotic entry. It is clear that the early *Drosophila* embryo at least, has constitutively high levels of an homologous mitotic activator in the form of maternally-supplied *string*$^{+}$ message (Section 1.1). This may contribute to the relative insensitivity of mitotic initiation in this system to the presence of unreplicated DNA.

It is possible therefore that embryos from *mus101*^{D2} mothers have a temperature sensitive defect in DNA replication and the abnormal nuclear divisions described in Section 4.1.2 are the consequence of an attempt by the mitotic apparatus to segregate unreplicated DNA. Remarkably similar abnormally-condensed mitotic nuclei were observed in syncytial embryos where replication had been inhibited with aphidicolin (Raff and Glover 1988).

The hypothesis, discussed above, that *mus101* mutant cells enter mitosis with incompletely replicated DNA does not agree well with the finding in both yeast and mammalian cells that the initiation of mitosis is extraordinarily sensitive to the presence of DNA containing lesions such as unrepaired single-stranded breaks or gaps. If such sensitive checkpoint mechanisms do indeed operate in the *Drosophila* gangliar cells, it would not be sufficient for *mus101* mutations to interfere only with DNA replication or repair. Nor would a mutation altering only the timing of mitotic entry be able to produce the characteristic metaphase chromosomes with undercondensed heterochromatin in neuroblasts. In cells with a sensitive S-M checkpoint, a mutation preventing complete replication should only be able to produce abnormally condensed mitotic chromosomes if the mutation also interferes with the checkpoint mechanism itself. Consequently it would probably be unwise to reject the hypothesis that the *mus101*$^{+}$ product might act at the S-M checkpoint simply because this product is required even in "permissive" pre-blastoderm embryos. It is now clear that embryonic nuclear cycles don't simply lack the components of the S-M checkpoint mechanism (Dasso and Newport 1990). I therefore believe that the *mus101* product is either, as suggested by Gatti et al. (1983), required purely in chromosome packaging, or is required in the mechanism linking mitotic initiation with DNA replication.

6.4 What is the effect of inhibiting chromatid disjunction and anaphase segregation on chromosome condensation?

A second checkpoint connects the extent of chromosome condensation to the disjunction of sister chromatids. It is known from studies with colcemid, colchicine, 2-mercaptoethanol and microsurgery of spindles that inhibition of spindle assembly in sea urchin embryos results in chromatin hypercondensation and delayed sister chromatid separation (Sluder 1979, Sluder and Begg 1983, Sluder et al. 1986). Activation of the $p34^{cdc2}$ protein kinase at the G2-M transition results in chromosome condensation. The hypercondensation of chromosomes in cells delayed at metaphase is likely to result from stabilisation of $p34^{cdc2}$ kinase activity. For example, depolymerisation of the mitotic spindle of human cells with the drug, nocodazole, arrests them in a mitotic state with a high level of $p34^{cdc2}$ protein kinase activity (Draetta and Beach 1988). The mechanism coupling $p34^{cdc2}$ kinase activity to spindle organisation has not yet been fully described but much attention has been focused on the type B mitotic cyclins. It is thought these cyclins may have several roles, activating $p34^{cdc2}$ kinase and bringing about reorganisation of microtubules as the mitotic spindle. A role for cyclins in coupling $p34^{cdc2}$ kinase inactivation to anaphase chromatid separation has been suggested because proteolytic degradation of cyclin B correlates with the onset of sister chromatid disjunction (Minshull et al. 1989).

The processes of chromosome condensation and microtubule reorganisation by active forms of $p34^{cdc2}$ have been separated using *Xenopus in vitro* extracts (Verde et al. 1989) and a conditional mutation in *S. pombe* cyclin *cdc13-117* (Hagan et al. 1988, Moreno et al. 1989). In *cdc13-117* mutants chromosome condensation occurs and cells arrest with a stable high level of cdc2 protein kinase activity. Microtubules, however, remain in an interphase-like configuration suggesting that while *cdc13-117* is competent to activate and maintain cdc2 kinase activity, the $p34^{cdc2+}$/pcdc13-117 complex is inactive in the pathways of microtubule reorganisation and cyclin degradation. Booher and Beach (1987) have shown that $cdc2^{+}$ overexpression will complement the *cdc13-117* mutation so the spindle-reorganising function of the cyclin is likely to act via $p34^{cdc2}$.

The mechanism by which $p34^{cdc2}$ protein kinase activity is held at a high level during mitotic arrest is not understood. One mechanism has been characterised which maintains cells at meiotic metaphase II. Cytostatic factor (CSF) of *Xenopus* eggs comprises a protein kinase, c-*mos* (Sagata et al. 1989, reviewed by Hunt 1989), the activity of which prevents cyclin degradation. *c-mos* can indeed phosphorylate cyclin B. Roy et al. (1990) have speculated that this phosphorylation ensures the stability of the cyclin, preventing the inactivation of cyclin-associated $p34^{cdc2}$ (M-phase promoting factor or MPF). Whether this is a mechanism common to other cell types is not clear. CSF activity has not been detected after fertilisation in *Xenopus* (Meyerhof and Masui 1979) and c-*mos* RNA is absent or expressed at extremely low levels after gastrulation

in cycling somatic cells (Sagata et al. 1988, 1989). It is theoretically possible that mitotic arrest is maintained by an analogous, uncharacterised protein kinase in somatic cells with disrupted spindles. In a tentative model of this kind an opposing protein phosphatase would permit $p34^{cdc2}$ protein kinase inactivation and drive mitotic progression. The observations of Picard et al. (1989) show that, in starfish oocytes, eggs and embryos and in *Holothuria* (sea cucumber) oocytes, inhibition of protein phosphatase 1 (PP1) at metaphase will delay or prevent $p34^{cdc2}$ kinase inactivation. Thus PP1 is also required to terminate M-phase kinase activity in these systems.

The regulation of cyclin stability by phosphorylation is potentially rather complicated. Felix et al. (1990b) have shown that in *Xenopus* activated egg extracts it is the protein kinase activity of starfish $p34^{cdc2}$ which triggers the destruction of cyclin and hence the inactivation of the kinase complex. These authors do not however favour a model in which $p34^{cdc2}$ kinase phosphorylates cyclin directly to bring about its destruction so these results are not necessarily contradictory.

Once the $p34^{cdc2}$ protein kinase has been inactivated at the onset of anaphase, it is not clear whether there are further checkpoints linking the processes of chromatid segregation, chromosome decondensation and nuclear envelope reformation or to what extent these processes rely on coordinate regulation by protein phosphatases.

It seems likely that PP1 is required in the correct regulation of spindle organisation or function. The unusual, collapsed and disorganised spindle structures seen in whole ganglia of *Drosophila PP1 87B* mutants could conceivably result in the mitotic delay and chromatin hypercondensation observed via such a feedback pathway. However, as stated above, the chromosome condensation process is also likely to be abnormal in PP1 mutants. Also, many of these mutant spindles must function at least in early mitosis because overcondensed chromosomes are correctly placed at the metaphase plate.

6.5 *PP1 87B* is essential for mitosis in *Drosophila*.

I have described mutations in the type 1 protein phosphatase gene, *PP1 87B*. One, $ck19^{e211}$, completely abolishes expression of this gene. Another, $ck19^{hs46}$, which is phenotypically indistinguishable, eliminates the major *PP1 87B* transcripts. Such null mutants may still express PP1 activity provided by one or more of the other *PP1* genes (Dombrádi et al. in prep.). I have also identified a mutant allele, $ck19^{e078}$, which is also rescued by germline transformation with the wild type *PP1 87B* gene but which has some residual function in that neuroblasts from larvae of genotype $ck19^{e078}$/Df(3R)E-079 are mitotically normal with well developed imaginal discs. Thus I conclude that the mitotic abnormalities seen in the null mutants $ck19^{e211}$ and $ck19^{hs46}$ prevent larval neuroblast and imaginal cell proliferation. If all of the *Drosophila* PP1 isoenzymes are expressed in mitotically active tissue and differ only in relative abundance then we may further conclude that there is a threshold level of activity required for normal

mitotic progression. Even if the other PP1 isoenzymes are expressed in mitotically active larval tissues their contribution is insufficient to complement total loss of *PP1 87B* function. We do not yet know if this is the case, but *PP1 87B* transcription is not developmentally regulated (Dombrádi et al. 1989) and transcripts are ubiquitously present in the third instar larva (C. H. Girdham, JMA and D.M. Glover, unpublished results). The other PP1 genes might show differential developmental or tissue-specific expression. Tissue specific expression of isoforms of other proteins is well documented in *Drosophila*. For example, mutations in the male-specific β-tubulin gene lead to defects in male meiosis and spermiogenesis even though functional isoforms of β-tubulin are found in other tissues (Kemphues et al. 1982, Fuller et al. 1987). Null mutations in the other *PP1* iso-genes and assays of activity in isolated tissues will be needed to address this possibility experimentally. Despite the similarity of the predicted amino acid sequences of the *Drosophila melanogaster* PP1 isoenzymes (Dombrádi et al. unpublished), there also remains the possibility that the isoenzymes are not equivalent either in activity or in their ability to bind specific regulators. Although transformation of flies mutant in one PP1 gene with chimeric PP1 constructs or extra doses of another iso-gene may help to investigate this possibility, biochemical studies on null mutants or of iso-enzymes expressed *in vitro* may prove to be more informative.

Although $ck19^{e211}$ is a non-conditional zygotic null mutation it must be borne in mind that the cells of homozygous larvae may differ in the residual level of protein contributed by their heterozygous mothers. Thus some heterogeneity of terminal phenotype is to be expected. Although it is clear that a sub-population of cells continue to cycle normally in late larvae bearing *PP1 87B* null mutations, some (hyperploid cells) have evidently been through one or more cycles with less residual PP1 than that required for normal mitosis. It is at this stage in development in these cells, therefore, that we observe the earliest departure from normality, caused by the mutations. Because cells of mutant larvae may undergo a number of mitotic cycles in the presence of diminishing PP1 activity it might be anticipated that the cumulative effects of the insufficiency of this enzyme in the regulation of a number of cellular processes would eventually prevent entry to mitosis, a major energy-requiring event. Surprisingly, we found a high mitotic index and cells with disjoined but highly overcondensed chromatids, both characteristic of defective regulation of the later stages of mitosis. It is possible that cells are rendered incapable of completing mitosis by having grown under conditions of reduced PP1 activity. However, it is clear that in the processes of exit from mitosis, many substrates are dephosphorylated. It seems likely, therefore, that this period of the cell cycle demands a relatively high level of protein phosphatase activity, and that it is this requirement which leads to the defects seen in PP1-deficient mitotic cells.

In contrast, genetic analysis of the cell cycle of fungi has concentrated most fruitfully on conditional mutations producing a characteristic terminal phenotype in

largely homogeneous populations of cells. It is nevertheless possible to see some similarities between the mitotic phenotypes of null mutations in the *Drosophila PP1 87B* and conditional lethal mutations in the PP1 genes of *S. pombe* and *A. nidulans*.

6.5.1 PP1 mutations in Aspergillus, Schizosaccharomyces and Drosophila disrupt a subset of mitotic processes.

The success of a mitotic division depends on the coordination in space and time of multiple serial and parallel molecular events. In all three species, PP1 mutations result in an elevated mitotic index. I infer from this that mutant cells enter mitosis but spend longer in a mitotic state than the wild type. The *dis2-11*^{cs} mutation of *S. pombe* results in the initiation of mitosis at the nonpermissive temperature "apparently...with normal timing". Cells with "strongly condensed" chromosomes then accumulate and progressively lose viability (Ohkura et al. 1988, 1989). I found an accumulation of mitotic cells with overcondensed chromosomes to be the predominant cytological phenotype associated with mutations in *Drosophila PP1 87B*. When *A. nidulans bimG-11*^{ts} mutant conidia are shifted to nonpermissive temperature, the chromosome mitotic index rises to a peak approximately tenfold higher than wild type but then declines as nuclei undergo at least one further cycle of chromatin decondensation and recondensation without completion of nuclear separation (Doonan and Morris 1989).

Abnormal mitotic spindle organisation is seen in PP1 mutants of all three species. *dis* mutant spindles in *S. pombe* lack the "blob-like" ends of the early spindle. Ohkura et al. (1988) suggest this might reflect an absence or malfunction of kinetochore microtubules. Also, in this mutant parts of the spindle associated with the condensed chromosomes do not undergo the normal telophase disassembly. As in other animal cells, the spindle microtubules of *Drosophila* are structurally organised in a more complex way than those of fungi. The lack of *PP1 87B* in the *Drosophila* mutant results in a similarly complex set of unusual structures including multipolar spindles, disorganised microtubules surrounding overcondensed chromosomes, and unusually densely aggregated spindle microtubules bearing metaphase-like chromosomes. These latter spindles have an abnormal distribution of microtubules associated with the spindle pole. *bimG-11* mutant spindles are abnormally bent, and short, indicating that spindle elongation does not occur at the nonpermissive temperature. *dis2* spindles do elongate but sister chromatids disjoin in fewer than 2% of mitoses studied. Without this elongation step, whole chromosomes are distributed unequally between the two ends of the cell in an anomalous anaphase. It is not clear whether chromatids disjoin in the *bimG* mutant mitosis. At the second mutant mitosis after temperature shift "two clumps of closely apposed, condensed chromatin" are observed implying partial partitioning of the nucleus (Doonan and Morris 1989). Although this is evidence that *bimG* mutant chromatids disjoin it is not necessarily evidence that they do so during mitosis. Indeed, studies of acentric double minute chromosomes in mammalian cells provide an example

of chromatids unable to disjoin in mitosis, which nevertheless do so in G1 before re-replication (Takayama and Uwaike 1988). The presence of aneuploid and polyploid nuclei in the *Drosophila* mutant is indicative of defective chromosome segregation, which might result from the unusual spindle structures. The polyploid multipolar cells seen are evidence that prevention of nuclear separation by mutation of *PP1 87B* does not necessarily preclude the continuing replication DNA and centrosomes.

6.5.2 PP1 mutations also affect other vital processes.

It is clear that the PP1 mutations which have been studied to date are pleiotropic, disrupting mitotic processes but also affecting other cellular functions. This is not surprising given that PP1 functions in multiple biochemical pathways in mammalian cells (for review see Cohen 1989). In addition to exhibiting abnormal mitosis, *bimG-11* mutants do not establish developmental polarity at nonpermissive temperature (Doonan and Morris 1989). *dis2-11* mutants even under permissive conditions are hypersensitive to caffeine, are unable to form homologous diploids, and lose a minichromosome at an elevated frequency (Ohkura et al. 1988, 1989). I found that $ck19^{e211}$/Df(3R)E-079 larvae were of superficially wild type size and behavior. Salivary gland chromosomes in these mutant larvae appear normal with respect to banding pattern and degree of chromatid replication. The simplest inference is that PP1 is not required in chromatid replication or that less activity is required for replication than for mitosis. This inference would be invalid were the iso-genes differentially expressed in larval tissues.

Mutations in *PP1 87B* probably do impair other vital processes at later developmental stages. There are two lines of evidence for this. Firstly, while $ck19^{e078}$ is undoubtedly a lethal mutation in *PP1 87B* because it can be rescued by P elements bearing the wild type gene, $ck19^{e078}$/Df(3R)E-079 larvae show little evidence of abnormal mitosis (see above). These mutants never complete pupation. Secondly, Reuter et al. (1986, 1987) have reported that several chromosomes bearing alleles of *ck19* have the ability to suppress the variegation of euchromatic genes displaced by chromosomal rearrangements to a locus adjacent to a region of heterochromatin. The basis for heterochromatic position-effect variegation is not well understood but is likely to be a reflection of the higher order structure of interphase chromatin on gene expression (Tartof et al. 1984, Eissenberg 1989, Section 1.4). The number of mutations with the property of suppressing or enhancing variegation is large and it is now clear that these genes may act in distinct pathways. For example, transformation with extra copies of the wild type zinc finger protein gene $Su\text{-}var(3)7^{+}$ complements the suppression due to *Su-var(3)7*, $Su\text{-}var(2)1^{01}$ and $Su\text{-}var(3)3^{03}$ but not that due to $Su\text{-}var(3)6^{01}$ (Reuter et al. 1990). There is evidence for allelism of *ck19* and *Su-var(3)6*. Not only does the suppression of position-effect variegation co-segregate with *ck19* lethality but also the lethality of trans-heterozygotes between variegation-suppressing *ck19* chromosomes and the semi-lethal $Su\text{-}var(3)6^{01}$ correlates well with the magnitude of the suppression of

variegation in these flies. The possibility that *PP1 87B* might be important in regulating interphase chromatin structure is intriguing given its role in mitotic chromosome condensation. However, the regulation of interphase and mitotic roles could be very different. It remains to be proven that it is mutations in *PP1 87B* itself which affect heterochromatic position-effect variegation. It will now be possible to use the *PP1 87B* transformants to attempt to influence variegation in wild type, enhancer, and suppressor mutants, and so to clarify this interpretation.

6.5.3 Evolutionary conservation of PP1 catalytic subunits reflects a multiplicity of protein-protein interactions.

Type 1 protein phosphatases are amongst the most highly conserved homologous proteins known. 92% of the amino acid residues are identical in the *Drosophila* and rabbit skeletal muscle enzymes. It is likely that the interaction of the enzyme with multiple regulatory subunits and substrates has placed a constraint on evolutionary sequence divergence. While the catalytic subunit has broad substrate specificity *in vitro*, the actual substrates dephosphorylated *in vivo* will be limited by the activity and location of PP1 specified by regulatory proteins. The best characterised of these are the phosphoprotein inhibitors I-1 and I-2 (Huang and Glinsmann, 1976, for review, see Cohen 1989). Inhibitory activity of I-2 oscillates in a cell-cycle-dependent manner (Brautigan et al. 1990) which suggests a mechanism which could modulate the timing of PP1 activity during the cell cycle.

PP1 is concentrated in the nucleus (Kuret et al. 1986, Ohkura et al. 1989, Cohen 1989). The mechanism for this nuclear localisation is unknown. However, G and M subunits have been identified which bind PP1 catalytic subunits to glycogen particles and myofibrils respectively. If analogous subunits localise PP1 within the mitotic nucleus, then the structural state of the nucleus could determine the sites of phosphatase activity.

It is important to note that the alleles of *dis2* and *bimG* that affect mitosis in *S. pombe* and *A. nidulans* are not null mutations. Indeed, abolition of gene function by disruption of $dis2^{+}$ or $bimG^{+}$ is apparently not sufficient to produce abnormal mitosis because of functional complementation by other PP1 genes. The conditional mutations studied by Doonan and Morris (1989) and Ohkura et al. (1989) are not dominant, but nevertheless interfere with mitotic progression, perhaps by misregulation of PP1 activity. This could result from an abnormal interaction of the catalytic and regulatory subunits (for discussion see Cyert and Thorner 1989). In contrast, a null mutation in *PP1 87B* does cause abnormal mitosis, an observation most simply explained as a consequence of insufficient catalytic activity. However, it remains possible that loss of *PP1 87B* catalytic subunit permits excess regulatory subunits to interfere with the function of the other PP1 isoenzymes. If so, then mutations such as $ck19^{e211}$ which

abolish the catalytic subunit could be more deleterious than mere loss of *PP1 87B* catalytic activity.

What then is the role of PP1 in mitosis? This phosphatase might be needed as one step in the inactivation of $p34^{cdc2}$ protein kinase after metaphase (Sections 1.3, 6.4). Alternatively, the mitotic arrest or delay found in PP1 mutants could result from failure to dephosphorylate proteins that are phosphorylated due to $p34^{cdc2}$ kinase activity. Dynamic turnover of phosphate on specific proteins might also be important for some mitotic processes. For example, reorganisation of interphase microtubules as a mitotic spindle in starfish embryos requires the activity of both $p34^{cdc2}$ protein kinase and the okadaic acid sensitive protein phosphatases PP1 and/or PP2A (Picard et al. 1989). The balance of kinase versus phosphatase activity may also influence the density of chromatin packing. If neuroblasts are blocked in a metaphase-like state, for example by treatment with colchicine, chromosomes continue to condense. The strongly overcondensed chromosomes seen in the *Drosophila PP1 87B* mutants without drug treatment are, however, more highly condensed than those seen in wild type neuroblasts blocked for 2h in colchicine or in the metaphase-like arrested cells of *asp* mutants (Ripoll et al. 1985) or *l(1)d.deg4* (Gatti and Baker 1989). Other *Drosophila* mutants have been described in which chromosomes are found to be as extremely overcondensed as they are in *PP1 87B* mutants. Mutants of this class include *l(1)d.deg3* and *l(1)d.deg10* (Gatti and Baker 1989).

My analysis of the phenotype of neuroblasts from *PP1 87B* null mutant larvae leads me to the conclusion that the phosphatase encoded by *PP1 87B* is required for the execution of steps in mitotic pathways subsequent to metaphase. It is quite possible that the protein phosphatase is directly required in the control of both chromatin packaging and spindle dynamics or we may be observing the effects of an alteration in the timing or coordination of mitotic processes in the absence of this essential regulatory enzyme.

6.6 Prospects for genetic analysis of chromatin.

The identification of a protein phosphatase required in spindle organisation, chromosome decondensation and for exit from mitosis may enable us to identify proteins hyperphosphorylated in cells lacking the activity of this phosphatase. Some of these proteins are likely to be those whose state of phosphorylation determines the condensation state of chromatin.

My identification of a null mutation in the *PP1 87B* gene will allow us to test the effect *in vivo* of genes mutagenised *in vitro*. These mutant proteins could be expressed and assayed *in vitro* before being introduced into *Drosophila* by germline transformation. The similarity of the PP1 genes in different species will allow us to test homologous mutations in yeast and flies. For example, the *bim*G temperature sensitive mutation could be introduced into *Drosophila*. It would be particularly interesting to investigate the effect of conditional (temperature sensitive) *PP1 87B* mutations on

chromosome condensation in *Drosophila* by shifting the temperature at different times in the cell cycle. The use of *in vivo* labelling techniques such as injection into embryos of fluorescent histones (Minden et al. 1989) in combination with temperature sensitive mutations such as *mus101*^{D2} will permit us to examine the earliest defects in chromosome condensation and decondensation exhibited by these mutations. It would also be interesting to investigate the expression of zygotic *mus101* by determining at what time in development the paternal genome is competent to rescue the lethal temperature sensitive maternal effect of *mus101*^{D2}.

Further genes encoding proteins essential for the organisation of mitotic chromosomes might also be obtained by strategies other than the relatively slow process of screening *Drosophila* mitotic mutants followed by molecular cloning of the loci by chromosomal walking. Loci might be isolated by interspecific complementation of cytologically abnormal yeast mutants or by identification of cloned non-histone protein genes with lethal mutations resulting in a cytologically abnormal mitotic phenotype. Antibodies raised against the proteins encoded by these genes will be valuable tools with which to probe chromosome structure.

As the collection of genes required for chromosome packaging grows larger, it may be possible to separate those with a role in interphase chromatin organisation from specialised mitotic functions. In this respect it would be interesting to examine the effect on mitotic chromosomes of lethal mutations first identified by their dominant modifying effect on position-effect variegation (Section 1.5). Genetic analysis of dosage dependence and gene interactions between loci required for chromosome condensation and decondensation should also help us to arrive at a more complete understanding of the complex and dynamic organisation of the eukaryotic chromosome.

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[illegible] action of the proportionality of the breakage frequency to chromatin mass, regardless of its arrangement in blocks.

by H. J. Muller, Amherst College.

(Abstract)

The finding that the breakage frequency in heterochromatic regions is inordinately greater, in relation to the number of "active genes" contained, than that in euchromatic regions, led to the hypothesis (Muller and Painter, 1932) that the heterochromatic region contained a great number of "inactive genes". On the contrary, the evidence (Muller and Gershenson, 1935) that the mitotically visible part of the heterochromatic regions consists of very few genes producing large "blocks", became important to measure the relative breakage frequencies more precisely, especially since previous determinations by genetic or cytological means were admitted to be very faulty. The present genetic tests for translocations overcome the difficulties caused by male-sterility effects of X and Y and lethality of X by attached-X daughters of irradiated males (Males were irradiated and crossed to attached-X females containing autosomal inversions in all arms and the daughters tested for translocations. Instead of X-Y autosome translocations being not more than a third as frequent (or less) as autosome-autosome translocations (see Muller, 1935), they are now found to be at least as frequent, just as the breakage frequency depended on total amount of chromatin present regardless of number of genes. This agreement, if not accidental, would imply that a can cause breakage at any point in the indivisibility of the chromosome.

It is to be noted that the percentage of female sterility after crosses of irradiated males to non-attached-X females is almost entirely explained by the frequency of Y breaks followed by Y-translocations, thus (2) of the following by loss of all or part of the Y.

½ F₁ male sterility in crossing

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