SUPPRESSORS OF POSITION-EFFECT VARIEGATION AND THE *cdc2Dm* GENE IN *DROSOPHILA MELANOGASTER*

By

NIGEL JOHN MIATT CLEGG

B.Sc.(Hon.), Queen's University, 1983
M.Sc., Queen's University, 1986

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Department of Zoology)

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

January 1992

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Zoology

The University of British Columbia
Vancouver, Canada

Date 23/11/92

ABSTRACT

Suppressor of Position-Effect-Variegation (*Su(var)*) mutations in *Drosophila melanogaster* are believed to identify genes that participate in the establishment or maintenance of heterochromatic domains. A cytogenetic analysis of region 31 of the *Drosophila melanogaster* polytene chromosome map was undertaken to clarify the number and position of several previously identified *Su(var)* mutations. Ten deficiencies were used to divide the 31 region into 15 separate subintervals. Results of this analysis suggest that there is a single *Su(var)* locus (*Suvar(2)1*) in the 31A-B region. Two recessive suppressors of position-effect variegation reside in the 31E-32A region. A fourth locus, *Su(var)216*, was positioned in region 31E.

Additional mutations were sought throughout the 31 region. In total, one hundred and twenty-one new EMS, gamma-irradiation, and *P* element induced mutations were tested for complementation and mapped using deficiencies. None of the mutations had a *Su(var)* phenotype, but 8 alleles failed to complement the lethal phenotype associated with the *Su(var)216* chromosome.

A *P* element induced allele of *Su(var)216* was cloned and sequenced. The *P* is adjacent to *cdc2Dm*, the *Drosophila* homologue of the fission yeast *cdc2* gene. The kinase encoded by *cdc2* is required for proper progression through the cell cycle. The lethal phenotype of *Su(var)216* can be rescued by an

ectopically placed *cdc2Dm* gene construct; however, the *Su(var)* phenotypes are not rescued. Deficiency mapping of *Su(var)216* with a *cdc2Dm* gene construct in the genetic background suggests that the *Su(var)216* and *cdc2Dm* mutations may be tightly linked (<0.5 cM) but separable.

Six EMS induced missense mutations of *cdc2Dm* were sequenced. One mutation, $cdc2^{E1-4}$, is located within the PSTAIR sequence of *cdc2Dm*, a region believed to interact with cyclins. A second mutation, $cdc2^{D57}$ is within a region highly conserved amongst kinases. Hemizygous $cdc2^{D57}$ mutants die as embryos if they inherit the mutation from their mothers, but die as larvae if they inherit the mutation from their fathers. Most of the other mutant alleles of *cdc2Dm* die predominantly during the pupal stage.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF BIOCHEMICAL ABBREVIATIONS

APS	ammonium persulphate
bp	base pairs
BCIP	5-bromo-4-chloro-3-indolyl phosphate
DTT	dithiothreitol
EDTA	ethylene-diaminetetra-acetic acid
EMS	ethylmethane sulfonate
kb	kilobase pairs
lambda-dil	100 mM NaCl, 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$
LM	10 g/l tryptone, 5 g/l yeast extract, 2 g/l $MgCl_2$, 5 g/l NaCl
MOPS	3-[N-morpholino]propanesulfonic acid
NBT	nitro-blue tetrazolium
nt	nucleotides
PEG	polyethylene glycol 8000
pfu	plaque forming units
PVP	polyvinylpyrrolidine
SSC	standard saline citrate (0.15 M sodium chloride, 0.015 M sodium citrate)
TE	10 mM Tris-HCl (pH 8.0), 1mM EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactoside

LIST OF GENETIC ABBREVIATIONS

Balancers

CyO	*Curly derivative of Oster In(2LR)O, dp^{lvI} Cy pr cn^2*
SM1	Second Multiple *In(2LR)SM1, al^2 Cy cn^2 sp^2*
TM3	*In(3LR)TM3, ry^{RK} Sb*
TM6	*In(3LR)TM6, ss^- Ubx^{67b}*

Mutants

b	*black* (2-48.5); body colour
cn	*cinnabar* (2-57.5); eye colour
Cy	*Curly* (2-6.1); wings curled upward
Gla	*Glazed*; ommatidia a smooth sheet; pericentric inversion on Chromosome 2
w^{m4}	*In(1)w^{m4}*; variegated for *white*
Ly	*Lyra* (3-40.5); lateral margins of wing excised
nw^D	narrow-Dominant (2-83); long, thin wings
P[ry^+ 2-3](99B)	abbreviated *Δ2-3(99B)*; *P* element insertion that activates other elements in the soma and germline (see Robertson et al., 1988)
pr	*purple* (2-54.5); eye colour
Pin^{Yt}	*Pin-Yellow tip* (2-107.3); distal third of thoracic bristles thin and yellow
ry^{506}	*rosy* (3-52); eye colour
S	*Star* (2-13); irregular eye facets, eye reduced
Sp	*Sternopleural* (2-22); sternopleural bristles increased in number

Sb	*Stubble* (3-58.2); short thick bristles
Sb^{V}	*Stubble-Variegated*; associated with $T(2;3)Sb^{V}$ =*T(2;3)41A-C;88;89B*: displays position effect variegation
Tft	*Tuft* (2-53.2); extra bristles on mesothorax

Acknowledgements

I wish to thank my supervisor, Dr. T. Grigliatti, and Dr. G. Spiegelman for advice and financial support. I also thank members of my committee, Drs. H. Brock, R. MacGillivray, and D. Moerman for helpful discussions.

I would like to thank M. O'Grady for assisting with molecular aspects of the project, and R. Mottus for assisting with the genetics. At some time or another, virtually every member of the Fly Group participated in the genetic analysis of the 31 region and I am grateful for their efforts.

I am particularly indebted to Hugh Brock, Ian Whitehead, and Don Sinclair. In many discussions and a brief collaborative effort with Hugh, I've learned how research can and should work. Ian and I collaborated on numerous aspects of this project including genetic screens, a cytological analysis of the 31 region, and the analysis of transcription units in 31E. Many tedious evenings of virgin collecting were relieved by our discussions. Don Sinclair's enthusiasm, knowledge, and willingness to discuss *and* test ideas were inspirational. Much of the work described here was built on his efforts.

Finally, none of the work would have been completed without the understanding and support of my wife Kathy.

GENERAL INTRODUCTION

In the interphase nucleus of a typical eukaryotic cell such as that of *Drosophila melanogaster*, approximately 1.4 X 10^8 base pairs of DNA measuring over 30 centimetres in length must be folded to occupy a spherical nucleus only 10 μm in diameter. This remarkable compaction is thought to involve several hierarchical levels of chromatin organization. The lowest level of organization consists of approximately 145 bp of DNA wrapped around a histone octamer (consisting of one H3-H4 tetramer and two histone H2A-H2B dimers) to form a nucleosome. Adjacent nucleosomes are separated from each other by roughly 30-50 bp of linker DNA (McGhee and Felsenfeld, 1980). A second, less well defined level of organization is the subsequent folding of the DNA/histone complex into a helical or solenoid-like 30 nm fiber (Felsenfeld and McGhee, 1986). These first two levels of folding result in roughly a 40-fold compaction of the DNA. To attain the 100- to 200-fold compaction required to package the DNA complement into the nucleus, additional levels of organization are required. These additional levels of organization are poorly understood, but in most current models the chromatin fiber is sequestered into a series of discrete and topologically independent domains (Jackson, 1991). The establishment and maintenance of these higher levels of organization requires additional non-histone chromosomal proteins (NHPs).

The organization of chromosomes into highly compact

structures influences the utilization of the genetic material. This is best exemplified by experiments involving histone gene stoichiometry. In *in vitro* assays, if nucleosomes are formed on genes prior to the assembly of a transcription complex that includes either RNA polymerase I (Schlissel and Brown, 1984) or RNA polymerase II (Workman and Roeder, 1987), then the genes become resistant to transcription. In *S. cerevisiae*, changes in histone stoichiometry alter transcription patterns (Clark-Adams *et al.*, 1988), and nucleosome depletion increases transcription initiation *in vivo* (Han and Grunstein, 1988). Nucleosomes may also repress transcription if they are positioned at specific sites on the chromosome (Roth *et al.*, 1990).

In addition to simply blocking the access of transcription factors to DNA, protein complexes that condense DNA may also have more subtle and specific effects on gene regulation. For example, sequences in the amino terminal domain of the histone H4 gene of *Sacharomyces cerevisiae* are necessary to repress (Kayne *et al.*, 1988) or to activate (Durrin *et al.*, 1991) the transcription of specific genes *in vivo*. Hence, histones are multifunctional.

NHPs involved in establishing and maintaining chromosomal architecture are also likely to have both global and gene-specific effects on transcriptional regulation. NHPs constitute approximately 1/3 of the mass of chromatin, yet how they interact to form higher orders of structure is largely

unknown. Although NHPs are now being identified at a rapid rate, the sheer numbers and potential interactions between proteins as they assemble into functional chromatin complexes pose a problem for the biochemical study of chromatin structure and function. In multicellular organisms, most studies of NHPs have focused on the isolation of proteins which remain associated with DNA following various types of biochemical extraction (*e.g.* Alfageme *et al.*, 1980). This biochemical approach has been very successful in identifying a subset of proteins that associate with DNA, but in many instances the biological function of the proteins is unclear.

A complementary approach to the *in vitro* biochemical analysis of chromatin structure in multicellular organisms is to identify genetically genes that affect chromatin condensation. With the exception of dominant mutations in the tandemly reiterated histone genes, mutations that affect chromatin condensation are likely to arise in genes that encode NHPs or proteins that modify either NHPs or histones. A genetic analysis of such mutations may reveal the developmental consequences of impaired or altered chromatin structure. Also, interactions between mutations at different loci may reveal previously unsuspected relationships between components of chromatin. A further advantage to the genetic approach is that some mutations, such as those induced by transposable elements, can facilitate gene cloning. Thus, genetical and biochemical studies can proceed in concert.

This thesis is concerned with genes that affect two cytologically differentiable states of chromatin compaction: euchromatin versus heterochromatin. Euchromatin decondenses during interphase and contains the majority of transcriptionally active gene species while heterochromatin fails to decondense during interphase and is considered, for the most part, to be transcriptionally quiescent. Herein I describe studies to clone one of several *Suppressor-of-position-effect-variegation* (*Su(var)*) genes located in region 31 on the *Drosophila melanogaster* polytene chromosome map. These genes are believed to help to maintain or establish heterochromatic and/or euchromatic domains. The hypothesis that *Su(var)* genes encode NHPs or modifiers of NHPs that affect chromatin condensation is discussed in Chapter 1, a survey of the current literature on position-effect variegation and its modifiers. A cytogenetic analysis of a region that contains several genes that suppress position-effect variegation is described in Chapter 2. In Chapter 3, the transposon tagging and cloning of the putative suppressor *cdc2Dm*$^{Su(var)216P}$ is presented. Finally, Chapter 4 describes the phenotype of mutants at the *cdc2Dm* locus and the relationship of *cdc2Dm* to suppressors of position-effect variegation.

CHAPTER 1

LITERATURE REVIEW

A. EUCHROMATIN AND HETEROCHROMATIN

Eukaryotic chromatin assumes its most condensed state in metaphase chromosomes; however, this state is quite transient in most tissues. During telophase, some chromosomal regions unravel to form euchromatin, while others retain a dense staining, compact morphology in the interphase nucleus. The latter regions constitute heterochromatin.

In *Drosophila*, mitotic chromosomes possess conspicuous heterochromatic regions adjacent to their centromeres. These regions are best visualized during prophase before euchromatic chromatin becomes condensed. In *Drosophila melanogaster*, heterochromatin comprises the proximal half of the acrocentric X chromosome, the proximal quarters of each arm of the metacentric second and third chromosomes, and the entire Y chromosome. Most of the fourth chromosome is also considered to be heterochromatic. These heterochromatic regions replicate later in S phase than euchromatin (Barigozzi *et al.*, 1966).

In interphase nuclei in the somatic tissues of most organisms, euchromatin is generally a very loosely packaged entity which is morphologically diffuse and hence is easily defined or delineated. An exception to this general rule is the polytene chromosomes from the salivary glands of mature larvae of dipteran insects such as *Drosophila*. During polytenization in *Drosophila* euchromatin undergoes up to ten

rounds of DNA replication without chromatid asynapsis or cytokinesis. The result is a set of euchromatic chromosome arms with a characteristic banded morphology. Extensive biochemical and genetic data accummulated over the last half-century indicate that the majority of genes are located in this polytenized material.

The heterochromatin of polytene chromosomes can be subdivided into two cytologically different categories: α- and β-heterochromatin (Heitz, 1934). Alpha-heterochromatin surrounds the centromere of each chromosome and mingles with similar regions of other chromosomes to form the chromocenter. Beta-heterochromatin is the poorly banded material which connects the chromocenter to the polytene chromosome arms (Heitz, 1934). The two types of heterochromatin also have distinct biochemical and biological properties. Alpha-heterochromatin is not polytenized (Rudkin, 1969; Gall *et al.*, 1971). It consists predominantly of satellite DNA sequences and contains few, if any, transcribed genes. In contrast, β-heterochromatin -- like euchromatin -- is endoreduplicated (Gall *et al.*, 1971; Lakhotia, 1974). It is transcriptionally active (Biessman *et al.*, 1981; Miklos *et al.*, 1984; Devlin *et al.*, 1990), although it contains fewer unique genes than euchromatin (see Hilliker *et al.*, 1980). Beta-heterochromatin contains a higher concentration of middle repetitive elements than either euchromatin or α-heterochromatin (Spradling and Rubin, 1981; Healy *et al.*, 1988; Devlin *et al.*, 1990).

The formation of heterochromatin takes place during early embryogenesis. During the initial, very rapid nuclear divisions in the syncitial blastoderm, the nuclei appear homogeneous (Mahowald, 1963); however, by cycle 10-13 heterochromatin is visible using Hoechst 33258 staining (Foe and Alberts, 1985). The appearance of heterochromatin in division cycle 10 of *Drosophila melanogaster* is correlated with the recruitment of heterochromatic proteins encoded by maternal mRNAs (James *et al.*, 1989). The cytogenetic distinction between heterochromatin and euchromatin is also correlated with the acquisition of transcriptional competence in division cycle 10 (Edgar and Schubiger, 1986).

B. POSITION-EFFECT VARIEGATION

General Genetics and Cytology

Position-effect variegation (PEV) is the inactivation of a gene by an adjacent chromosomal rearrangement. In its most common form, PEV involves euchromatic genes which are inactivated by a genetic rearrangement with a breakpoint in a heterochromatic region. If one chromosome bears a mutant allele (*g*) and the other the relocated (*R*) wild-type allele (g^+), then the heterozygote $R(g^+)/g$ will show a mosaic of wild-type and mutant gene expression within the tissue affected by this gene. In contrast, an $R(g)/g^+$ will be wild-type.

This type of variegated position effect has been observed in a wide variety of organisms, including mice (Cattanach,

1974) and plants (Catcheside, 1947). Hence, PEV seems to be a phenomenon that is widespread among eukaryotes. Nonetheless, it has been given the most attention by fruit-fly geneticists, who have been studying it for nearly a century (see Karpen and Spradling, 1990). Therefore, with rare exceptions, this survey will confine itself to drosophilids and predominantly *Drosophila melanogaster*.

That an observed variegation is caused by a position effect can be demonstrated in two ways. The first is by reversion of the variegated gene inactivation phenotype with reversion of the rearrangement (*e.g.* Hinton and Goldsmith, 1950). The second is by recovering a wild-type phenotype when a mutant allele is exchanged for the inactivated allele by crossing over between the locus and the breakpoint (*e.g.* Judd, 1955). The latter result demonstrates that the gene inactivation resulting from PEV does not result from gene loss or mutation, at least in the meiotic stem cells.

In *Drosophila*, most euchromatic and heterochromatic sequences appear susceptible to, or capable of causing, PEV. Only *ebony* (Brosseau, 1970) and the Bithorax Complex (E.B. Lewis, cited in Henikoff, 1990) have not yielded variegating alleles upon a search. When euchromatic genes variegate, the rearrangement breakpoint associated with, and presumably causing, PEV is typically in α-heterochromatin (Tartof, *et al.* 1989).

Unique genes that normally reside in β-heterochromatin

are also able to variegate in response to a change in chromatin environment (Hessler, 1958; Wakimoto and Hearn, 1990). Heterochromatic loci display mosaicism when moved adjacent to distal euchromatin and thus this form of variegation is different from the more common form of PEV in that it does not involve α-heterochromatin. Two examples of genes which variegate under these circumstances are *light* (*lt*) which is normally located in 2L heterochromatin (Hessler, 1958; Wakimoto and Hearn, 1990) and *cubitis interruptus* (*ci*) on chromosome 4 (Stern and Kodani, 1955). In these instances, for reasons unknown (but see Wakimoto and Hearn, 1990), the variegating rearrangements are restricted to those with breakpoints in the very centric or distal portions of the euchromatic region of the chromosomes. Only rarely has PEV been associated with euchromatin/euchromatin rearrangement breakpoints. One such case is in *Drosophila hydei* (van Breugel, 1988).

There is usually a polarity of gene inactivation associated with PEV. Whenever an affected locus is several bands from the rearrangement breakpoint, intervening loci are also affected. An example is the translocation $T(1;4)w^{m258-18}$ (Demerec and Slizynska, 1937: cited in Cohen, 1962). In this case, the *roughest* eye-morphology locus is closer to the variegation-inducing rearrangement breakpoint than the *white* eye-colour locus. In eyes displaying mosaicism, the rough patches of the eye are larger than, and completely include,

all areas of the eye that are white. In this variegating system, whenever the *white* gene is repressed, *roughest* is always inactivated. The order of gene repression reflects the gene order on the chromosome suggesting a polarized spreading effect of an inactivating substance along the chromosome.

The polarized spreading effect associated with PEV is correlated with a cytological disruption of the banding pattern in polytene chromosomes. This "heterochromatization" is suggestive of a spread of heterochromatin along the chromosome. Using translocation $T(1;4)w^{m258-21}$, which breaks closer to the *white* gene (in 3C2) than to the *Notch* gene (in 3C7), Hartmann-Goldstein (1967) showed that band 3C2 was clearly visible in more salivary gland cells than was 3C7, and was always visible when 3C7 was visible. The extent of cytological variegation correlated well with the extent of *white* and *Notch* variegation in adults. In *Drosophila hydei*, this spreading effect has also been observed cytologically for heterochromatic genes which variegate near euchromatic breakpoints (Hess, 1970).

Exceptions to the rule that inactivation of more proximal loci accompanies the inactivation of loci further from the breakpoint are rare. Clark and Chovnick (1986) reported a gene that fails to variegate although it lies closer to a rearrangement breakpoint than a variegating *rosy* allele. However, subsequent studies have refuted these claims (A. Chovnick, pers. comm.).

Variegating loci have been observed up to 80 polytene chromosome bands distant from the associated rearrangement breakpoint (Schultz, 1950). Since each cytologically visible band on a polytene chromosome contains, on average, 20-30 kilobases of DNA (Spierer *et al.*, 1983), over 1500 kilobases of DNA separate the affected locus from the breakpoint. In most cases, however, the observed distances are not so great. The distance over which the inactivation extends seems to depend on the particular variegation inducing region and the particular euchromatic region involved (Spofford, 1976).

The Timing of Position-effect Variegation

For some variegating rearrangements, such as $T(1;4)w^{m258-18}$, the mosaicism is so fine grained that the final decision whether or not the relevant locus is to be active must occur late in development. Nonetheless, in several instances investigators have argued for an early inactivation event. For example, embryos hemizygous for variegating rDNA cistrons ($In(1)sc^{S1}$/O embryos) synthesize significantly less rRNA during the first five hours of embryogenesis, resulting in 14% less rRNA in newly hatched larva than in controls (Puckett and Snyder, cited in Spofford, 1976). Based on the data of Noujdin (1936) and several developmental studies (Bryant, 1970; Garcia-Bellido and Merriam, 1969 and 1971), Spofford (1976) hypothesized that the time of decision of *y ac* variegation in $In(1)sc^{8}$ was early in development: perhaps as early as the

cellular blastoderm stage. The *roughest* locus, which variegates when adjacent to *In(1)rst*^{3}, may also be inactivated as early as the time of blastoderm formation (Spofford, 1969; Spofford, 1976).

Later estimates of the timing of the inactivation event are derived from cell lineage analyses in the eye. For the variegating rearrangement *T(Y;5)pe*^{m1} in *Drosophila virilis*, the boundaries between mosaic patches of *peach (pe)* and *pe*$^{+}$ eye tissue (Baker, 1967) are very similar to the outlines of clonal twin spots generated by somatic crossing over in first-instar larvae (Baker, 1967). This suggests that the time of inactivation is no later than the first larval instar. Similar results have been obtained for variegating eye-colour phenotypes in *Drosophila melanogaster* (Baker, 1967; Becker, 1960 cited in Baker, 1967). Studies of twin spots in a variegating background have, in general, confirmed that inactivation of the *w* locus in *Drosophila melanogaster* occurs in the first larval instar (Baker, 1967; Janning, 1970). In addition to delimiting the developmental interval during which the transcriptional fate of a variegating allele is determined, these cell lineage studies demonstrate that the inactivation decision must be clonally inherited because progenitor cells undergo many more rounds of division before an eye is formed.

Molecular Aspects of Position-effect Variegation

For *rosy* (Rushlow *et al.*, 1984), *Hsp70* (Henikoff, 1981), *Sgs4* (Kornher and Kauffman, 1986), and *brown* (Henikoff and Dreesen, 1989), the gene inactivation associated with PEV has been shown to result in a reduction in nascent transcript accumulation. This apparent reduction could be a consequence of reduced accessibility of the template DNA to transcription factors. Alternatively, in polytene tissues, it might reflect a reduced number of gene copies. Position-effect variegation associated with euchromatic loci requires that the affected locus be adjacent to a rearrangement breakpoint in heterochromatin. Since α-heterochromatin is under-replicated in polytene tissues, the variegating phenotype and indistinct cytogenetic morphology associated with variegating loci might be a consequence of underreplication. Using cloned DNA sequences, the degree of replication at variegating loci has been compared to that at non-variegating euchromatic loci. No significant underreplication was detected for the *white* gene (Hayashi *et al.*, 1990), the heatshock locus (Henikoff, 1981), or the *rosy* locus (Rushlow et al., 1984). However, the *Sgs4* gene is underreplicated in $T(1;4)w^{m258-21}$ (Kornher and Kauffman, 1986), as is the *yellow* gene in *Dp(1;f)1187* (Karpen and Spradling, 1990). Thus, in some instances, PEV is correlated with underreplication in polytene cells. Although underreplication might explain variegation of some loci in polytene tissues, it cannot explain variegation in diploid

tissues. The pigment cells of the eye are not thought to be polytene or polyploid (Karpen and Spradling, 1990), yet eye-colour genes variegate. Similarly, germline tissue is not polyploid, yet the *nod* locus is subject to PEV (Zhang and Hawley, 1990).

Gene transcription near euchromatin/heterochromatin rearrangement breakpoints may be repressed because of altered accessibilty of the chromatin to transcription factors. *In situ* hybridization with cloned probes suggests that in polytene chromosomes the DNA of a variegating *white* allele is less accessible to a molecular probe than is a white allele distant from the rearrangement breakpoint (Hayashi *et al.*, 1990). Thus, PEV may be a consequence of an altered chromatin conformation.

Biochemical studies have also focused on the structure of rearrangement breakpoints associated with PEV. Tartof *et al.* (1984) cloned three rearrangement breakpoints associated with variegation at the *white* locus. w^{m51b} and w^{mMc} display fine-grained mosaicism suggestive of a late determinitive event, while w^{m4} is coarsely mottled with large clonal patches of *w* tissue suggesting an early decision. In all three cases the rearrangement is very close to the *white* gene. w^{m51b} and w^{mMc} share nearly identical euchromatic breakpoints, which may be of importance in determining their fine-grained pattern of variegation. When w^{m4} was reinverted to re-activate the *w* gene, adjacent repetitive sequences were still associated with the

white gene. This result suggested that the phenomenon which causes PEV originates not at the breakpoint itself, but deep in heterochromatin from whence it is propagated. However, Reuter *et al.* (1985) have re-activated the white gene in a similar fashion and have shown that such "wild-type" revertants often do still variegate if a strong modifier of PEV (see "Modifiers of PEV") is present in the genetic background. Tartof has claimed that the revertants induced in his laboratory do not respond to modifiers of PEV (T. Grigliatti, pers. comm.). Thus, if specific DNA segments are required for PEV, they are unknown.

Modifiers of Position-effect Variegation

Higher temperatures usually suppress PEV, while lower temperatures enhance the variegating phenotype (Gowen and Gay, 1934). Crowding of larvae during early development also enhances variegation (Hinton, 1949). Flies reared at pH 2.6 develop more slowly than flies reared at higher pH. Since this treatment also enhances *white* eye-colour variegation in *In(1)w*^{m4} , Michailidis *et al.* (1988) have suggested that many environmental effects are a consequence of prolonged development. They further speculate that prolonged development is responsible for the enhancement of PEV by some agents which interfere with DNA synthesis (Schultz, 1956).

Some chemical agents, however, have effects which are separable from their effects on development time. For example,

both butyrate and proprionate suppress *white* inactivation associated with *In(1)w*^{m4}, although they significantly prolong development (Mottus *et al.*, 1980; Rushlow *et al.*, 1984).

The mechanisms whereby exogenous chemicals influence PEV are ill-defined. Sodium butyrate is hypothesized to induce suppression by inhibiting histone deacetylation, thereby ameliorating alterations in chromatin structure at or near variegating loci (Mottus *et al.*, 1980). However, while sodium butyrate does affect chromatin compaction (Annunziato *et al.*, 1988), it also affects several other cellular processes (see Boffa *et al.*, 1981; Christman *et al.*, 1980). Another chemical agent, DMSO, which has no known effect on histone modification, also suppresses PEV (cited in Michailidis *et al.*, 1988).

Several genetic factors have been shown to modify PEV. Extra heterochromatin, located elsewhere in the genome, affects the expression of variegating genes. The presence of a Y chromosome suppresses variegation; its absence enhances it (Gowen and Gay, 1934). Thus, XXY females and XYY males are almost wild-type for loci near a variegating rearrangement, while XO males show more extreme variegation than XY males (Spofford, 1976). Deletions and duplications for autosomal heterochromatin have similar effects on PEV (Spofford, 1976). In the case of the Y chromosome, an analysis of numerous large deficiencies suggest that suppression is a function of the amount of Y heterochromatin in the genome and that it is not

attributable to any discrete region of the Y chromosome (Dimitri and Pisano, 1989).

Deletion of histone genes suppresses variegation (Moore *et al.*, 1979; Moore *et al.*, 1983; Khesin and Bashkirov, 1979), as do deletions for numerous other genetic loci (*e.g.* Wustmann *et al.*, 1989). These modifiers are discussed in detail in the section entitled "Su(var)s and E(var)s".

C. SUPPRESSORS AND ENHANCERS OF POSITION-EFFECT VARIEGATION

There are many euchromatic loci which can be mutated to generate suppressors *(Su(var)s)* (Spofford, 1967; Reuter and Wolff, 1981; Sinclair *et al.*, 1983; Reuter *et al.*, 1986) or enhancers *(E(var)*s) of PEV (Sinclair *et al.*, 1989; Reuter and Wolff, 1981; Locke *et al.*, 1988). The greatest number of modifiers has been inferred from the deficiency/ duplication mapping of haplo- and triplo-abnormal loci. From a study of 12 mutations which identified 4 independent loci, Locke *et al.* (1988) proposed that 20-30 dosage-sensitive loci existed. However, Wustmann *et al.* (1989) have suggested that there may be as many as 120-150 loci, since 38 haplo-dependent modifiers of PEV were identified in approximately 30% of the autosomal complement. This number is in good agreement with estimates based on cytogenetic analyses of modifiers of PEV in regions 87C (Henikoff, 1979), 86-88 (Reuter *et al.*, 1987), and 24D4-25F2 (Szidonya and Reuter, 1988).

The 42 known dosage-dependent loci can be divided into

four classes: (1) haplo-abnormal suppressor loci with a triplo-abnormal enhancer function (2) haplo-abnormal enhancer loci with a triplo-abnormal suppressor function (3) haplo-abnormal enhancer loci, and (4) haplo-abnormal suppressor loci (Locke *et al.*, 1988; Wustmann *et al.*, 1989). To explain the dosage-dependence of so many loci affecting the same phenomenon, Locke *et al.* (1988) elaborated upon earlier hypotheses which suggested that modifiers of PEV encode chromatin proteins or modifiers of chromatin proteins (Zuckerkandl, 1974; Spofford, 1976; Sinclair *et al.*, 1983). They suggested that these loci encode proteins which participate in a large macromolecular complex, chromatin, and that the observed dosage effects were a result of the law of mass action. Specifically, a large assemblage such as chromatin would be exquisitely sensitive to small changes in the concentration of any one constituent, since any such alteration would drive the assembly/disassembly of chromatin away from its normal equilibrium. Since only eight loci are both haplo- and triplo-abnormal, these loci are likely to play pivotal roles in the PEV phenomenon.

In addition to duplication/deficiency analyses, a large number of *Su(var)* and *E(var)* mutations have been isolated which affect single loci (Reuter and Wolff, 1981; Reuter *et al.*, 1986; Sinclair *et al.*, 1983). Reuter and his colleagues have identified 12 dominant *Su(var)* mutations on the second chromosome and 11 on the third, as well as two *E(var)*

mutations (see Wustmann *et al.*, 1989). Many of the mutations described by Sinclair *et al.* (1983) are likely to represent additional alleles of these loci. A comprehensive study of allelism between these two sets of mutants is in progress (T. Grigliatti, pers. comm.). In general, there is a good correlation between *Su(var)* and *E(var)* mutants, and dosage-sensitive genes which modify PEV (summarized in Wustmann *et al.*, 1989), suggesting that most *Su(var)* and *E(var)* mutations are amorphs or hypermorphs.

Su(var) and *E(var)* mutations affect PEV generally since they suppress or enhance the inactivation of loci associated with different rearrangements (Hayashi *et al.*, 1990; Reuter *et al.*, 1982; Sinclair *et al.*, 1989, 1991). The extent to which they modify PEV is allele specific. One mutation might suppress inactivation of the *white* gene in $In(1)w^{m4}$ by 90%, while another might suppress the inactivation by only 50%. The effectiveness of a particular *Su(var)* or *E(var)* mutation can vary depending on the rearrangement involved. Thus, a strong suppressor of *white* gene inactivation in $In(1)w^{m4}$, could be a weak suppressor of *Stubble* gene inactivation in $T(2;3)Sb^{v}$. Finally, genetic modifiers of PEV do not always display the same trends in their ability to affect variegating loci. One *Su(var)* mutation might suppress inactivation of *white* better than inactivation of *brown*, while another might have more profound effects on *brown* variegation than on *white* (*e.g.* Hayashi *et al.*, 1990).

Mutations at some, but not all, *Su(var)* loci are sexually dimorphic, each sex suppressing the same variegating locus to a different extent (Hayashi *et al.*, 1990; Reuter *et al.*, 1986; Sinclair *et al.*, 1991).

It remains uncertain whether or not the majority of genetic modifiers of PEV are essential for normal development. In one study, most suppressor mutants isolated on the third chromosome were homozygous lethal or sterile (Reuter *et al.*, 1986), while in another, all were homozygous viable (Sinclair *et al.*, 1983). The homozygous viable mutants may simply be hypomorphic alleles of essential loci, or they may represent different, non-essential loci.

Only mutations in *Su-var(2)1* and *Su-var(3)* have been tested for their effects in the germ-line (Szabad *et al.*, 1988). Germ-line recombination studies with mutant alleles *Su-var(2)1*^{01} and *Su-var(3)*^{03} indicate that wild-type functioning of both *Su-var(2)1* and *Su-var(3)* is required for normal development of the germ-line as well as for the soma.

The most extensively studied *Su(var)* locus is *Su-var(2)1*, located in region 31 on the polytene chromosome map. Alleles of *Su-var(2)1* display a general effect on PEV (Reuter *et al.*, 1982; Hayashi *et al.*, 1990; Sinclair *et al.*, 1991), and reduce the cytological heterochromatization associated with variegating rearrangements (Reuter *et al.*, 1982; Hayashi *et al.*, 1990). The *Su-var(2)1*^{01} allele increases the *in vitro* transcriptional capacity of polytene chromosomes in intersexes

(Khesin and Bashkirov, 1979), suggesting that it also affects euchromatic regions. Larvae heterozygous or homozygous for the *Su-var(2)1*^{01} allele also exhibit significant hyperacetylation of histone H4 and an increased accessibility of DNA to endogenous endonucleases (Dorn *et al.*, 1986). These findings suggest that *Su-var(2)1* encodes a modifier of an NHP such as a histone de-acetylase (Dorn *et al.*, 1986), or an NHP which alters the accessibility of heterochromatin to modifiers such as acetylases.

Su-var(2)1 is also sensitive to known modifiers of PEV. Mutant alleles exhibit strongly reduced viability as homozygotes or transheterozygotes when grown on medium containing sodium butyrate (Reuter *et al.*, 1986; Sinclair *et al.*, 1991). They also interact with the Y chromosome, such that XXY; *Su-var/Su-var* progeny die but X/O; *Su-var/Su-var* progeny survive. In contrast, alleles of *Su-var(3)1*, *Su-var(3)2* and *Su-var(3)9* are not sensitive to sodium butyrate or Y heterochromatin (Reuter *et al.*, 1986). Thus, modifiers of PEV only modify the phenotypes of mutations at some *Su(var)* loci. Presumably these altered phenotypes are the result of additive effects on chromatin assembly or compaction.

Cloned Genes

Two unique genes that affect PEV have been cloned. HP1 was originally identified as a gene encoding a heterochromatin binding protein (James *et al.*, 1986). Subsequently, two

allelic *Su(var)* mutations were found to have lesions in the gene (Eissenberg *et al.*, 1990). *Su(var)205* (Sinclair *et al.*, 1983) contains a G to A transition at the first nucleotide of the last intron of HP-1, causing missplicing of the mRNA. The HP1 sequence of the *Su(var)2-5* mutant contains a nonsense mutation. The phenotypic effects of a splicing defect are hard to predict, but nonsense mutations usually result in loss of protein activity. Since a loss-of-function mutation in HP1 suppresses PEV, while a duplication for the gene enhances variegation (Wustmann *et al.*, 1989), HP1 is a haplo- triplo-abnormal locus with respect to PEV.

The HP1 protein has sequence similarity with the protein encoded by the *Polycomb* (*Pc*) gene (Paro and Hogness, 1991). Like HP1, Polycomb protein is also a component of chromatin (Zink and Paro, 1989), and possibly part of a heterochromatin-like complex (see Gaunt and Singh, 1990; see Paro, 1990). The region of similarity between the two proteins is 37 amino acids long; and within this chromo domain (chromatin organization modifier) 24 amino acids are precisely conserved. Since both HP1 (T.C. James, cited in Singh *et al.*, 1991) and *Pc* (Paro, 1990) proteins fail to bind DNA, the chromo-domain could be involved in protein-protein interactions which permit packaging in heterochromatin or heterochromatin-like complexes (Singh *et al.*, 1991).

The *Polycomb* gene is a member of the *Polycomb*-Group, a set of genes which act as dosage-sensitive, negative

regulators of homeotic genes (Jurgens, 1985). Thus, PEV and the negative regulation of homeotic gene expression might represent analagous mechanisms of gene repression. In fact, some members of the *Polycomb*-Group are modifiers of PEV (D. Sinclair, N. Clegg, T. Grigliatti, and H. Brock, submitted). Hence, some modifiers of PEV may act not only as general regulators (*i.e.* influencing the euchromatin/heterochromatin decision), but also as regulators of specific, developmentally important genes.

Using a DNA probe from the region of HP-1 that encodes the chromo domain (the chromo box), Singh *et al.* (1991) isolated murine and human cDNAs with sequence similarity to the *Drosophila* gene. These clones may represent transcripts from genes with analagous modes of action to the HP1 gene in fruitflies. Cross-hybridization was also detected to the DNA from other plant and animal species. Since one of these species, *Caenorhabditis elegans*, has no cytologically visible heterochromatin (Wood, 1988), chromo domains might be involved in other instances of heritable gene repression (Singh *et al.*, 1991).

The other cloned modifier of PEV is *Suvar(3)7*. Deletion of one copy of *Suvar(3)7* suppresses variegation of the *white* gene in *In(1)w^{m4}*, but duplications of the locus enhance variegation of *white* (Reuter *et al.*, 1987). Reuter *et al.* (1990) deficiency mapped the suppressor locus, then transformed wild-type fruitflies with pieces of DNA within the

appropriate deficiency interval. A DNA fragment containing the *Suvar(3)7* locus was identified by its ability to enhance *white* variegation in *In(1)w*^{m4}. *Suvar(3)7* encodes a 932 amino-acid deduced protein sequence with 5 potential DNA-binding zinc-fingers of the Cys_2-His_2 type. Unlike the zinc-fingers found in transcription factors, the *Suvar(3)7* motifs are separated from each other by 40-107 amino-acids. The zinc-fingers of *Suvar(3)7* may serve to bind and draw together relatively distant DNA sequences for packaging, perhaps at scaffold attachment sites (Reuter *et al.*, 1990).

The cloning of *Su(var)* genes extends the scope of genetic studies on PEV and in the future will be of cardinal importance in a genetic/biochemical dissection of chromatin structure.

CHAPTER 2

INTRODUCTION

The genetics and cytology of region 31 on the left arm of chromosome 2 has not been intensively studied; however, several suppressor of position-effect variegation (*Su(var)*) mutations have been localized to this region (Sinclair *et al.* 1983, 1991; Wustmann *et al.* 1989; Reuter *et al.*, 1982). The most extensively researched *Su(var)* locus (see "Literature Review"), *Suvar(2)1*, has been deficiency mapped to 31A-D (Reuter *et al.*, 1982; Wustmann *et al.*, 1989; Sinclair *et al.*, 1991). At least 13 alleles of this locus have been reported.

Suvar(2)1 alleles display a range of phenotypes. Some mutants are homozygous lethal, while others are only semi-lethal. Heteroallelic combinations of *Suvar(2)1* mutations are also semi-lethal. This results from a marked reduction in the numbers of males. The survival of heteroallelic females is only slightly affected, but such females lay no eggs. The same sexual dimorphism is observed amongst mutation-bearing hemizygotes: males die and females are infecund. However, unlike mutant hemizygotes, flies heterozygous for any two *Suvar(2)1* mutations also display a red-brown eye phenotype and held-out wings (Sinclair *et al.*, 1991; Brock, 1989). The difference in phenotype between mutant hemizygotes and flies bearing heteroallelic combinations of *Suvar(2)1* alleles suggests that mutations at the *Suvar(2)1* locus may alter the function of the wild-type gene product (Sinclair *et al.*,

1991).

Three other dominant *Su(var)* mutations have also been mapped within region 31 using their suppressor phenotypes and secondary sterile or lethal phenotypes (summarised in Figure 1 and Table 1; Sinclair *et al.*, 1991). Recombination studies, based on suppressor phenotypes, place *Su(var)204* and *Su(var)207* 0.5-1.0 cM to the left (centromere-distal) of *Jammed*, while the lethal phenotype of *Su(var)216* maps to the right. All three mutations are uncovered by *Df(2L)J2*, which is deleted for region 31. Each mutation can be further positioned by deficiencies which partition region 31 into several genetic subregions. Both *Su(var)204* and *Su(var)207* map to the same interval delimited by the distal breakpoints of *Df(2L)J39* and *Df(2L)J77* (Figure 1). However, *Su(var)204* and *Su(var)207* fully complement each other. The mutation *Su(var)216* fails to complement *Df(2L)J27*, placing it in an entirely different deficiency interval (Figure 1).

Remarkably, *Su(var)216*, *Su(var)207*, and *Suvar(2)1*, display intergenic effects when combined in *trans*. These include male semi-lethality, female infecundity, and the red-brown eye-colour phenotype seen in some homozygotes (Brock, 1989; Sinclair *et al.*, 1991). The close proximity of these mutations on the chromosome and their phenotypic interactions might indicate some common origin or shared function (Sinclair *et al.*, 1983). Although most functionally related genes are not clustered, several exceptions have been reported (Karch *et*

Figure 1. Su(var) loci and the Sandler gene cluster

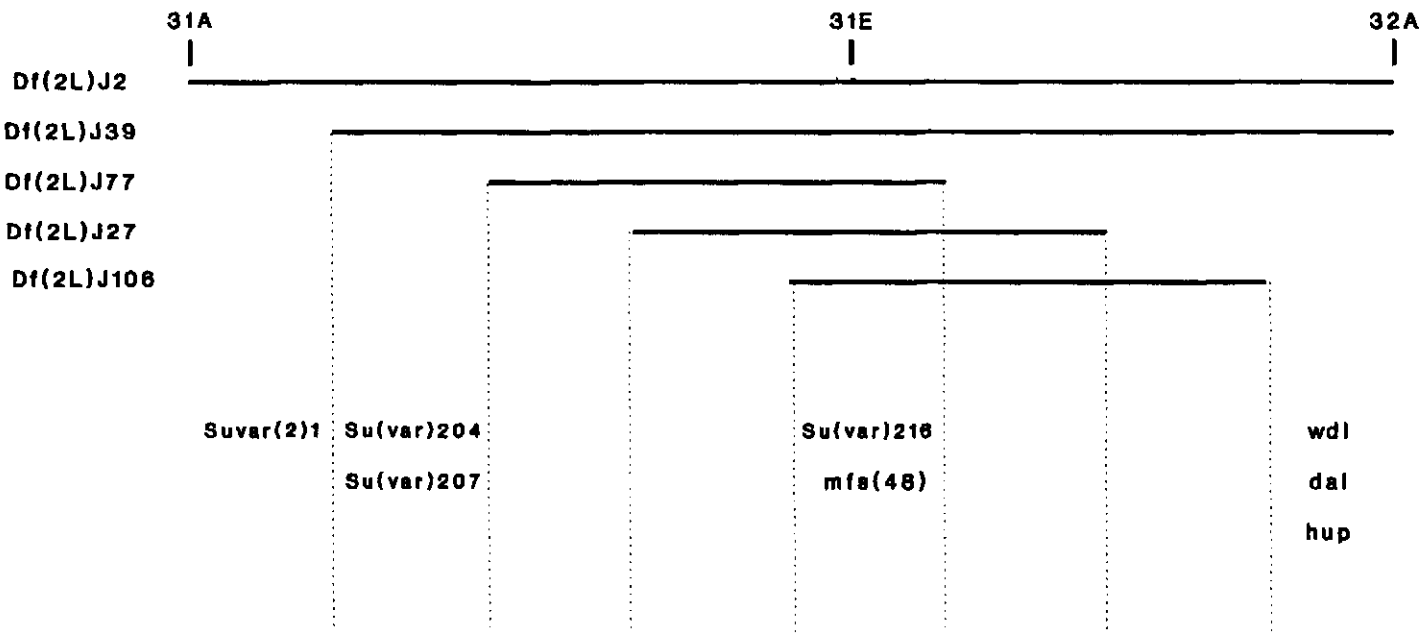


Table 1: Summary of mapping results involving region 31 deletions and *Su(var)* mutations. Data summary = male viability/female fertility; v= viable, l= lethal, sl= semi-lethal (<15% of expected progeny); f= female fertile, s= female sterile (no eggs produced).

Deletion	*Su(var)* mutation			
	Su(var)204	*Su(var)207*	*Su(var)216*	*Suvar(2)1*
Df(2L)J2	v/s	v/s	l/l	l/s
Df(2L)J39	v/s	l/l	l/l	v/f
Df(2L)J77	v/f	v/f	l/l	v/f
Df(2L)J27	v/f	v/f	l/l	v/f
Df(2L)J106	v/f	v/f	l/l	v/f

al. 1985; Kaufman *et al.*, 1980; Spradling *et al.*, 1980; Karlik *et al.*, 1984). Equally plausible, however, is that the dominant mutations represent a random sampling of the estimated 120-150 *Su(var)* loci (Wustmann *et al.*, 1989) in the *Drosophila* genome.

Two recessive suppressors of position-effect variegation (PEV), each represented by a single allele, have also been identified in region 31 (Sinclair *et al.*, 1991). One, *mfs48* (Sandler, 1977), is located in 31E while the other, *wavoid-like* (*wdl*; Sandler, 1977), is in the interval 31F-32A. The number and chromosomal distribution of recessive suppressors of PEV is not known; therefore, like the dominant suppressor mutations, the presence of two recessive *su(var)* loci in region 31 might be either coincidental or of functional significance.

The two recessive suppressors of PEV are part of another group of genes. Sandler and his colleagues (Sandler, 1977; Lindsey *et al.*, 1980) proposed that *daughterless* (*da*), *hold-up* (*hup*), *wavoid-like* (*wdl*), *mfs48*, *daughterless-abo-like* (*dal*) and *abnormal oocyte* (*abo*) constitute a cluster of functionally related genes in 31E-32B. Excluding *mfs48*, which cannot be tested, mutant alleles of these loci are all sensitive to the intracellular levels of heterochromatin (Sandler, 1977), a property shared by dominant modifiers of PEV (Reuter *et al.*, 1982; Sinclair *et al.*, 1991). Thus, some members of the Sandler cluster might participate in the establishment or

maintenance of the gross structure of chromatin.

All together, the available dominant and recesssive mutations suggest that as many as six loci affecting PEV might reside in region 31. A comprehensive genetic analysis of region 31 was undertaken with the following objectives: (1) to determine the number of *Su(var)* loci that exist in this region, (2) to isolate additional alleles of *Su(var)/su(var)* loci represented by single mutations, (3) to obtain null mutations at *Su(var)* loci for which there are putative gain-of-function mutations, and (4) to examine the nature of the genes flanking the *Su(var)/su(var)* loci.

This chapter extends the deficiency mapping of 64 mutations previously isolated in the Grigliatti laboratory (Figure 2; see Brock, 1989). In addition, it describes the isolation of 57 new mutations within a sub-interval of the cytogenetic region 31 delimited by the centromere-distal breakpoint of *Df(2L)J77* and the centromere proximal breakpoint of *Df(2L)J106*. The *Df(2L)J77-Df(2L)J106* sub-interval was intensively analysed because the suppressors *Su(var)216* and *mfs48* reside in that region (Brock, 1989; Sinclair *et al.*, 1991), and the region was sufficiently small to permit a thorough search for additional alleles at these loci.

MATERIALS AND METHODS

Mutant strains: The chromosomal deficiencies used in this study are listed in Table 2. *Df(2L)J2*, *Df(2L)J27*, and *Df(2L)J39* are described in Mange and Sandler (1973) and Sandler (1977). Flies bearing *Df(2L)J39* are female sterile, but not infecund. Both sexes display a *Minute* phenotype when heterozygous for *Df(2L)J39*. A stock was maintained by outcrossing *Df(2L)J39/SM5* or *Df(2L)J39/bw^{VDe2}* males to *bw^{VDe2}/SM5* females each generation. *Df(2L)J77* and *Df(2L)J106* were provided by J. Lengyel (see Salas and Lengyel, 1984).

The origins of mutants previously reported to be in region 31 are summarized in a composite table in the Results section (Table 3). Several recessive lethal and/or female sterile mutants were provided by L. Sandler: *da*, *dal*, *wdl*, *hup*, *mfs48* and *l(2)54* (see Mange and Sandler, 1973; Sandler, 1977). The mutant *da^{2}* was obtained from C. Cronmiller. The following female sterile mutants were provided by T. Schüpbach: *erratic* (*err*) *mat(2)earlyQM47*, *mat(2)synPJ50*, *trk*, *PI23*, *RU26*, and *DG25* (Table 3; see Schüpbach and Wieschaus, 1989). *Female-sterile-2-rosy-4*, (*fs(2)ry4*), which is female infecund, was provided by A. Spradling. *Su(var)204* and *Su(var)207* were induced in the Grigliatti laboratory (Sinclair *et al.*, 1983) and are described in detail in Sinclair *et al.* (1991). *Suvar(2)1^{01}* (Reuter *et al.*, 1982) was provided by G. Reuter.

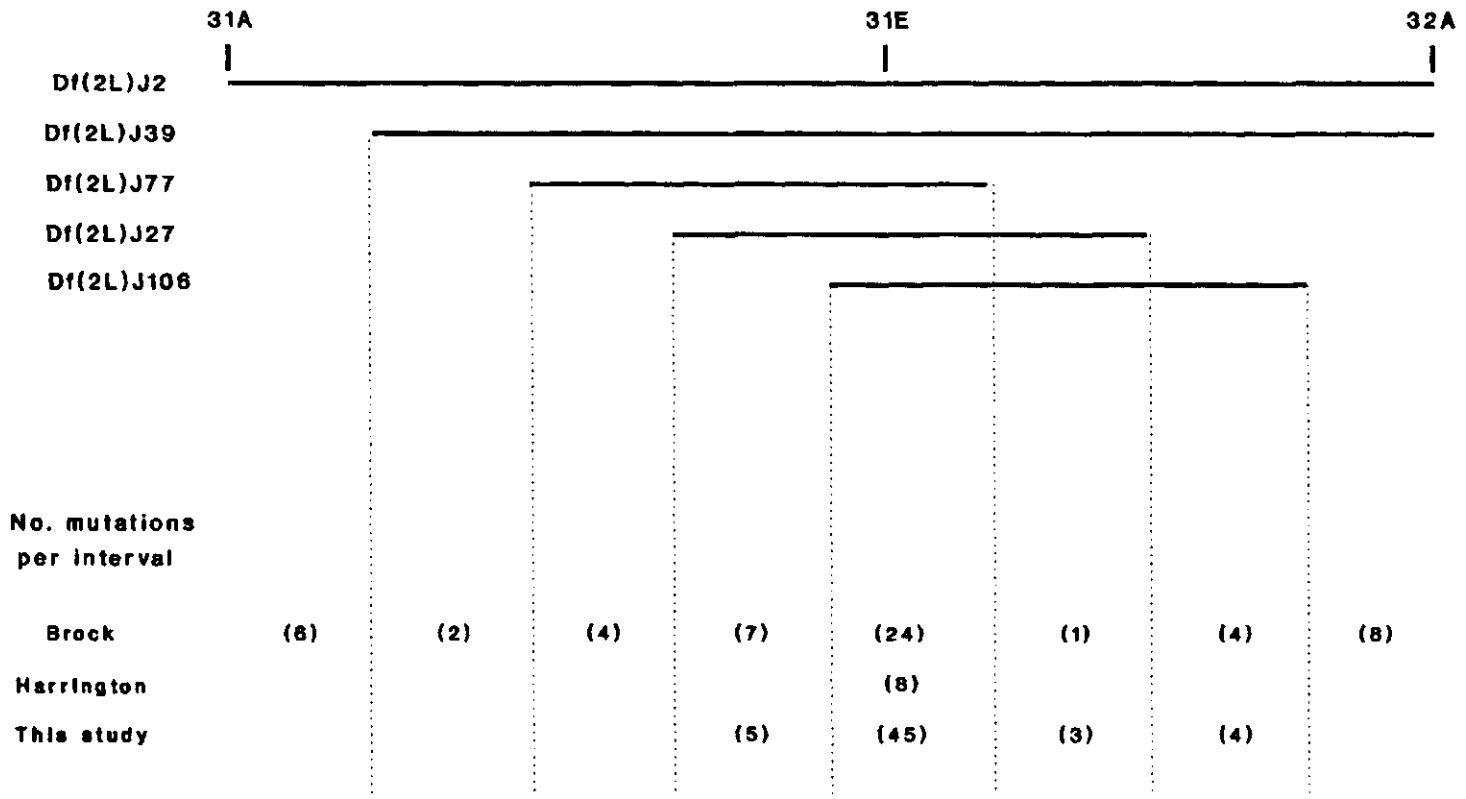
A summary of mutations isolated in the Grigliatti lab

between 1983 and 1988 appears in Figure 2. From 5000 second chromosomes tested, Sinclair, Kafer, Camfield, and Grigliatti isolated 16 gamma-radiation induced mutations which were lethal in *trans* with *Df(2L)J2* (cited in Brock, 1989). Brock (1989) isolated 40 ethyl methanesulphonate (EMS) induced mutations that were lethal in *trans* with *Df(2L)J2* from amongst 1484 second chromosomes. Harrington (1990) isolated 8 gamma-radiation induced mutations that were lethal in *trans* with *Df(2L)J27* from amongst 5000 chromosomes. Sinclair (unpublished) reverted the crumpled wing phenotype of the neomorphic mutation *Jammed-34e* (J^{34e}) according to the method of Salas and Lengyel (1984) and obtained 18 J^{+} strains.

Culture conditions: Fly cultures were maintained at 22^{0} on corn-meal sucrose Drosophila medium supplemented with 0.04% Tegosept as a mould inhibitor. Except where indicated, experiments were performed at 25^{0}.

Eye pigment assessment: The ability of a mutation to suppress the inactivation of the white gene in the variegating rearrangement $In(1)w^{m4}$ was assessed visually. $In(1)w^{m4}/Y$; *mutant/CyO* males were mated to $In(1)w^{m4}/In(1)w^{m4}$; *+/+* females. Mutation bearing progeny with pigment levels less than approximately 70% of wild-type levels were scored as non-suppressors. Progeny were observed in parallel with $In(1)w^{m4}/In(1)w^{m4}$ flies which had 10-30% of wild-type pigment levels, and with suppressed flies previously shown (Brock, 1989) to have approximately 70% pigment.

Figure 2. Summary of hemizygous lethal mutations

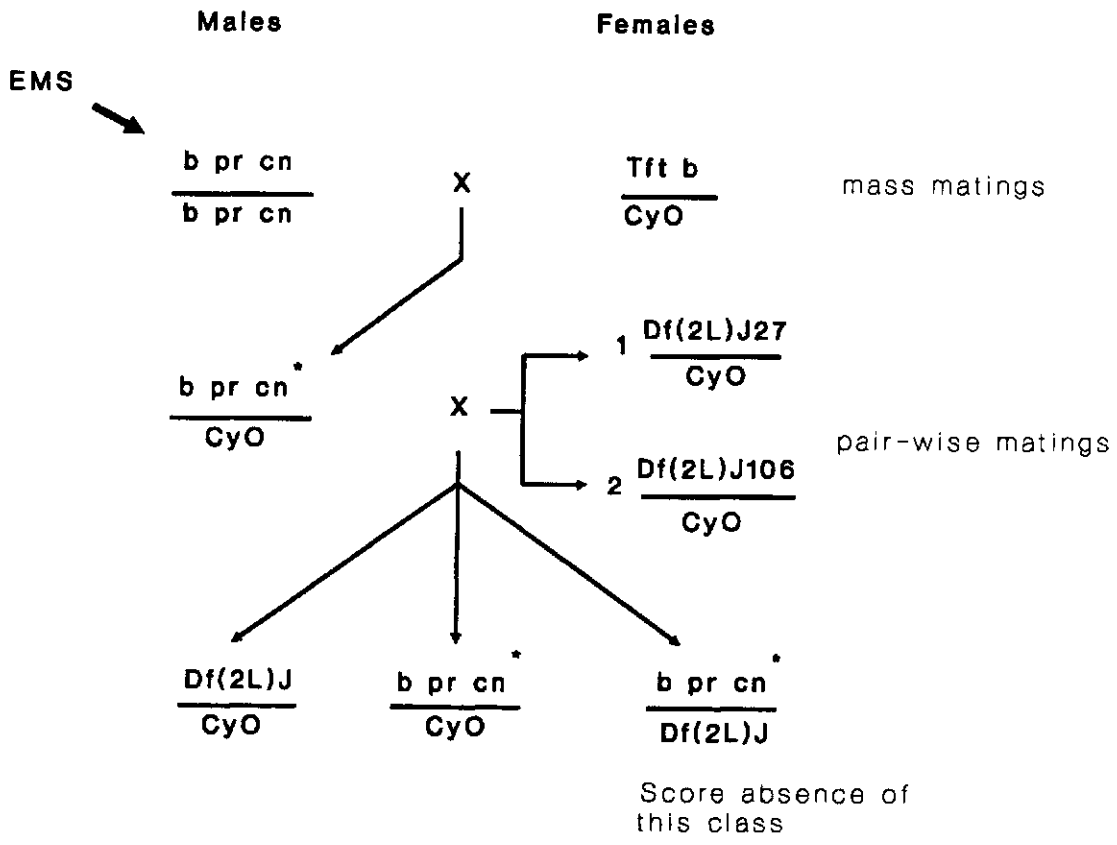


Cytology: Males bearing balanced chromosome rearrangements were crossed to wild-type Oregon-R females and the offspring raised at 18^0. Salivary glands from third instar larvae were dissected in *Drosophila* saline and fixed in 45% acetic acid. The chromosomes were then squashed in acetic acid, water and lactic acid (3:2:1), or stained in lacto-aceto-orcein (Yoon *et al.*, 1973) and then squashed. Chromosomes were examined under phase contrast optics and interpreted according to the revised map of Bridges (Lefevre, 1976).

Ethyl methanesulphonate (EMS) mutagenesis: Two independent sets of experiments were conducted to isolate EMS induced mutations (Fig. 3). In both sets of experiments, homozygous *b pr cn* males were mutagenized with ethyl methanesulfonate (0.025 M) by the method of Lewis and Bacher (1968). These males were mated *en masse* to *Tft/In(2LR)CyO, dp^{lvI} Cy pr cn^2* virgin females at 22^0 (Figure 3). Male progeny with a *b pr cn* chromosome balanced over *CyO* were identified by a curly-wing phenotype and *pr cn* eyes. Each male was individually mated at 29^0 to 3-5 virgins from stocks of either *Df(2L)J27/CyO* or *Df(2L)J106/CyO* (two separate experiments). The presence of a recessive lethal mutation on the marked chromosome was indicated by the absence of the *b pr cn/deficiency* (straight-winged) class of flies amongst the progeny. To determine whether any of the mutants were temperature sensitive, the tests were repeated at 22^0.

Hybrid dysgenesis screen: The isolation of *P* transposable

Figure 3. EMS mutagenesis screen.

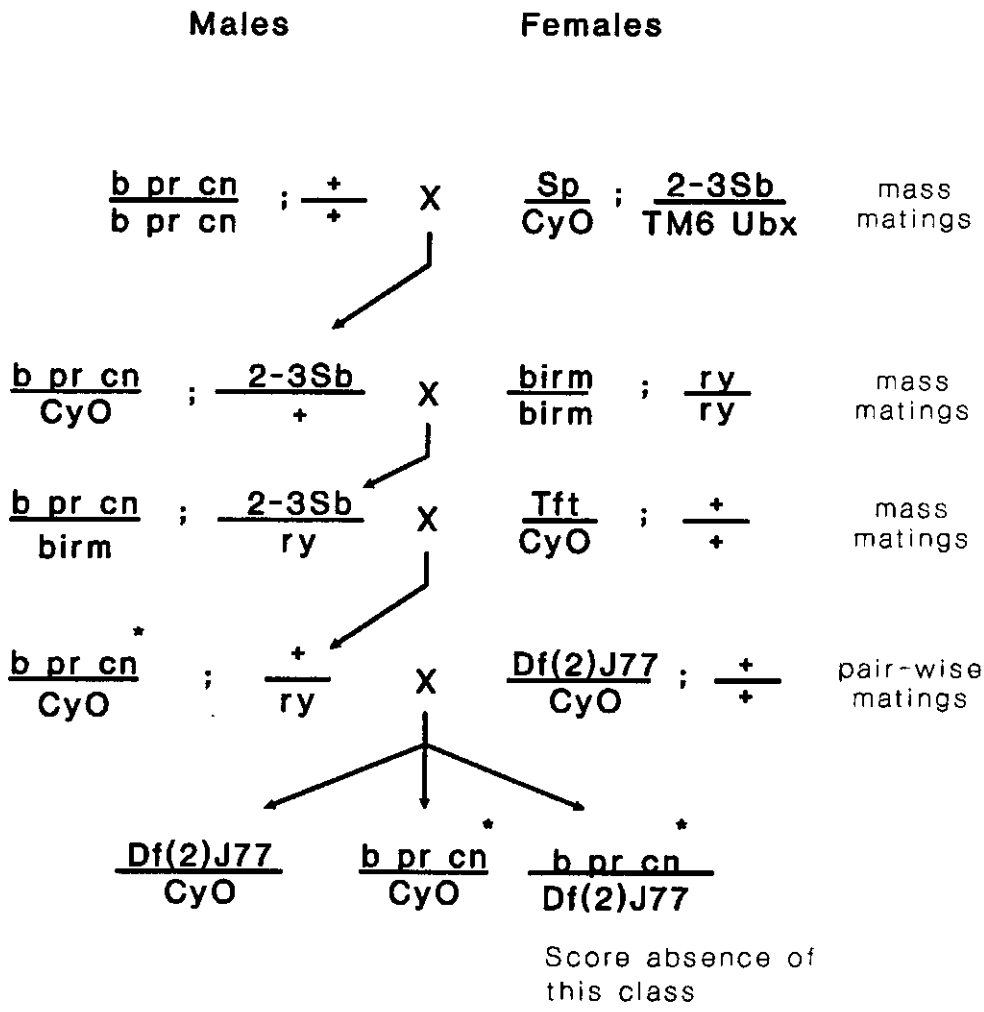


element induced mutations in region 31 was accomplished as follows (Figure 4). Homozygous *b pr cn* males were mated *en masse* to *Sp/CyO; Sb Δ2-3/TM6, Ubx* females. Males of the genotype *b pr cn/CyO; Sb Δ2-3/+* were mated at 18^0 to females homozygous for the Birmingham second chromosome and ry^{506}. The *Δ2-3* locus provides an active source of *P* transposase, while the Birmingham chromosome carries 13 partially deleted *P* elements. Males of genotype *b pr cn/Birm2; Sb Δ2-3/*ry^{506} were mass mated at 18^0 to *Tft/CyO* females. *b pr cn/CyO* male progeny were then individually mated to 3-5 *Df(2L)J77/CyO* females. A lethal mutation was indicated by the absence of *b pr cn/Df(2L)J77* flies in the final cross.

Deficiency mapping and complementation tests: First, each EMS, gamma-ray, and *P* element lethal or sterile mutation was localized to a sub-interval of cytogenetic region 31 by its failure to complement a battery of deficiencies for viability or fertility.

Once this was accomplished, mutations within each sub-interval were tested for allelism via *inter se* complementation analysis. Pairs of mutants, maintained as heterozygotes with *CyO*, were mated. Failure to complement was based on the absence of Cy^+ flies amongst at least 50 F_1 progeny, or the detection of a sterile or visible phenotype amongst Cy^+ flies. For lethal mutations which complement each other, 33% of the total progeny were expected to be Cy^+. At least 50 flies were scored to ascertain that two mutations complemented each

Figure 4. P element mutagenesis screen.



other. When a deficiency sub-interval contained a large number of mutants, a single representative allele from each complementation group identified in early rounds of crosses was used as a tester strain in subsequent crosses. For some sterile mutations, failure to complement a single allele of a series was used as the criterion for inclusion in the complementation group.

Lethal phase analysis: The principal time at which hemizygous mutants die was determined by mating *mutant/+* males to *Df(2L)JR16/CyO* females. After 72 hours, the parents were transferred to bottles with plain agar medium covered with a smear of yeast paste. After several hours of egg deposition, the eggs were transferred to thin strips of construction paper and counted. The paper strips were placed on the surface of regular cornmeal/agar medium. Two to three days later, the construction paper was removed from the vial and the unhatched eggs were counted. Light eggs displayed no discernable development and were assumed to be unfertilized. Dark eggs represented dead embryos. The difference between the number of hatched eggs and the number of pupae indicated the extent of larval death. Pupal death was determined by counting the un-eclosed flies.

RESULTS

Cytogenetics

The region deleted by *Df(2L)J2* defines the physical bounds of this analysis. *Df(2L)J2* extends from 31A to 32A, a region that encompasses approximately 43 bands. Several smaller deficiencies further subdivide this segment of the chromosome into a number of sub-regions (Figure 1 and Figure 5). In polytene chromosomes the banding pattern within regions 31A and 31F is distinctive, and rearrangement breakpoints can be determined with reasonable accuracy. In contrast, the banding pattern in the 31B-E interval is particularly unclear (see Lefevre, 1976). In view of this difficulty, there is some uncertainty associated with the assignment of deficiency breakpoints. The approximate locations of the deficiency breakpoints are shown in Figure 5 and Table 2.

In addition to the previously identified deficiencies *Df(2L)J2*, *Df(2L)J39*, *Df(2L)J27*, *Df(2L)J77*, and *Df(2L)J106)*, six additional deficiencies were identified amongst the gamma-radiation induced *J* revertants isolated by Sinclair (see Materials and Methods). *Df(2L)JR1*, *Df(2L)JR3*, *Df(2L)JR4*, *Df(2L)JR11* *Df(2L)JR16*, and *Df(2L)JR17* were all lethal with numerous complementation groups (see below). *Df(2L)JR11* itself was female sterile and was not investigated in detail. The approximate cytological breakpoints of these deficiencies are shown in Figure 5.

To order the relative breakpoints of the 10 deficiencies

Figure 5. The cytological extent of deficiencies in region 31. Dashed lines indicate the uncertainty associated with each breakpoint. Deficiency names have been shortened for clarity; full names appear in the text.

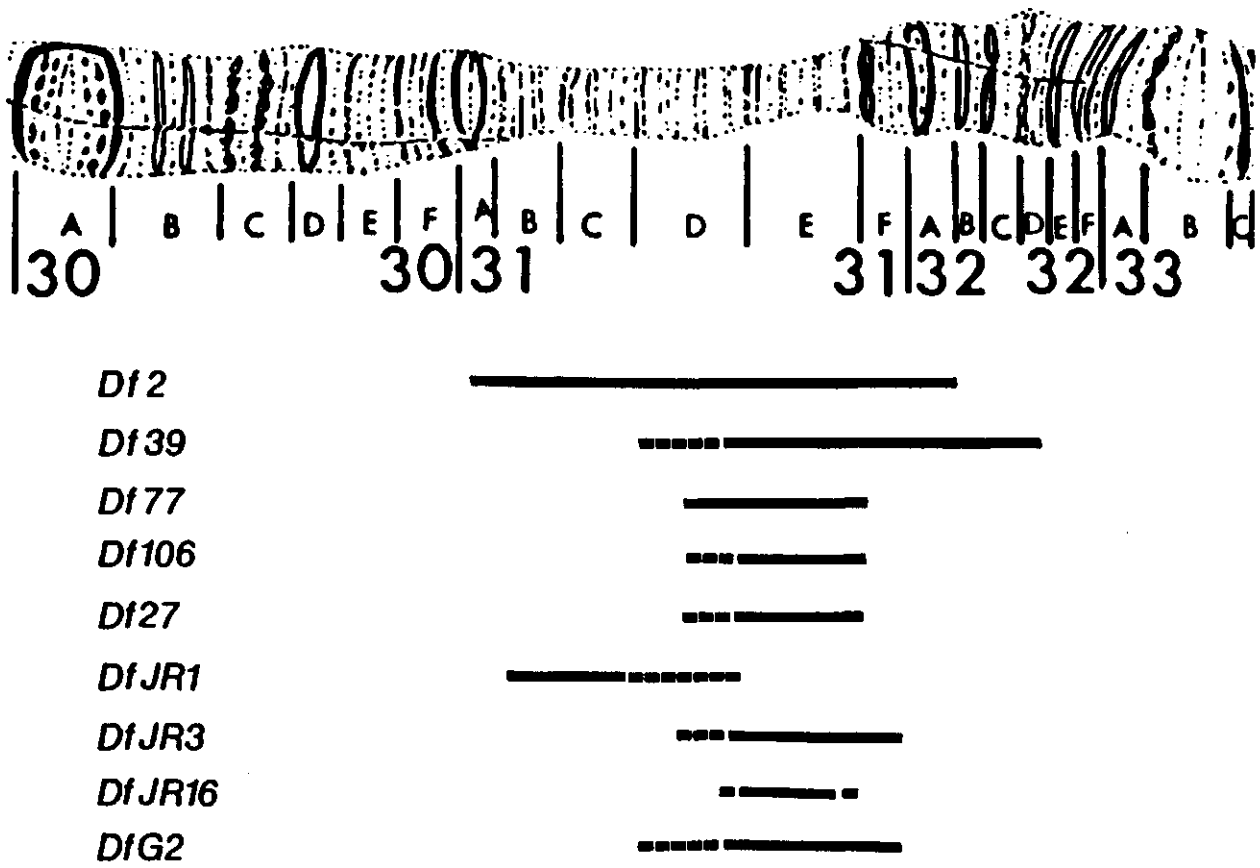

A B C D E F A B C D E F A B C D E F A B C D
30 30 31 31 32 32 33
Df 2
Df 39
Df 77
Df 106
Df 27
Df JR1
Df JR3
Df JR16
Df G2

TABLE 2. Cytological limits and origins of chromosomal rearrangements.

Rearrangement	Reference	Comments
Df(2L)J2	Mange and Sandler (1973)	Df(2L)31A3;32A
Df(2L)J39	Mange and Sandler (1973)	Df(2L)31D;32B female sterile
Df(2L)J27	Mange and Sandler (1973)	Df(2L)31D-31E
Df(2L)J77	Salas and Lengyel (1984)	Df(2L)31D;31E
Df(2L)J106	Salas and Lengyel (1984)	Df(2L)31D;31E
Df(2L)JR1	Sinclair; This study	Df(2L)31B;31D
Df(2L)JR3	Sinclair; This study	Df(2L)31D;31F
Df(2L)JR16	Sinclair; This study	In(2L)30C-D;31E associated with a deficiency in 31E
Df(2L)JR17	Sinclair; This study	not visible
Df(2L)G2	Sinclair; This study	Df(2L)31D;31F
Df(2L)JR11	Sinclair; This study	female sterile

more accurately, genetic and cytological data were compared. Alleles from each complementation group within region 31 were deficiency mapped (Appendix 1), resulting in the identification of 15 small deficiency sub-intervals (Figure 6, Figure 7, Figure 8).

The *Df(2L)JR1* chromosome complements *da*, but fails to complement *fs(2)ry4* (Figure 6; Appendix 1). These loci have been mapped by *in situ* hybridization to 31E (Cronmiller *et al.*, 1988) and 31B (Spradling, pers. comm.; N. Clegg and I.P. Whitehead, unpublished), respectively. Thus, *Df(2L)JR1* must extend as far as 31B, but does not delete sequences in 31E. Contrary to the cytological analysis of Sandler (1977), *Df(2L)J39* cannot extend past 31B, since *Df(2L)J39/fs(2)ry4* females lay eggs, but hemizygous *Df(2L)JR1/fs(2)ry4* flies do not. *Df(2L)JR3* and *Df(2L)JR4* delete the same complementation groups. Cytologically, *Df(2L)JR3* appears to be similar to *Df(2L)J106*, but complementation data (Figures 6, 7, and 8) indicate slight differences. The *Df(2L)JR3* chromosome is deleted for 31E, but normal pairing of homologues is consistently disrupted throughout the region 30D-31E. Thus, the deficiency may be associated with a small, paracentric inversion in this region. *Df(2L)JR17* is not cytologically visible.

A single deficiency, *Df(2L)G2*, was isolated in a gamma-irradiation screen for additional alleles of *Su(var)216* (data not shown). It deletes a large portion of the 31 region.

Figure 6. Loci distal to Df(2L)J27. (), no. of alleles isolated; [], the probable location of Su(var)207.

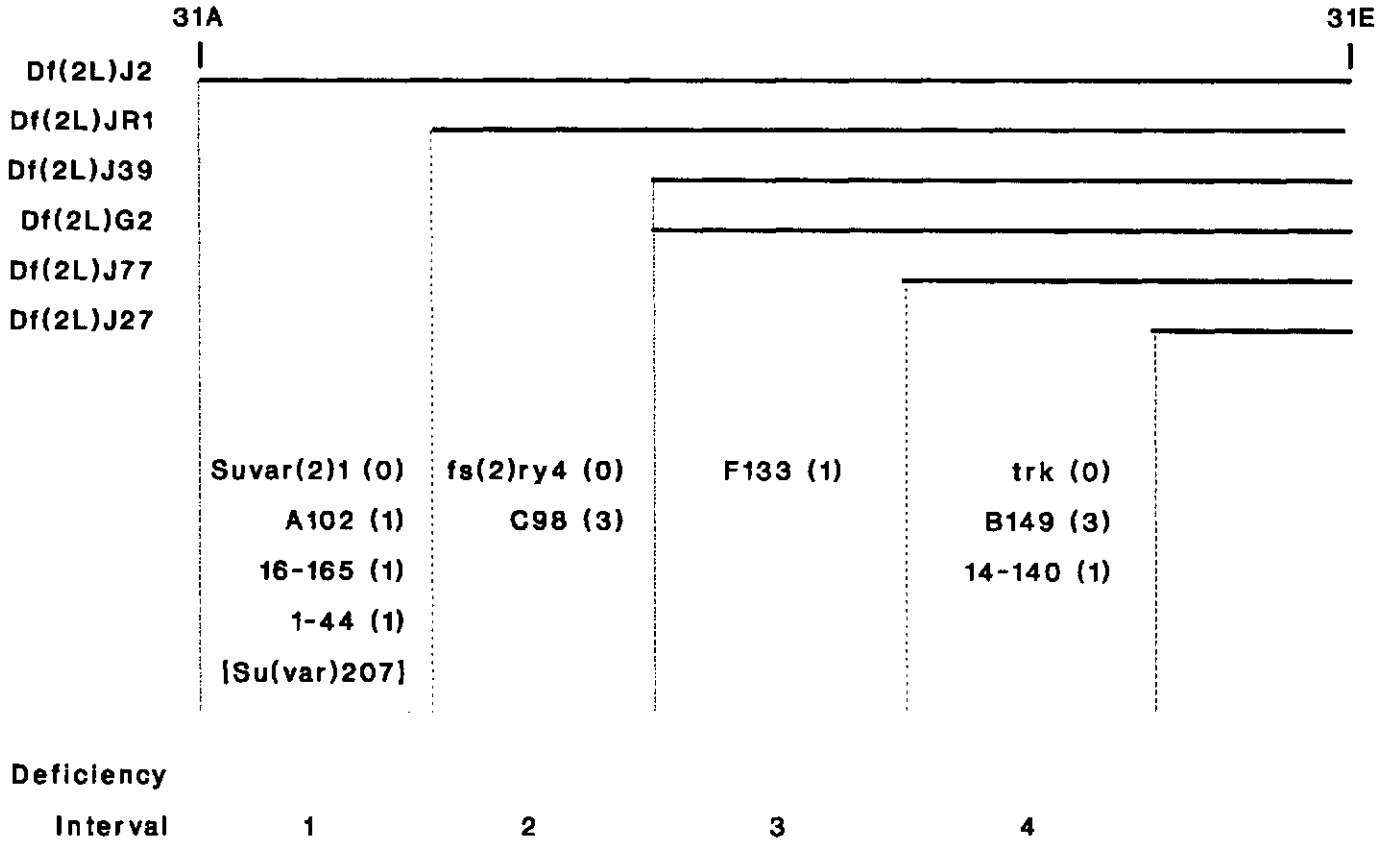


Figure 7. Loci uncovered by Df(2L)J27. The numbers in parentheses indicate the number of mutations recovered at each locus

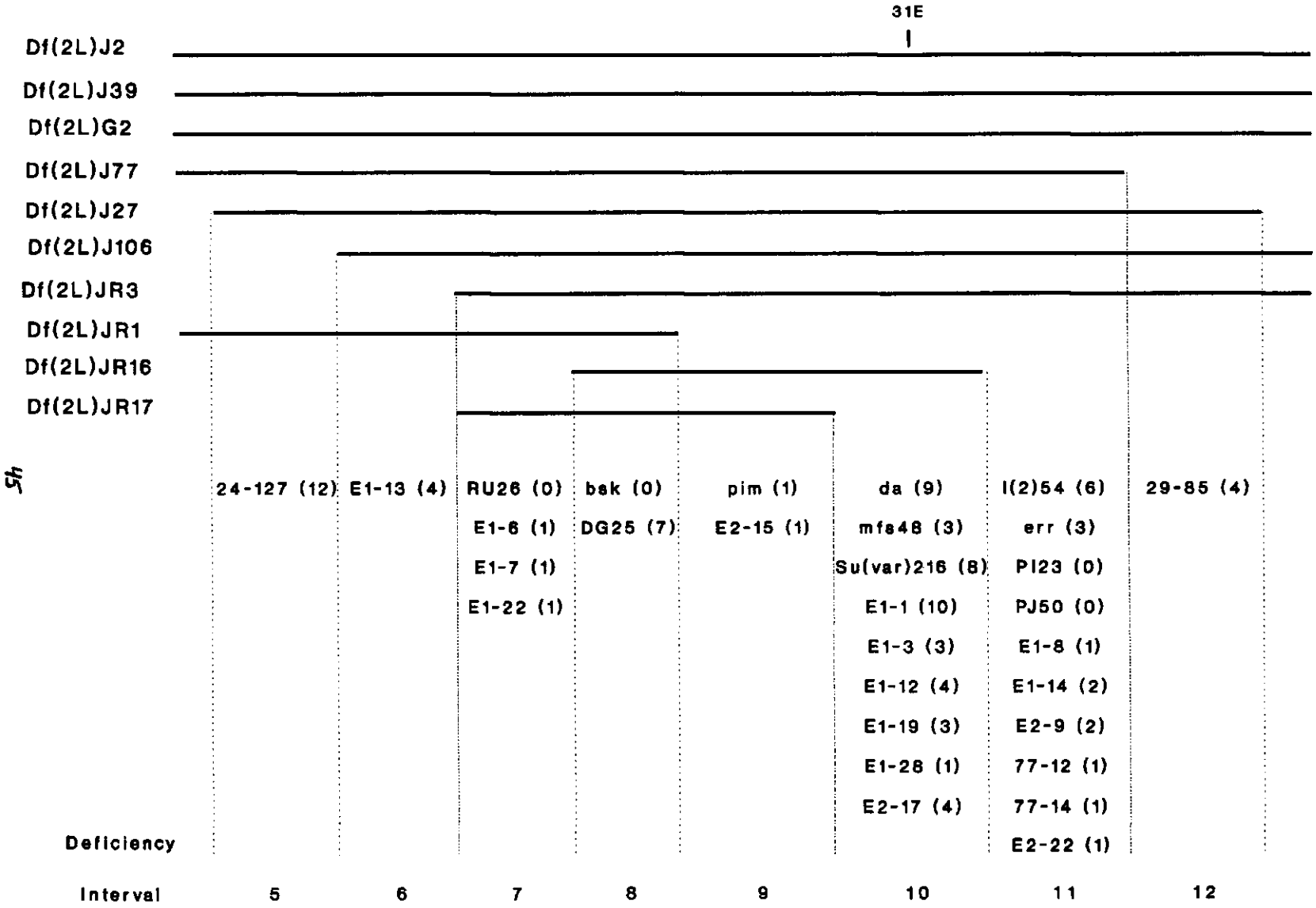
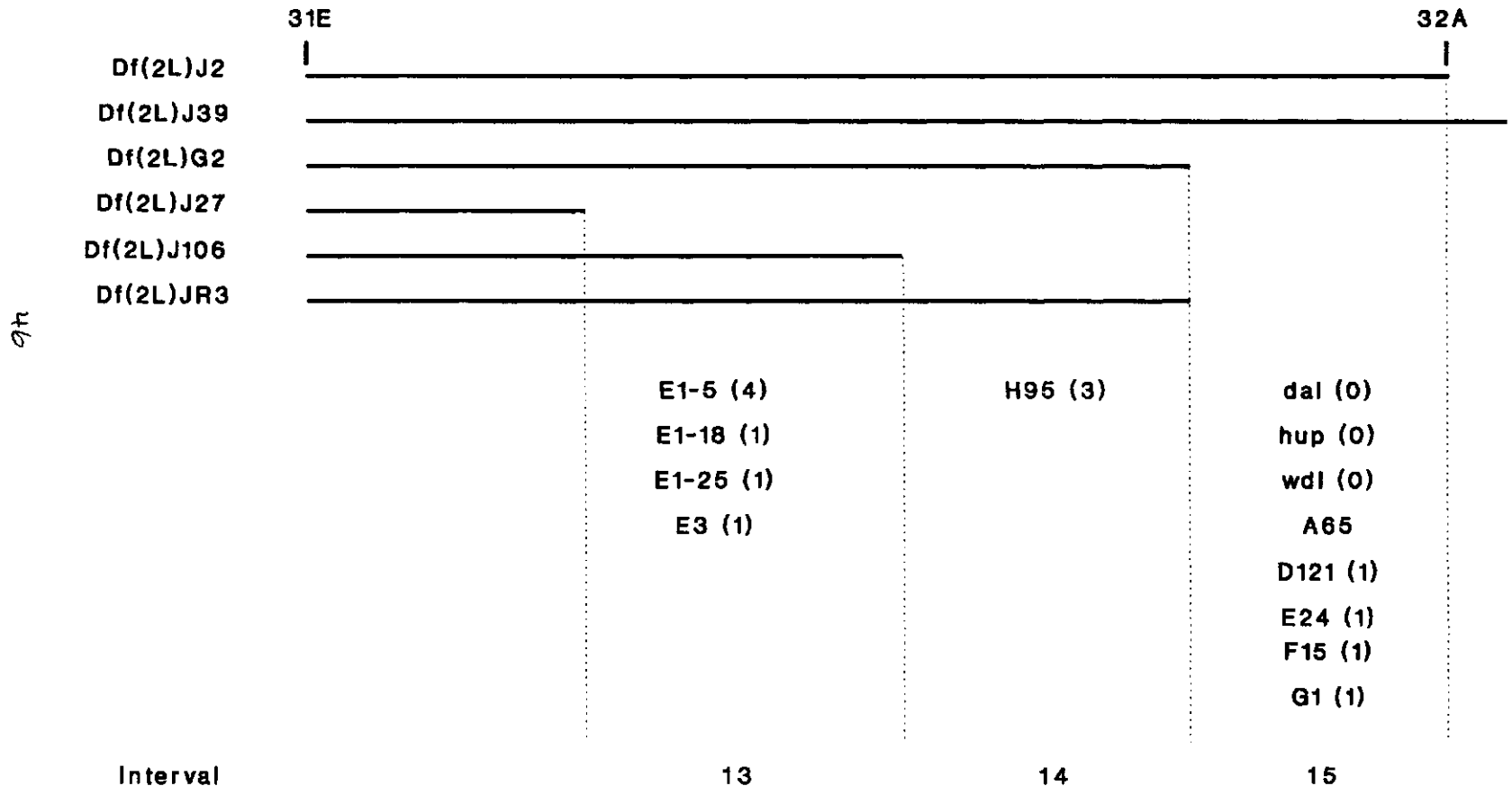


Figure 8. Loci centromere proximal to Df(2L)J27.



Genetically, it uncovers the same mutations as the centromere distal end of *Df(2L)J39*.

Genetic screens

This genetic characterization of region 31 incorporates data from previous screens (see Materials and Methods) in addition to the results from three new screens. The results of the latter are described below.

Five thousand EMS-mutagenized second chromosomes were examined for new hemizygous lethal mutations within the bounds of *Df(2L)J106* (Screen 1). Twenty-seven mutations were recovered. From 10000 EMS mutagenized chromosomes an additional 26 mutations were recovered that were lethal in *trans* with *Df(2L)J27* (Screen 2). Finally, 4 hemizygous lethal *P* element induced mutations which fail to complement *Df(2L)J77* were isolated from amongst 12500 mutagenized chromosomes (Screen 3). The frequencies of isolation of mutations were 0.0054, 0.0026, and 0.0003 for Screens 1, 2, and 3, respectively.

The new mutants, which were originally identified as hemizygous lethals at 29^0, were retested and found to be lethal at 25^0. Mutants isolated in Screen 2 were also tested at 22^0, but no temperature-sensitive mutations were detected. No sex-specific mutants were recovered, nor were any dominant suppressors of PEV.

Brock (1989) described the isolation of 16 gamma-ray

induced mutations and 39 EMS induced mutations which failed to complement *Df(2L)J2* at 29^0. Amongst the EMS induced mutants, two mutations (*E20* and *A65*) were temperature sensitive: hemizygotes raised at 25^0 survived more frequently than flies raised at 29^0.

The mutations isolated in each of the above screens were placed into complementation groups along with all other mutations previously shown to map in region 31.

General complementation

In total, one hundred and twenty-one hemizygous lethal mutants were assigned to 43 different complementation groups (Figures 6, 7, and 8). Thirty-seven were new alleles of 7 different mutations identified in other studies (Table 3); the remainder are distributed amongst 36 new complementation groups (Figures 6, 7, and 8). In addition, 10 hemizygous viable revertants of the J^{34e} mutant allele were recovered amongst the 18 *J* revertants recovered by Sinclair (see Materials and Methods). Since these revertants are hemizygous viable, and they do not affect any known complementation groups other than *J*, they are likely to be "point mutation" revertants.

There are at least 12 loci from the region for which no new mutant alleles were recovered (Table 3). Several of these loci were originally identified by criteria other than hemizygous lethality. These include alleles of the maternal

Table 3. Previously described mutations in region 31.

Mutation	Comments	Reference	No. New Alleles
Suvar(2)1	hemizygous male lethal, female sterile	Reuter *et al., 1982*	0
Su(var)204	hemizygous female sterile	Sinclair *et al.*, 1983	0
Su(var)207	hemizygous lethal	Sinclair *et al., 1983*	0
Su(var)216	hemizygous lethal	Sinclair *et al.*, 1983	8
bsk	zygotic lethal; cuticle defects	Nüsslein-Volhard *et al.*, 1984	0
da	pleiotropic zygotic lethal	see Cline, 1989	9
dal		Sandler 1977	0
DG25	homozygous mothers lay small eggs	T. Schüpbach, pers. comm.	7
err	hemizygous lethal; maternal effect sterile	Schüpbach and Wieschaus, 1989	3
fs(2)ry4	female sterile; P element insert	A. Spradling, pers. comm.	0
hup	wings held up	Sandler, 1977	0
J	neomorphic mutation; wings crumpled	see Lindsley and Grell, 1968	10
l(2)54	hemizygous lethal; see *mat(2)earlyQM47*	Sandler, 1977	5
mat(2) synPJ50	homozygotes arrest in syncitial blastoderm	Schüpbach and Wieschaus, 1989	0
mat(2) earlyQM47	maternal effect sterile; presyncitial arrest in homozygotes	Schüpbach and Wieschaus, 1989	(see l(2)54

Mutation	Comments	Reference	No. New Alleles
mfs48	hemizygous inviable; centriole segregation defect in spermatids	Lindsley *et al.*, 1980	3
PI23	hemizygous sterile;	T. Schüpbach, pers. comm.	0
pim		Nüsslein-Volhard *et al.*, 1984	1
RU26	homozygous females lay collapsed eggs	T. Schüpbach, pers. comm.	0
trk	hemizygous female sterile; terminal group mutant	Schüpbach and Wieschaus, 1986	0
wdl	wavy wings	Sandler, 1977	0

effect female sterile mutants *PI23*, *RU26*, *trk*, and *mat(2)synPJ50*, all of which are located in the intensively mutagenized region between the distal breakpoint of *Df(2L)J77* and the centromere proximal breakpoint of *Df(2L)J27* (Figure 7). However, additional alleles of the homozygous sterile mutants *mat(2)earlyQM47*, *err*, and *DG25* were identified (Figure 7), indicating that these loci have essential functions not restricted to the female germline.

In accord with detailed studies of other regions (see Lefevre and Watkins, 1986), the total number of complementation groups identified in the region 31A-32A (53) roughly corresponds to the total number of bands deleted by *Df(2L)J2* (43).

As in other studies (*e.g.* Lasko and Pardue, 1988), the pattern of complementation associated with loci in each of the deficiency intervals was generally simple. For non-complementing mutations, either there were no surviving transheterozygotes, or the flies displayed a visible or sterile phenotype. In a few cases, there were deviations from this simple complementation pattern, with one or more mutations displaying apparent intragenic complementation. These cases probably involve haplo-specific lethal mutations with sufficient residual activity to permit interallelic complementation (Nash and Janca, 1983). They are noted below, as specific deficiency intervals are discussed.

Suppressor loci distal to *Df(2L)J27*

Three dominant *Su(var)* loci (*Suvar(2)1*, *Su(var)204*, and *Su(var)207*) have been mapped by recombination and deletion analyses centromere distal to *Df(2L)J27* (see Figure 1). Complementation analysis with the EMS and gamma-radiation induced alleles cited in Brock (1989) did not reveal any new lethal alleles of these loci. An additional screen of 20000 chromosomes also failed to detect any P element induced recessive lethal alleles of *Su(var)214*, although several *trans*-acting second site enhancers of position-effect variegation were recovered (Whitehead and Clegg, data not shown).

Mapping with new deficiencies indicates that *Su(var)207* must be assigned to a new location. Flies of genotype *Df(2L)JR1/Su(var)207* are viable and fertile (Appendix 1), yet *Df(2L)J39/Su(var)207* flies die and *Df(2L)J2/Su(var)207* flies are female sterile and male semi-lethal (Table 1). Since *Df(2L)JR1* deletes *J* and extends further towards the telomere than *Df(2L)J39* (Figure 6), the lethal phenotype of *Df(2L)J39/Su(var)207* must map centromere proximal to *J*. This is not consistent with recombination mapping which positions *Su(var)207* to the left of *J* (Sinclair, unpublished). The right breakpoint of *Df(2L)J39* extends further toward the centromere than that of *Df(2L)J2*. Therefore, a likely explanation of the genetic and recombination data is that the lethal mutation is a second site mutation on the *Su(var)207* chromosome which maps

outside the confines of *Df(2L)J2*, but which is within the region deleted by *Df(2L)J39*. Since *Df(2L)J2/Su(var)207* females are sterile, the *Su(var)207* mutation has been re-assigned to the deficiency interval centromere distal to *Df(2L)JR1*.

Deficiency mapping results suggest *Su(var)204* is not in its previously reported position (Brock, 1989). Female flies of genotype *Df(2)J2/Su(var)204*, *Df(2L)J39/Su(var)204*, or *Df(2L)G2/Su(var)204*, are all sterile. In contrast, females of genotype *Df(2L)JR1/Su(var)204* are fertile. *Df(2L)JR1* deletes *J*, and extends beyond the centromere distal breakpoint of *Df(2L)J39*; therefore, the female sterile mutation on the the *Su(var)204* chromosome cannot be located to the left of *J*. Recombination mapping, however, positions the *Su(var)* suppressor phenotype to the left of *J*. The female sterile mutation resides within region 31, centromere proximal to *Df(2L)J106*, but its location has not been investigated further. Hence no phenotype other than suppression is directly attributable to *Su(var)204*, and the mutation cannot be positioned by deficiency mapping.

Non-suppressor loci distal to *Df(2L)J27*

Six new recessive lethal complementation groups were identified between the centromere distal breakpoint of *Df(2L)J2* and *Df(2L)J27* (Figure 6). The distribution of alleles amongst the various screens performed in the region is listed in Appendix 2. With the exception of *C98* and *B149* each

complementation group contains a single allele. The complementation pattern amongst *B149* alleles was previously determined by Brock (1989). All three *C98* alleles were lethal in pair-wise combinations (Appendix 3).

No recessive lethal alleles of *trk* or *fs(2)ry4* were recovered. A *trk* mutation provided by T. Schüpbach was mapped to deficiency sub-interval 4 (Figure 6) based on hemizygous female sterility.

The *fs(2)ry4* locus was also deficiency mapped based on hemizygous female infecundity rather than recessive lethality. *fs(2)ry4* complements *Df(2L)G2* for fertility. It also complements *Df(2L)J39*, based on the observation that *Df(2L)J39/fs(2)ry4* females lay eggs. *Df(2L)J39/fs(2)ry4* females lay eggs that fail to hatch, whereas *fs(2)ry4/Df(2L)J2* and *fs(2)ry4/Df(2L)JR1* females are infecund.

Loci uncovered by *Df(2L)J27*

The majority of mutants described in this study fail to complement *Df(2L)J27*. The region spanned by *Df(2L)J27* has been divided into 8 sub-intervals by all the available deficiencies (Figure 7). The origin and distribution of mutations amongst the region 31 screens is given in Appendix 2 and salient aspects are described below.

Sub-interval 5 : One large complementation group, *24-127*, deficiency maps to this interval. The recessive lethal mutation *24-127* was induced by gamma-irradiation (cited in

Brock, 1989) and is associated with a pericentric inversion with breakpoints in 31D and region 51. Eleven mutations fail to complement the *24-127* mutation. *Inter se* crosses have been performed between two subsets of mutants, and mutant *A141* was tested with both subsets (Table 4). Amongst the first set of crosses, only one combination of alleles was viable and fertile. In crosses between mutations *E56* and *A63* approximately 25% of the expected number of heteroallelic progeny survive to adulthood. In the second set of crosses *A141/E2-13* progeny also survive. These complementation patterns are probably a result of alleles encoding partially functional products which, although mutant, can rescue the lethal phenotype. Thus, our analysis identifies only a single locus associated with the left breakpoint of the inversion associated with *24-127*.

Sub-interval 6: Four lethal mutations mapped within this subinterval and all four fail to complement each other (Table 5), thereby identifying a single complementation group designated the *E1-13* gene.

Sub-interval 7: Three newly described complementation groups, plus a fourth defined by the single *RU26* mutation (mapped on the basis of hemizygous female sterility) isolated by T. Schüpbach, have been identified in this region.

Sub-interval 8: Two independent complementation groups, *bsk* and *DG25*, have been positioned in this segment. This region may also include a third locus, namely, that defined by *J*

Table 4. Complementation data for mutations in the same deficiency interval as the mutation *24-127*. Ratios represent the number of *Cy* to straight-winged progeny recovered from the cross *mutant1/CyO* X *mutant2/CyO*

	A63	*A141*	*C35*	*E56*	*H30*	*E15*
24-127	94:0	176:0	118:0	104:0	228:0	460:0
A63	-	154:0	156:0	437:51	148:0	86:0
A141		-	294:0	102:0	158:0	n.d.
C35			-	165:0	180:0	n.d.
E56				-	218:0	n.d.
H30					-	n.d.
E15						-

	A141	*E2-1*	*E2-12*	*E2-13*	*E2-32*	*E2-42*
24-127	176:0	242:0	399:0	339:0	580:0	211:0
A141	-	226:0	138:0	210:77	199:0	249:0
E2-1		-	262:0	328:1	225:0	n.d.
E2-12			-	294:0	n.d.	343:0
E2-13				-	245:0	164:0
E2-32					-	n.d.
E2-42						-

Table 5. Complementation matrix for the *E1-13* locus.

	E1-13	*E1-17*	*G2-5*	*13-117*
E1-13	-	110:0	236:0	206:0
E1-17		-	207:0	229:0
G2-5			-	138:0
13-117				-

mutations. No new alleles of *bsk* were recovered. The original *bsk* mutation (Nüsslein-Volhard *et al.*, 1984) was mapped on the basis of hemizygous lethality.

The original *DG25* mutant is homozygous sterile, laying tiny eggs (T. Schüpbach, pers. comm.). When this mutant is crossed to *Df(2L)J27*, less than 40% of the expected number of hemizygous flies survive and they are female sterile. Seven putative alleles of this locus were isolated: 4 were EMS induced and three were gamma-ray induced (Appendix 2). Only five of the new mutations have been studied in detail. Their pattern of complementation is presented in Table 6. In *inter se* crosses, these five alleles produce a spectrum of abnormalities. Heteroallelic mutants have wavy-wings with a wax-like appearance and are female sterile and semi-lethal (Table 6). Some allelic combinations have rough eyes and/or missing or damaged macrochaetes. Mutant hemizygotes that eclose have the same phenotypes as heteroallelic mutants.

Two less well studied mutations, *E2-5* and *E2-21*, complement the other *DG25* alleles for viability and fertility, but not for other visible phenotypes. Both mutations cause a wavy, waxy wing phenotype when heterozygous for any of the other *DG25* alleles. Also, when crossed to *Df(2L)JR16*, a few hemizygous mutants eclose. These hemizygotes have rough eyes and waxy wings similar to those observed amongst *DG25* alleles. These phenotypes suggest that *E2-5* and *E2-21* are weak alleles of *DG25*, although their complete lethality in *trans* with each

Table 6. Complementation pattern amongst alleles of the *DG25* locus. Ratios above the matrix diagonal represent the proportion of *Cy* to straight-winged progeny in crosses between mutants heterozygous for *CyO*. The sterility (S) or fertility (F) of heteroallelic mutant females is noted beneath the matrix diagonal.

	23-127	*29-142*	*25-159*	*D22*	*DG25*	*C93*	*13-117*
23-127	-	60:10	597:22	98:3	503:97	226:12	92:0
29-142	S	-	375:12	157:38	n.d.	98:18	125:84
25-159	S	S	-	62:19	n.d.	273:19	46:22
D22	S	S	S	-	n.d.	160:33	331:148
DG25	S	n.d	n.d.	n.d.	-	n.d.	423:184
C93	S	S	S	S	n.d.	-	234:101
13-117	S	F	F	F	F	F	-

Cross	Progeny
23-127 X *Df(2L)J27*	424:24
29-142 X *Df(2L)J27*	464:57
25-159 X *Df(2L)J27*	1134:26
D22 X *Df(2L)J106*	145:0
DG25 X *Df(2L)JR1*	448:0
C93 X *Df(2L)J106*	232:11
13-117 X *Df(2L)J27*	779:0

other is hard to reconcile with this view.

One allele of *DG25*, *23-1273*, is lethal in *trans* with *13-117*. The mutation *13-117* is an allele of the *E1-13* complementation group, which is located in deficiency subinterval 6 (Figure 7). While *13-117* is lethal in *trans* with *23-127*, flies of genotype *Df(2L)JR17/13-117* and *Df(2L)JR3/13-117* are viable and fertile. Flies heterozygous for *23-127* and other alleles of *E1-13* are viable, suggesting that *23-127* and *13-117* may share a common second site mutation outside region 31. Alternatively, the two mutations may interact in some unknown fashion to cause a lethal phenotype.

The *A8* mutation, which is not included in the alleles shown in Figure 7, may also be an allele of the *DG25* locus. Expression of this mutation is temperature dependent; at 25^0 this mutation complements the *23-127* allele, but at 29^0 the heterozygote has a wavy-winged phenotype.

The precise location of the *J* locus remains unknown. It should lie within the region of overlap shared by all of the *J*-derived deficiencies; however, *Df(2L)JR16* still expresses *J* in some genetic backgrounds. This suggests that the distal breakpoint of this deficiency may be adjacent to *J* rather than simply deleting it. New sequences juxtaposed to the locus might periodically inactivate *J* creating a euchromatic position effect, although this does not explain why a Jammed phenotype is rarely observed in some backgrounds. Conservatively, *J* must be located in the interval between the

distal breakpoint of *Df(2L)JR3* and the proximal breakpoint of *Df(2L)JR1* (sub-intervals 7+8). Its close proximity to *DG25*, which also imparts a recessive wing phenotype, may indicate some connection between the loci. *Inter se* crosses between these two sets of mutants did not produce a lethal or any strong visible phenotype. However, *J* itself is hemizygous viable. Thus, the absence of any phenotype in *DG25/J* individuals is not conclusive.

No hemizygous lethal mutations of *J* were recovered in any of the region 31 EMS-mutagenesis screens. Amongst the 18 gamma-induced mutations that revert the *J* phenotype, six are associated with deficiencies in region 31; and two others segregate only in males, suggesting that they are *T(Y;2)* translocations. The remaining revertants are hemizygous viable and fertile. Thus, the *J* product may not be essential for viability. The two potential *Y;2* translocations may be useful in positioning *J*, but were not examined cytogenetically.

Sub-interval 9: There are two loci in this segment. One is the zygotic lethal mutation *pim* (Figure 7). A single allele of *pim* was recovered. Unlike the original allele, which causes embryonic death (Nüsslein-Volhard *et al.*, 1984), pim^{E1-15} hemizygotes die predominantly during the larval stages (Table 7). A few hemizygous progeny even survive to adulthood at 22^0. The *E2-15* mutation, which defines the second locus within this sub-interval, causes predominantly embryonic lethality in hemizygotes (Table 7).

Table 7. Lethal phases for mutations which fail to complement *Df(2L)JR16*. Mutant hemizygotes should represent 25% of the progeny from the cross *mutation/+* X *Df(2L)JR16/CyO*.

Mutant X *JR16/CyO*	% Embryonic Lethality	% Larval Lethality	% Pupal Lethality	% Mutant Adults
E1-1^{E2-16}/+	3 (5/247)	28 (68/247)	5 (10/247)	0
E1-3/+	23 (106/459)	6 (26/459)	0	0
E1-15/+	5 (18/439)	18 (79/439)	0	5 (17/342)
E2-17/+	1 (4/426)	28 (119/426)	3 (9/426)	0
E2-15/+	28 (107/388)	5 (19/388)	1 (3/388)	0
E2-43/+	2 (4/353)	26 (91/353)	3 (3/353)	0

Sub-interval 10: The interval defined by the proximal breakpoints of *Df(2L)JR17* and *Df(2L)J16* contains 9 identified loci. Lethal phases have been determined for several mutations within this interval (Table 7). Amongst these mutations, only *E1-3* is lethal in embryos as a hemizygote.

Five of the complementation groups in this region, *E1-3*, *E1-12*, *E1-19*, *E1-28*, and *E2-17* all have simple complementation patterns (Appendix 2). The largest complementation group in the region is represented predominantly by alleles which fail to complement (Table 8); however, a few flies do survive in some heteroallelic combinations (Appendix 3). All heteroallelic survivers are viable and fertile without any obvious mutant phenotypes.

Three of the complementation groups within this region, *da*, *Su(var)216*, and *mfs48*, were of special interest as known or potential suppressors of PEV:

da: Nine new *da* alleles were isolated. Two are hemizygous lethal but homozygous viable. A third, da^{E1-21}, is essentially hemizygous lethal, but produces a few viable and fertile progeny (Table 9). None of the alleles is a suppressor of PEV. One second chromosome bearing an allele of *da* is lethal in combination with a chromosome bearing an allele of *l(2)54*. The two loci are separable by deficiency mapping and the effect is allele specific. This suggests that the two mutants may share a common lethal mutation outside region 31.

Table 8. Complementation matrix for alleles of the *B35* locus. The ratio indicates the number of *Cy* to straight winged progeny from the cross *mutant1/CyO* X *mutant2/CyO*.

	B35	*E2-16*	*13-83*
B26	465:0	n.d.	284:0
E1-1	240:0	240:27	n.d.
E2-16	95:0	-	617:0
E2-31	138:0	290:0	268:2
E2-38	104:0	244:0	n.d.
G2-3		118:0	
G2-7		156:0	
13-47	631:0	n.d.	
13-83	193:0	617:0	-

Table 9. Complementation matrix for *da* alleles. Ratios represent the proportion of *Cy* to straight-winged progeny recovered from the cross *mutant1/CyO* X *mutant2/CyO*.

	da^{2}	*E2-35*	*E2-24*
F75	310:0	61:0	129:0
G2-10	66:0	n.d.	148:0
77-11	201:0	59:0	146:0
E1-21	53:10	126:4	n.d.
E1-26	152:0	69:0	n.d.
E2-20	536:0	65:0	103:0
E2-24	119:0	57:0	-
E2-30	64:0	206:0	122:0
E2-35	137:0	-	n.d.

Cross	Progeny
E1-21 X *Df(2L)J27*	166:5
E1-21 X *Df(2L)J106*	107:0
E1-21 X *Df(2L)J77*	176:15
E1-21 X *Df(2L)JR16*	447:25
E1-21 X *E1-26*	385:11

Su(var)216: Eight new mutations were recovered that are lethal with the original *Su(var)216* chromosome. All 8 were EMS induced. None is a dominant suppressor of PEV. The genetics of these mutations are discussed in Chapter 4.

mfs48: Three new alleles of this recessive suppressor of PEV were recovered. Each allele is hemizygous lethal, but heteroallelic survivors from *inter se* crosses all display the short bristle phenotype observed by Lindsley *et al.* (1980). Flies bearing all heteroallelic combinations suppress PEV (I.P. Whitehead, personal communication). One of the alleles was induced in a *P* element mutagenesis screen (Screen 3), while the other two alleles were EMS induced.

Sub-interval 11: Ten genes have been positioned to this interval. Four of the complementation groups are represented by single mutations (*E2-22*, *E1-8*, *77-12*, *77-14*). Three of these mutants (*E1-8*, *77-12*, and *77-14*) display reduced viability when crossed pairwise. Hence, they may actually be weak alleles of the same locus. Two of the mutations, *77-12* and *77-14*, were induced in a *P* element mutagenesis scheme. Since these genes may contain *P* elements, they may be useful for further cytogenetic and molecular studies of the region.

No new alleles of *PI23* or *PJ50* were isolated. Both mutations were mapped to deficiency sub-interval 11 based on hemizygous female sterility. Three new hemizygous lethal alleles of *err* were recovered. One of these alleles, *A76*, is weak. It is not lethal with the original *err* mutation, but

this combination is female sterile at 29° (Appendix 3). No visible phenotypes were observed amongst these mutant progeny.

One gene in this region, *l(2)54*, was of special interest because it, along with *da* and *mfs48*, was proposed to be part of a gene cluster (Sandler, 1977). The homozygous female sterile mutation *mat(2)earlyQM47* (Schüpbach and Wieschaus, 1989) fails to complement *l(2)54* (Sandler, 1977). Several alleles of *l(2)54* are viable in heteroallelic combinations with *mat(2)earlyQM47* (Table 10). In each instance, mutant adults have (with variable expressivity) disorganized eye facets and wings held up over the body in a very sharp "V" formation. Approximately 50% of mutants with rough eyes also have scalloped wings. All surviving heteroallelic combinations of mutations are female sterile. Thus, the *l(2)54* product is required for both viability and female fertility.

Loci proximal to *Df(2L)J27*

Thirteen loci map between the centromere proximal breakpoints of *Df(2L)J27* and *Df(2L)J2*. Their cytogenetic distribution is shown in Figure 8. Four complementation groups, *E1-5*, *E1-18*, *E1-25*, and *E3*, map to an interval between the centromere proximal breakpoints of *Df(2L)J27* and *Df(2L)J106* (sub-interval 13). Three of the loci are represented by a single mutation. The fourth is represented by four alleles which are lethal in heteroalleic combinations (Appendix 3).

Table 10: Complementation pattern of alleles of *l(2)54*. The allele *mat(2)earlyQM47* is abbreviated to *QM47*. Ratios above the matrix diagonal are the proportion of *Cy* progeny relative to straight-winged progeny in a cross between mutants heterozygous for *CyO*. Female sterility (S), where applicable, is noted below the matrix diagonal.

	QM47	*H113*	*C70*	*G2-4*	*E1-2*	*E1-16*
l(2)54	317:0	438:0	345:0	126:0	313:0	209:0
QM47	-	770:52	n.d.	279:12	274:143	165:33
H113	S	-	520:0	68:0	203:0	118:0
C70	n.d.		-	180:0	164:0	354:0
G2-4	S			-	54:0	433:0
E1-2	S				-	309:0
E1-16	S					-

In sub-interval 14, there is a single locus represented by three alleles. All three alleles are hemizygous lethal (Brock, 1989), but are semi-lethal in heteroallelic combinations (Appendix 3). Survivors have etched tergites and are male and female sterile.

Eight complementation groups map to deficiency sub-interval 15. Five are represented by single alleles that have no phenotypes other than hemizygous lethality (Brock, 1989).

The *hup*, *wdl* and *dal* mutations have previously been reported to map in the region deleted by *Df(2L)J2*, but centromere proximal to *Df(2L)J106* (Sinclair *et. al.*, 1991). Neither *hup* nor *wdl* display their respective "wings held up" or "wavy wing" phenotypes with *Df(2L)JR3* or *Df(2L)G2*. *hup* and *dal* were not included in the complementation analysis because they are only weakly inviable, but *wdl* was included because it is also hemizygous semi-lethal. No new alleles of *wdl* were recovered.

DISCUSSION

This study adds region 31-32A to a growing number of regions which have been intensively characterized at the genetic level. Throughout the region deleted by *Df(2L)J2*, 136 mutations were deficiency mapped and tested for complementation. Included in this number were 57 new hemizygous lethal mutations which map between the distal breakpoint of *Df(2L)J77* and the proximal breakpoint of *Df(2L)J106*. Thirty-five new complementation groups have been described, and 15 previously described loci have been more precisely mapped within region 31.

Three new alleles of the recessive suppressor *mfs48* were recovered. Similarly, eight new alleles of *Su(var)216* were isolated. The new alleles of *Su(var)216* all fail to suppress, a point which will be elaborated on in Chapter 4 when the genetics of the putative *Su(var)216* alleles are discussed. The genetics of *mfs48* is being investigated in detail by I.P. Whitehead.

Genetic recombination experiments position the *Suvar(2)1*, *Su(var)204*, and *Su(var)207* mutations very close to each other (32-35 cM; Sinclair *et al.*, 1983). Initial deletion analyses suggested that they were separate loci. However, dominant mutations are difficult to map by deficiencies because secondary, recessive phenotypes must be ascribed to the same locus. The preliminary conclusion that *Su(var)207* and *Suvar(2)1* are separate loci is now in doubt. Complementation

analysis with a new deficiency, in combination with previous recombination data, suggests that *Su(var)207* and *Suvar(2)1* map to the same small deficiency interval. *Su(var)207* is viable in *trans* with *Df(2L)JR1*, but maps to the right of *J* by recombination. Assuming that the hemizygous sterility of the *Su(var)207* chromosome is a true secondary phenotype of the suppressor mutation, these data can only be reconciled if *Su(var)207* is located distal to *Df(2L)JR1*.

Two observations suggest that *Su(var)207* is an allele of *Suvar(2)1*. First, *Su(var)207* and alleles of *Suvar(2)1* interact. Transheterozygotes display a red-brown eye phenotype, reduced male viability, and female infecundity characteristic of heteroallelic combinations of *Suvar(2)1* alleles. Second, like *Su(var)207*, alleles of *Suvar(2)1* are infecund in *trans* with *Df(2L)J2*. Previously, the lethality of *Su(var)207/Df(2L)J39* was attributed to an exacerbation of the sterility phenotype caused by the large semi-lethal deficiency. However, this interpretation, in conjunction with recombination data, would position *Su(var)207* in the region deleted by *Df(2L)JR1*. Since the deficiency and *Su(var)207* complement, the *Df(2L)J39* results are probably caused by a second site recessive lethal mutation on the *Su(var)207* chromosome.

Deficiency mapping in combination with previous recombination data also suggests that the initial localization of *Su(var)204* may have been incorrect. A second site lesion,

which on the basis of deficiency mapping appears to be located to the right of *J*, can account for the sterility of *Su(var)204* females over *Df(2L)G2* and *Df(2L)JR3*. *Su(var)204* has been recombination mapped to the left of *J* (Sinclair, unpublished). Hence the suppression of position-effect variegation and the sterility phenotypes are separable. The *Su(var)204* mutation has no other secondary phenotypes which can be used to ascertain its true location. A duplication for region 31 cannot be used to map the *Su(var)* because the only extant duplication acts as an enhancer (Sinclair *et al.*, 1991). This enhancer, like most others, ameliorates the suppression phenotype of numerous distantly located *Su(var)* mutations. At present the only way to locate *Su(var)204* appears to be by recombination mapping with very tightly linked markers.

The suggestion that there are tightly linked second site mutations on both the *Su(var)204* and *Su(var)207* chromosomes underscores the need for extreme care in interpreting *Su(var)* phenotypes or recessive phenotypes associated with any dominant mutant allele. Until further detailed mapping experiments are performed, a conservative interpretation of the available deficiency mapping data is that there is a single dominant suppressor locus in 31A-B. By this interpretation, region 31 may contain four genes that affect PEV: *Suvar(2)1*, *Su(var)216*, *mfs48*, and *wdl*. However, data presented in Chapter 4 suggest that *Su(var)216* is also an allele of *Suvar(2)1* and that the lethal phenotype of the

suppressor-bearing chromosome is caused by a tightly linked second-site lethal mutation in the 31E region. *Suvar(2)1* is separated from the recessive suppressor loci *mfs48* and *wdl* by numerous complementation groups; and mutations in the intervening complementation groups do not cause dominant suppression of PEV. Furthermore, *wdl* and *mfs48* map to opposite sides of *da* (D. Sinclair, unpublished). Mutations in the *da* gene do not suppress PEV. Thus, this study suggests that suppressors of PEV in region 31 are dispersed among genes with other functions.

Sandler and his colleagues (Sandler, 1977; Lindsley *et al.*, 1980) proposed, by analogy with the Bithorax Complex (Lewis, 1979), that regions 31 and 32 contain a cluster of functionally related genes that interact with heterochromatin and that act early in development. Two members of the proposed cluster (*mfs48* and *wdl*) are recessive suppressors of PEV. Because we have not identified any new homozygous viable mutants, we do not know if additional recessive *su(var)* loci reside in the 31E-32A region. More important, however, is the question of whether there exists a functional cluster of genes. It is interesting in this regard that flies bearing heteroallelic combinations of *l(2)54* display a held-up wing phenotype similar to that of *hup*. They might, therefore, have similar functions. Nonetheless, deficiency and complementation mapping indicate that the proposed cluster of related genes would have to be very large: *wdl* is separated from *da* and

mfs48 by at least 15 complementation groups. Additional loci probably map between *wdl*, in region 31, and *abo*, a distant member of the cluster in region 32. Further analyses will be necessary to test the clustering hypothesis, but such a large number of functionally related essential loci would be extraordinary.

CHAPTER 3

INTRODUCTION

Throughout the past decade, genetic observations have suggested that many loci associated with supression of PEV encode non-histone chromosomal proteins or proteins associated with chromatin assembly or modification. A direct test of this hypothesis is to clone *Su(var)* loci and use biochemical assays to determine the function of the gene product. During the time period in which this thesis was progressing, two *Su(var)* genes were cloned. DNA sequence analysis of both *Su(var)* genes suggests that they encode NHPs, thus substantiating one portion of the original hypothesis. Additional genes need to be characterized to begin a comprehensive biochemical study of chromatin-associated proteins and their effects on gene transcription and chromosome architecture.

This chapter describes a *P* element mutagenesis screen for hemizygous lethal mutations in region 31. One mutation recovered from this screen was lethal with the *Su(var)216* chromosome. The *P* element transposon associated with this lethality was cloned and the adjacent DNA was sequenced to determine the location of flanking transcription units. The *P* element was located in the untranslated 5' sequence of the *cdc2Dm* gene, a gene which encodes a serine/threonine kinase required for progression through the cell cycle (Lehner and O'Farrell, 1990b), and which has histone H1 as one of its targets for phosphorylation.

Although suppressors of PEV are frequently thought of as NHPs, some *Su(var)* genes might encode products which modify NHPs or are involved in the biochemical pathways that lead to chromatin assembly. In fact, a phosphatase 1 gene of *Drosophila* may be a suppressor of PEV (Axton *et al.*, 1990). This gene is also implicated in the control of mitotic progression, suggesting a link between PEV and the cell cycle. The properties of *cdc2Dm* are discussed in this context.

MATERIALS AND METHODS

Genetics

A genetic screen was performed to identify *Su(var)* genes in region 31 (Figure 9). Males from three strains with *P* elements (OK, Harwich, and π_2) were mated, in separate sets of experiments, to homozygous *b pr cn* females without *P* elements (M strains). In such crosses, there is a marked increase in the frequency of *P* element transposition within the germline of the F_1 males and females. Thus, the mobile elements serve as a mutagen in the F_1 hybrids. Mutagenized *b pr cn* second chromosomes were captured over a balancer chromosome in a *P* element-containing strain. *P* element strains contain a repressor that reduce the frequency of transposition events, thereby stabilizing the new sites of *P* element insertion. Male progeny from such crosses were individually mated to 3-5 *Df(2L)J2/CyO* females. The appropriate chromosomes, from lines that were hemizygous lethal in *trans* with *Df(2L)J2*, were re-balanced in a *P* element containing background to prevent further *P* element excision events. Subsequently, the mutations were tested for failure to complement individual *Su(var)* genes that map under *Df(2L)J2*.

Putative *Su(var)* mutants were out-crossed for several generations to progressively replace Chromosomes 1, 2, and 3 with homologues that did not contain *P* elements. The protocol is illustrated in Figure 10. *Lyra* (*Ly*) is a dominant third

Figure 9. P element screen

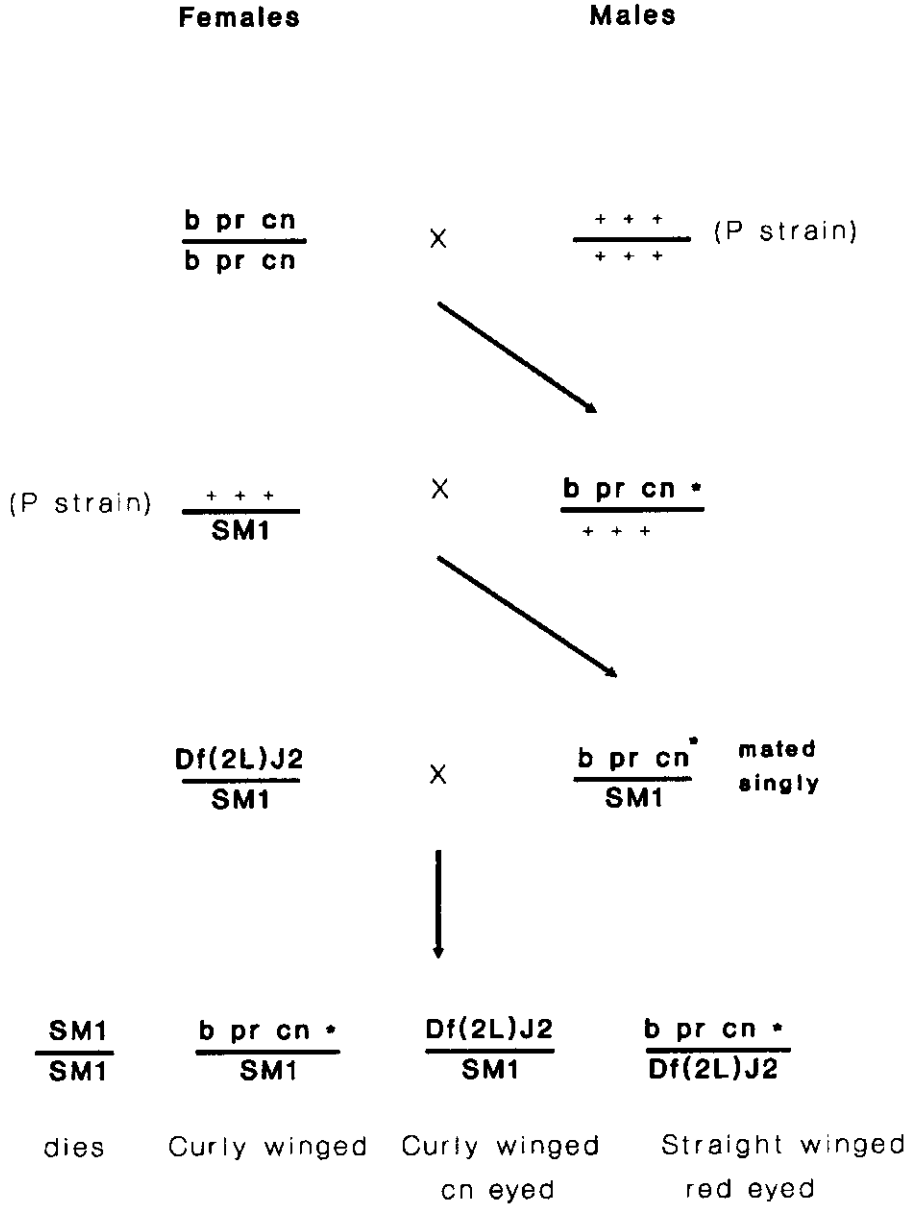
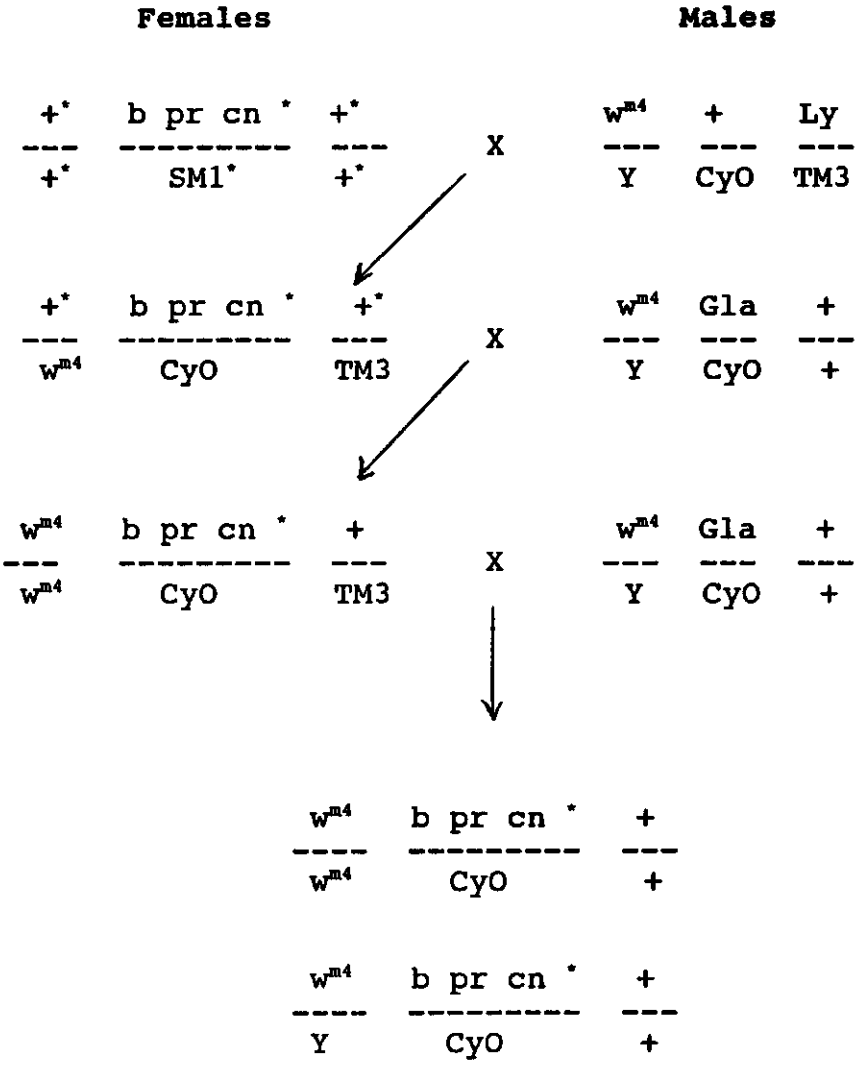


Figure 10. Genetic crosses to isolate the *OK15A* second chromosome.



chromosome mutation that causes a wing-shape change. *TM3* and *CyO* are multiply inverted third and second chromosomes marked with the dominant mutation *Stubble* and *Curly*, respectively. *Sb* causes a short, thick bristle phenotype; *Cy* causes a curly wing phenotype. In addition to providing phenotypic markers to score chromosome segregation, balancer chromosomes (*TM3* and *CyO*) were necessary to prevent meiotic recombination in females. Male *Drosophila melanogaster* do not undergo meiotic recombination.

Molecular Biology

In situ Hybridization to Polytene Chromosomes

In situ hybridization to *Drosophila* salivary chromosomes with biotinylated DNA probes was performed essentially according to Whiting *et al.* (1987). Salivary glands from mature third instar larvae were dissected in 0.8% saline then moved to a droplet of 45% acetic acid for approximately 20 s. The glands were then incubated in fixative (1 part lactic acid: 2 parts distilled water: 3 parts glacial acetic acid) for 2-3 min. The glands were placed on a clean microscope slide, covered with an ethanol-washed (air dried) coverslip, and squashed. The slides were frozen in liquid nitrogen, then the coverslips were removed using a sharp razor blade. The slides were immediately placed in cold (-50^0) ethanol which was left to gradually warm to room temperature. The slides were heated for 30 min at 70^0 in 2X SSC, then dehydrated in

pre-warmed 70% and 95% ethanol for 20 and 10 min, respectively. The ethanol solutions were pre-heated to 65° so that the slides slowly cooled to room temperature. The slides were stored at 4°.

A nick-translated probe (2 µg) was prepared using biotin-11-dUTP (Bethesda Research Lab.) and the BRL nick translation kit, according to the manufacturers instructions. Two-hundred micrograms of sonicated herring sperm DNA were added to the nick-translation mixture. The DNA was ethanol precipitated and resuspended in 117 µl of water. Immediately prior to use, the probe was heat denatured in boiling water for 5 min and plunged into an ice bath. The following was added to the denatured probe solution: 40 µl 10% dextran sulphate, 40 µl 20X SSC, and 4 µl 50X Denhardt's solution.

Immediately prior to hybridization with the DNA probe, the chromosomes on stored slides were denatured in 70 mM NaOH and washed in 70% ethanol for 4 min and 95% ethanol for 4 min.

Twelve microlitres of probe solution was placed on each slide, and covered with an acid-washed coverslip. The slides were placed in a sealed box with filter paper saturated in 2X SSC and the box was incubated at 58.5° for 6-12 hours. Once the hybridization was complete, the slides were washed twice in 2X SSC at 54° for 20 min and once in SSC at room temperature for 20 min.

The BRL BluGene kit was used for detecting the biotinylated probe. Slides were washed in Buffer 1 (0.1 M

Tris-HCl, pH 7.5, 0.15 M NaCl) supplemented with 1% BSA for one hour. Then they were drained and 200 µl of diluted streptavidin-poly(AP) conjugate (2 µl of BRL stock solution in 1.5 ml Buffer 1) was placed over the chromosomes. A coverslip was placed on each slide and the slides were placed in a sealed box with a buffer saturated towel. The box was left at room temperature for 2 hours.

The coverslips were removed by dipping the slides in Buffer 1. The slides were washed twice in 25 ml of Buffer 1 at room temperature for 10 min each, then once in Buffer 3 (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 0.05 M $MgCl_2$) for 10 min. The slides were drained. About 100-200 µl of dye solution (3.3 µl NBT in 0.75 ml Buffer 3 and 2.5 µl BCIP solution) were pipetted over each slide. A coverslip was placed over the dye solution and the slides were placed in a sealed box and incubated at room temperature overnight.

The coverslips were removed by dipping the slides in distilled water. The slides were washed in water for 3 hours. The chromosomes were photographed with water under the coverslip.

Isolation of Genomic DNA

High molecular weight DNA was purified by a modification of the procedure of Jowett (1986). Several hundred adult flies were flash-frozen in liquid nitrogen, then ground to a powder in a cooled mortar and pestle. The powder was transferred to

10 ml of lysis buffer (100 mM Tris-HCl (pH 8.0), 50 mM EDTA, 1% SDS, 0.15 mM spermine, 0.5 mM spermidine) containing 100 µg/ml proteinase K. After incubation at 65^0 for 2 hours, the mixture was extracted once with an equal volume of TE equilibrated phenol (pH 8.0), twice with phenol/chloroform (1:1), and once with chloroform. The aqueous phase was transferred to a fresh tube, and overlaid with two volumes of ice-cold 95% ethanol. DNA was spooled around a heat-sealed Pasteur pipette and re-dissolved in TE.

Isolation of Bacteriophage Lambda DNA

Bacteriophage lambda were isolated according to the plate lysate method of Fritsch (Maniatis *et al.*, 1982). Approximately 3 X 10^6 bacteriophages were mixed with 1.6 X 10^8 bacterial cells. After incubating the mixture for 15 min at 37^0, the cell suspension was diluted to 5 ml with LM. The liquid culture was incubated at 37^0 for 6-9 hours until the culture cleared. Bacterial debris was removed from the lysate by centrifugation. RNase A and DNase I were added to the supernatant, each to a final concentration of 50 µg/ml, and the mixture was incubated for 30 min at 37^0. An equal volume PEG solution (2.5 M NaCl and 20% (w/v) PEG in lambda-dil) was added, and the mixture was incubated at 4^0 for one hour. Precipitated bacteriophage particles were recovered by centrifugation at 10,000 rpm in an SS34 rotor for 30 min. The supernatant was poured off, and the tubes were drained in an

inverted position overnight. Any fluid remaining in the tube was absorbed with a paper towel. The bacteriophage pellet was resuspended in 0.4 ml of TE and transferred to a microfuge tube. Residual debris was removed by centrifugation at 8000*g*. Twenty microlitres of 0.5 M EDTA (pH 8.0) was added, and the solution was extracted twice with phenol/chloroform (1:1), and once with chloroform. Bacteriophage DNA was precipitated with 2.5 volumes of 95% ethanol. The DNA was pelleted by centrifugation, vacuum dried, and resuspended in TE.

Isolation of Plasmid DNA

Plasmid DNA was isolated by the alkaline lysis method. Small, medium and large scale isolations were performed. The bacteria pelleted from 2, 50, or 500 ml of LM (containing 0.005% ampicillin) were resuspended in 0.2, 2.5, or 10 ml of Solution 1 (50 mM glucose, 20 mM Tris-HCl (pH 8.0), 10 mM EDTA), respectively. One volume of Solution 2 (0.4 N NaOH, 2% SDS) was added, and the solution left at 4^0 for 10 min to lyse the bacteria. One-half volume of 3 M potassium acetate solution was added to the lysed bacterial suspension and the mixture was incubated for an additional 10 min at 4^0. To remove bacterial debris, small scale preparations were microfuged at 12000 xg for 5 min; medium and large scale preparations were centrifuged for 20 min in a Sorvall ss34 rotor at 8000 rpm and 15000 rpm, respectively. The supernatant was transferred to a new tube. DNA was precipitated directly

from the supernatant of large scale preparations by the addition of isopropanol (60% v/v). The supernatant from small and medium scale preparations was extracted once with phenol/chloroform (1:1); and then plasmid DNA was precipitated with 2 volumes of ice-cold 95% ethanol. The DNA was pelleted as previously described, then vacuum dried.

For small scale plasmid preparations the DNA pellet was resuspended in 50 µl TE, ready for use.

For medium scale preparations, the DNA was resuspended in 300 µl of TE. One microlitre of RNAse A (10 mg/ml) was added and the solution was incubated at 22^0 for 30 min. One half volume of 7.5 M ammonium acetate was added and RNAse was precipitated for 10 min at -80^0. After centrifugation (12000 xg for 5 min), the supernatant was transferred to a new microfuge tube. Plasmid DNA was precipitated with 2 volumes of ice-cold 95% ethanol. The DNA was pelleted (12000 xg for 10 min), dried under vacuum, then resuspended in 900 µl of TE. The DNA was again precipitated by the addition of 600 µl of PEG solution (20% PEG 6000, 2.5 M NaCl) and incubation at 4^0 for 1 hour. The plasmid DNA was pelleted (12000 xg for 10 min), dried, and resuspended in 100 µl of TE.

For large scale preparations, the DNA was resuspended in 8 ml TE buffer. Eight grams of CsCl and 0.8 ml of ethidium bromide (10 mg/ml in water) were added. The solution was poured into a Beckman polyallomer 16 X 76 mm Quick-Seal centrifuge tube and the tube was heat-sealed. Equilibrium

centrifugation was at 45,000 rpm for 36 h (at 22^0) with a Sorvall Tft65.13 rotor in a Beckman L8-80 ultracentrifuge. Supercoiled plasmid DNA was removed from the gradient using an 18 gauge needle attached to a 3 ml syringe. Ethidium bromide was extracted with *sec*-butanol, then the solution was diluted 3 times with water. Plasmid DNA was precipitated by the addition of 2 volumes of cold 95% ethanol. The DNA was pelleted by centrifugation, vacuum dried, and resuspended in TE.

DNA Restriction Digests and Gel Electrophoresis

For most plasmid and lambda phage digestions, 1-2 µg of DNA in 17 µl of water were mixed with 2 µl of the appropriate BRL core buffer and 1-2 µl of restriction endonuclease (3-10 units), then incubated at 37^0 for 3 hours. Some samples were incubated with heat-treated RNAse A (100 µg/ml, 15 min, room temperature) to hydrolyze contaminating RNA. Digestions were stopped by adding 0.2 volumes of loading buffer (6 M urea, 25% sucrose, 50 mM EDTA, and orange G dye). Genomic DNA (2-5 µg) was made more dilute (<100 µg/ml) and digested overnight, then ethanol precipitated and redissolved in 25 µl of TE.

Digested DNA was fractionated on 0.8-1.2% agarose gels containing Tris-acetate buffer (40 mM Tris-HCl, 5 mM sodium acetate, 1 mM EDTA, pH adjusted to 7.8 with acetic acid), and 0.1 µg/ml ethidium bromide. Gels were electrophoresed at 1.8-3.7 V/cm for 2-12 hours, then photographed by transmitted

ultraviolet light.

Transfer of DNA to Hybridization Membrane

DNA was transferred to Hybond-N nylon membrane according to a procedure adapted from Southern (1975). The gel was incubated in 0.4 N NaOH, 0.6 M NaCl for 30 minutes at room temperature with gentle agitation. Then it was neutralized in 1.5 M NaCl, 0.5 M Tris-HCl (pH 5.5) for 30 minutes with shaking. For blotting, the gel was placed on a pre-soaked Whatman 3MM paper wick suspended above a reservoir of 5X SSC transfer solution. A pre-wetted membrane was placed on the uppermost gel surface and overlaid first with dry Whatman 3MM paper and then with a thick stack of paper towels. A glass plate with a small weight on it was placed over the top to ensure even transfer. Transfer was complete within 6-12 hours, after which time the blot was washed in 2X SSC and dried at room temperature. DNA was permanently affixed to the membrane by exposure to a 254 nm ultra-violet light for 5 minutes.

Hybridization of Radiolabelled Probe to Transferred Nucleic Acids

Southern blots (DNA) were incubated in a sealed plastic bag with 10 ml of Hybridization Solution (6X SSC, 5X Denhardt's and 0.1% SDS: 100X Denhardt's is 2% BSA, 2% Ficoll, 2% PVP) for 2 hours at 65^0 with agitation. Radiolabelled probe was added and allowed to hybridize to the filter for 12-36

hours at 65° with shaking. High stringency washes were: three times 30 min in 0.1X SSC, 0.1% SDS at 65° with shaking. To detect sequences with a low degree of homology to the probe, low stringency washes were performed in Hybridization Solution for 2 h at 65°.

Labelling of DNA

DNA (50-200 ng) was radiolabelled to a specific activity of approximately 1.8 X 10^9 dpm/μg by random hexamer-primed DNA labelling (Feinberg and Vogelstein, 1983, 1984) using a Boehringer Mannheim Random Primed DNA Labelling kit.

Library Construction

Two micrograms of lambda EMBL4 DNA were digested in a 40 μl volume with an excess of *EcoRI* and *BamHI* (20 units). After 1 hour, the enzymes were inactivated by heating at 72° for 15 min. Sodium acetate was added to a final concentration of 300 mM, and the DNA was precipitated with 0.6 volumes of isopropanol for 15 min at room temperature. The precipitate was washed with 70% ethanol and vacuum dried.

These conditions excised the central stuffer region of the vector and exposed the *EcoRI* cloning site. Digestion with *EcoRI* and *BamHI* prevents the stuffer fragment from re-ligating into the vector in subsequent reactions. Since the very short *EcoRI-BamHI* linker fragments do not precipitate in the isopropanol step, the probability of a re-ligated stuffer

fragment is further reduced. Restriction endonuclease digested *Drosophila* genomic DNA was prepared as follows. Twenty micrograms of uncut genomic DNA was resuspended in 200 µl digestion buffer (100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, Tris-HCl (pH 7.5)) heated to 37°. Ten units of *EcoRI* were added and the time-course of digestion was monitored by removing 40 µl (2 µg) samples. Each sample was made 10 mM with respect to EDTA, and stored on ice until all samples were collected. The enzyme was then heat inactivated at 72° for 15 min. One half microgram of DNA from each sample was electrophoresed in a 0.3% agarose gel. The lane with the maximum intensity of fluorescence in the 15-20 kb range was determined, and the sample with the maximum number of molecules in that size range was prepared for ligation.

The partially digested genomic DNA was precipitated with ethanol, vacuum dried, and resuspended in phosphatase buffer (50 mM Tris-HCl (pH 9.0), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 1 mM spermidine). One half unit of calf alkaline phosphatase was added and the reaction was incubated at 37° for 1 h. The mixture was extracted sequentially with phenol, phenol/chloroform (1:1), and chloroform, then made 100 mM with respect to sodium acetate. The DNA was precipitated with 2 volumes of cold 95% ethanol.

Approximately 0.5 µg of genomic DNA partially digested with *EcoRI* and 2 µg of *EcoRI* digested vector were resuspended in 10 µl ligase buffer (10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$,

5 mM β-mercaptoethanol and 1 mM ATP). One unit of T4 DNA ligase (BRL) was added and the ligation proceeded overnight at 15°. Ligated DNA was packaged using a Gigapack Plus (Stratagene) packaging kit according to the manufacturer's instructions.

Screening of Libraries of Recombinant Bacteriophage Lambda

Four lambda-libraries were screened. An unamplified partial *EcoR1* library from *Drosophila* was constructed in *EMBL4* (Frischauf *et al.*, 1983), as described above. The host bacterium was *NM539*. The same bacterial strain was used to amplify a *Drosophila virilis* partial *Sau3A* library (Dr. J. Tamkun) cloned in *EMBL3*. A λ*gt11* 0-12 hour embryonic cDNA library (Dr. J. Tamkun) was propagated on *Y1090hsdR*. A third instar imaginal disc cDNA library (Dr. G. Rubin) constructed in λ*gt10* was amplified on *C600hflA*.

Recombinant λ bacteriophage were propagated on *E. coli* strains prepared as follows. A single host-cell colony was grown overnight in LM, and then 5 ml were used to inoculate a fresh 50 ml culture. After 3 hours at 37°, the cells were pelleted in a bench-top centrifuge (10 min at 3000 xg) and resuspended in 25 ml of sterile 10 mM $MgSO_2$. These cells were viable for several weeks.

Approximately 3-5 x 10^4 pfu, suspended in 2-50 µl of lambda-dil (100 mM NaCl, 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$), were added to 100 µl of plating cells and incubated at

37° for 15 min. The bacteria were then mixed with 8 ml of molten 50° top agar (LM + 7 g/l Bacto-agar). The agar was poured onto the surface of a 150 X 15 mm LM agar plate (LM + 14 g/l Bacto-agar) and allowed to cool. Plates were incubated at 37° for 6-12 hours to allow the bacterial lawn to grow.

Lambda phage DNA was transferred from plaques on LM-plates to pre-cut nitrocellulose filters by a modification of the procedure of Benton and Davis (1977). Each plate with bacteriophage plaques was blotted with a dry nitrocellulose filter. The filter was peeled off the bacterial lawn, and placed in 0.4 N NaOH for 30 seconds. The filter was then washed for 60 seconds in 1.5 M NaCl, 0.5 M Tris-HCl (pH 5.5), and finally for 30 seconds in 2X SSC. Filters were air dried on Whatman 3MM paper and baked for 2 hours at 80°.

The filters were hybridized with a radiolabelled probe to identify DNA with sequence similarity. Several rounds of replating and re-probing at successively higher phage dilutions were necessary to purify each lambda clone.

Subcloning Bacteriophage and Cosmid DNA

Bacteriophage or cosmid DNA was digested with an appropriate restriction endonuclease and the DNA was size fractionated on an agarose gel. A gel slice containing the fragment to be subcloned was transferred to a microfuge tube, and the DNA fragment was purified using glass beads (Gene Clean; Bio 101) according to the manufacturer's instructions.

Fifty to five hundred nanograms of restriction endonuclease digested DNA was co-precipitated with 50 ng of linearized pUC19 with compatible ends. The DNA was resuspended in a 20 µl reaction volume containing 10 mM Tris-HCl (pH 8), 10 mM $MgCl_2$, 1 mM DTT and 1 mM ATP. One unit of T4 DNA ligase was added and the sample was incubated for 8-12 h at 16^0.

pUC plasmids were transfected by adding 3 µl of the ligation reaction mixture to 50-200 µl of thawed competent DH5-α (subcloning efficiency; Bethesda Research Laboratories). After 30 min on ice the cells were heat shocked at 37^0 for 20 s. The cells were placed on ice for 2 min, then diluted with 500 µl of LM and incubated at 37^0 for 1 h. Cells (50-100 µl) were spread over a 10 cm 0.005% ampicillin (w/v) LM-plate, whose surface had been coated with 50 µl of 2% X-gal. White colonies were selected as recombinants, and their inserts were screened by either mini-plasmid preparation or colony hybridization.

Blotting DNA from Bacterial Colonies

Plasmid or cosmid DNA from lysed bacterial colonies was blotted directly onto the surface of nitrocellulose filters by a modification of the procedure of Grunstein and Hogness (1975). A pre-cut nitrocellulose filter was placed over colonies grown on an ampicillin LM plate. The filter was peeled off and placed bacteria-side up on Whatman 3MM paper soaked with denaturing solution (0.5 M NaOH, 1.5 M NaCl).

After 5 min it was then transferred to neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl pH 8.0) for 5 min. Filters were dried and baked at 80^0 for 2 hours.

RNA extraction

RNA was extracted essentially according to Jowett (1986). Approximately 200 µl of 0-6 hour old embryos were flash-frozen in liquid nitrogen and ground to powder with a mortar and pestle. The powder was brushed into 4 ml of phenol saturated with 200 mM sodium acetate (pH 5.0) at 65^0. After vortexing, an equal volume of Extraction Buffer (150 mM sodium acetate (pH 5.0), 2% SDS) was added. The solution was incubated at 65^0 for 5 minutes, then allowed to cool to 22^0. Subsequently, 4 ml of chloroform was added, vortexed, and centrifuged to separate the aqueous and organic phases. The organic phase was removed, and the aqueous phase was re-extracted: first with an equal volume of phenol/chloroform (1:1), then with chloroform. RNA was precipitated overnight at -20^0 by the addition of 2.5 volumes of 95% ethanol. The RNA was pelleted, washed with 70% ethanol, then re-dissolved in diethylpyrocarbonate-treated water.

Poly(A)$^+$ RNA was purified from total RNA by chromatography on oligo(dT)-cellulose. Oligo(dT)-cellulose was equilibrated in sterile loading buffer (20 mM Tris (pH7.6), 0.5 M NaCl, 1 mM EDTA, 0.1% SDS) and poured into a disposable, sterile column. The column was washed sequentially with the

following: (1) 3 volumes of sterile water, (2) 3 volumes of 0.1 M NaOH and 5 mM EDTA, (3) 3 volumes of sterile water, and (4) 5 volumes of loading buffer.

Total RNA dissolved in water was heated to 65^0 for 5 min, then an equal volume of 2X loading buffer was added. The RNA solution was loaded onto the column followed by 10 volumes of loading buffer. Next 4 volumes of washing buffer (20 mM Tris (pH 7.6), 0.1 M NaCl, 1 mM EDTA, 0.1% SDS) were passed through the column. Poly(A)$^+$ was eluted from the column with 2-3 column volumes of sterile elution buffer (10 mM Tris (pH 7.5), 1 mM EDTA, 0.05% SDS). Sodium acetate (3 M, pH 5.8) was added to a final concentration of 300 mM and the RNA was precipitated with 2.5 volumes of ethanol at -70^0.

RNA Gel Electrophoresis and Northern Blotting

RNA was fractionated by size in a formaldehyde, denaturing gel. Two grammes of agarose, 20 ml 10X MOPS (0.2 M 3-(N-morpholino) propanesulfonic acid, 50 mM sodium acetate, 10 mM EDTA, adjusted to pH 7.0), and 174 ml diethyl pyrocarbonate (DEP) treated autoclaved water was added to a 500 ml flask. The agarose was melted by boiling, then the solution was cooled to 50^0. In a fumehood, 10.2 ml 37% formaldehyde was added to the agarose solution. The gel mixture was poured into an 20 X 15 cm gel tray and allowed to set for one hour.

Approximately 5 μg of poly(A)$^+$ RNA, resuspended in 5 μl

of water was mixed with 25 µl electrophoresis sample buffer (0.75 ml deionized formamide, 0.15 ml 10X MOPS, 0.24 ml formaldehyde, 0.1 ml water, 0.1 ml glycerol, 0.08 ml 10% (w/v) bromophenol blue. The sample was heated at 65° for 15 min. Ethidium bromide (1 µl of a 1.0 mg/ml solution) was added, and the denaturing gel was loaded.

The formaldehyde gel was immersed in 1X MOPS/EDTA, the sample wells were flushed with electrophoresis buffer, and the prepared samples were loaded. Electrophoresis was at 30 V at room temperature.

Following electrophoresis, the gel was soaked for two 20 min periods in 10X SSC at room temperature with shaking. The gel was blotted as previously described for DNA, except that the transfer solution was 10X SSC. RNA was fixed to the Hybond-N membrane (Amersham) by exposure to short wave radiation (254 nm) for 5 min.

Radiolabelled DNA probes were hybridized to Northern blots exactly as described for Southern blots.

DNA Sequence Analysis

Fragments of DNA were subcloned into pUC19, then sequenced using a modification of the procedure of Tabor and Richardson (1987). Four micrograms of double-stranded plasmid DNA template was denatured for 2 minutes at 65° in 400 mM NaOH, then made 300 mM with respect to sodium acetate. The DNA was precipitated with 2 volumes of ice-cold 95% ethanol and

pelleted by centrifugation. DNA primer (3 pmol) was annealed to the template for 20 minutes at 37^0, in 10 µl of Annealing Buffer (50 mM NaCl, 10 mM $MgCl_2$, 40 mM Tris-HCl (pH7.5)). The annealing reaction was diluted to a final volume of 13.5 µl by the addition of 1 µl of 0.1 M DTT, 0.5 µl of (α-^{35}S)dATP, and 2 µl of nucleotide mix (1.5 µM each of dGTP, dCTP, and dTTP). Following the addition of 2 µl (1.8 Units) of T7 DNA polymerase, the labeling mix was incubated at 22^0. After 8 min, 3.5 µl of the reaction mix was added to each of four microfuge tubes. Each microfuge tube contained 2.5 µl of a different termination mix: one for each of the four deoxyribonucleotides found in DNA. The termination mixes contained 80 µM each of three dNTPs (dATP, dCTP, dGTP, or dTTP) and 8 µM of the fourth dNTP. Termination reactions were performed at 37^0 for 10 minutes. Reactions were stopped by the addition of 4 µl of Stop Solution (95% formamide, 20 mM EDTA, 0.5% bromophenol blue, 0.5% xylene cyanol).

Sequencing reactions were loaded into sharktooth sample wells in a 33 X 40 X 0.04 cm 6% polyacrylamide/urea sequencing gel. Electrophoresis was at approximately 45 Watts for 1-6 hours. Ninety-nine millilitres of gel solution was prepared by mixing 15 ml of 40% (w/v) acrylamide stock (acrylamide:bisacrylamide; 19:1), 50 g of urea, 10 ml of 10X TBE (121.1 g/l Tris base, 55 g/l boric acid, 7.4 g/l Na_2EDTA, pH 8.3) and 35 ml of water. Polymerization was induced by the addition of 1 ml 10% (w/v) ammonium sulphate and 20 µl TEMED.

Directed Deletions

Exonuclease III directed deletions were performed using a protocol based on the procedure developed by Henikoff (1984). Five micrograms of plasmid DNA was double-digested with two restriction endonucleases to produce double-stranded DNA with one 5' protruding end and one 4-base 3'protruding end. The DNA was precipitated and re-dissolved in 57 µl of 66 mM Tris-HCl (pH 8.0), 6.6 mM $MgCl_2$, at 35°. Exonuclease III (325 Units) was added to increase the reaction volume to 60 µl. After a delay of 20 seconds, and every 30 seconds thereafter, 2.5 µl samples of this reaction were mixed with 7.5 µl of ice-cold S1 mix (40.5 mM potassium acetate (pH 4.6), 338 mM NaCl, 1.4 mM $ZnSO_4$, 6.6% glycerol, 3.5 units S1 nuclease) in separate microfuge tubes. When all of the desired samples had been collected, the reactions were transferred to 22° for 30 minutes. The S1 enzyme was inactivated by heat-denaturation at 70° for 10 minutes following the addition of 1 µl of stop mix (300 mM Tris-HCl (pH 8.0), 50 mM EDTA). To assess the extent of exonuclease III digestion, 2 µl (approximately 40 ng DNA) of each sample was run on a 0.8% agarose gel. After the addition of 0.2 units of Klenow DNA polymerase, the remainder of each reaction (9 µl) was incubated at 37° for 3 minutes. Next, 1 µl of dNTP mix (0.125 mM each of dATP, dCTP, dGTP, dTTP) was added to each tube. The tubes were incubated at 37° for an additional 10 minutes. Subsequent DNA ligations to vector and transformation were

performed as previously described.

RESULTS

Genetics

Fourteen thousand second chromosomes were screened for *P* element induced hemizygous lethal mutations in region 31 using the protocol in Figure 9. Four *P* element induced mutations were isolated. Three fully complemented the suppressors *Su(var)207*, *Su(var)204*, *Su(var)216*, and *Suvar(2)1*. (These stocks subsequently died of a mould infection). The fourth, *OK15A*, failed to complement *Df(2L)J27*, *Df(2L)J106*, and *Su(var)216*.

Hybridization of a cloned *P* element probe to squashes of *OK15A* salivary gland polytene chromosomes established the presence of a *P* element in region 31E (see Figure 13). However, *P* elements frequently transpose into a gene and then excise again, deleting adjacent sequences. Such mutations are not readily clonable, since no *P* element resides in the gene. To test for the presence of a functional *P* element at the *Su(var)216* locus, the lethal phenotype of *OK15A* was reverted according to the protocol in Figure 11. The third chromosome carries *Δ2-3(99B)*, a genetically engineered *P* element which does not itself transpose but which provides a very active source of transposase (Robertson *et al.*, 1988). Amongst 5000 progeny from the dysgenic cross, 38 survived in *trans* with *Su(var)216*. Thirty-six of these were sterile, but two, *R34* and *R28*, were fertile. Both of these putative revertants were also viable when hemizygous for region 31. Thus, a mobile *P* element

Figure 11. Phenotypic reversion of the homozygous lethality of the *Su(var)216^{P}* mutant. Male or female revertants have a black body (see Genetic Abbreviations).

Females		Males
$\frac{\text{Su(var)216}^{P}\ \text{b pr cn}}{\text{CyO}}$	X	$\frac{\text{Sp}}{\text{CyO}}\ ;\ \frac{\text{Sb }\Delta\text{2-3}}{\text{TM6}}$
$\frac{\text{Su(var)216 b lt rl}}{\text{CyO}}$	X	$\frac{\text{Su(var)216}^{P}}{\text{CyO}}\ ;\ \frac{\text{Sb }\Delta\text{2-3}}{+}$

$$\frac{\text{Su(var)216}^{PR}\ \text{b}\ +\ +\ \text{pr cn}}{\text{Su(var)216}\ \ \text{b lt rl}\ +\ +}$$

is associated with the lethal phenotype of *Su(var)216*.

The original *OK* strain contains approximately 30 intact or partially deleted *P* elements distributed over the full chromosome complement. The abundance of elements in *OK15A* made it difficult to identify the *Su(var)216*^{P} transposon; therefore, the X and third chromosomes were replaced with homologues without *P* elements. The genetic crosses are illustrated in Figure 10. Each cross was designed to introduce non-*P* element bearing chromosomes from males, since this causes a lower incidence of transposition than the reciprocal cross (see Kidwell, 1986).

In addition to reducing the total number of *P* elements in the stock, the crosses removed the second chromosome from a dysgenic background. Many *P* elements have a defective transposase gene and are only able to transpose if an alternative source of transposase is present. Thus, if fully functional *P* elements are removed from the genetic background, the remaining elements are not able to transpose. The loss of transposon mobility can be tested by crossing *P* element bearing males to non-*P* element bearing females at 29^{0}. Under these extreme conditions, dysgenic female progeny are sterile (see Kidwell, 1986). *OK15A* females were still fertile at 29^{0} after the replacement of both sets of X and third chromosomes, suggesting that none of the remaining *P* elements was capable of transposition.

Once the mutagenized third and X chromosomes had been

replaced, a Southern blot of digested *OK15A* genomic DNA was hybridized with a cloned *P* element. The pattern of bands obtained suggested that numerous *P* elements still remained in the stock (Figure 12). To reduce the number of elements further, the right arm of Chromosome 2 was replaced by genetic recombination. *Su(var)216^{P} b pr cn/S Sp Tft nw^{D} Pin^{Yt}* females were crossed to *Gla/CyO* males, and recombinant *Tft nw^{D} Pin^{Yt}/CyO* male progeny were collected and tested for the *Su(var)216^{P}* lesion. Each male was crossed to 4-5 females of genotype *Df(2L)J27/CyO*. In 150 such pair matings, two recombinants, *OK#1* and *OK#2*, were obtained that were lethal in *trans* with *Df(2L)J27*. Assuming that only a single cross-over event occurred, the entire right arm of the chromosome should have been replaced.

Approximately 5 DNA fragments homologous to a 900 bp *P* element probe are detectable on Southern blots of *EcoRI*-cut *OK#1* genomic DNA (Figure 12). A similar number of bands hybridize to a 500 bp *P* element probe when *OK#1* DNA is digested with *BamHI* and *XbaI*. *BamHI* and *XbaI* do not cut within the DNA of full length *P* elements (O'Hare and Rubin, 1983); this suggests that approximately 5 distinct *P* elements are present in *OK#1*.

The *OK#1* strain was re-tested for the presence of a *P* element in region 31 by *in situ* hybridization (Figure 13), and then an *OK#1* genomic DNA library was constructed.

Figure 12. *P* elements in *OK15A* derived strains. A. *P* elements on the second chromosome detected with a 950 bp *HindIII* fragment from pπ 25.1 (O'Hare and Rubin, 1983). B. *P* elements on the second chromosome after genetic recombination. The probe was a 500 bp *EcoRI/HindIII P* element fragment from HBΔ-89 (Simon *et al.*, 1985).

Panel A. Lane: 1. *SalI*

2. *BamHI*

3. *EcoRI*

4. *XhoI*

5. *XbaI*

6. *SstI*

Panel B. Lane: 1. *BamHI*

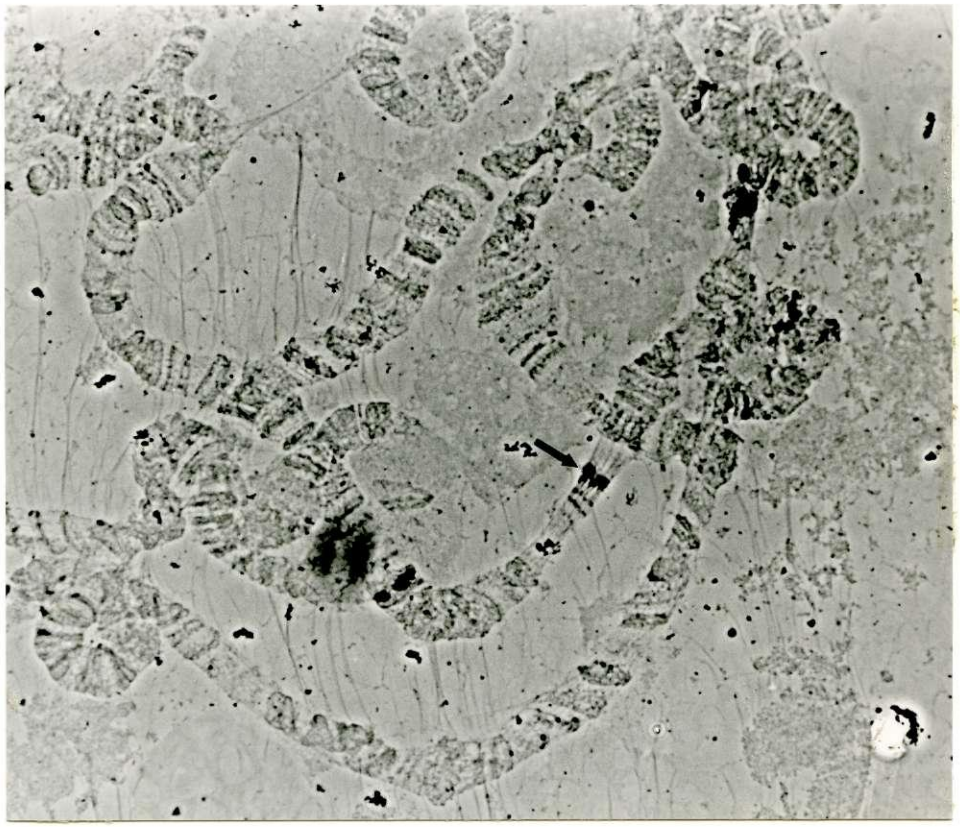
2. *XbaI*

3. *EcoRI*

A.

B.

Figure 13. Localization of a *P* element in the 31E region by *in situ* hybridization. An arrow indicates the site of *P* element hybridization.



Cloning a *P* element in Region 31E

A partial *EcoRI* library was constructed in *EMBL4* as described in Materials and Methods. The unamplified library contained approximately 7 x 10^5 recombinant phage, or 70 genomic equivalents of *Drosophila melanogaster* DNA, assuming an average insert size of 17 kb and equal representation of all sequences. A portion of the unamplified library (3 x 10^5) was screened with a radiolabelled *P* element probe. Twenty recombinant phage were plaque purified and their DNA isolated.

Since the chromosome bearing *Su(var)216*^{P} contained approximately 5 *P* elements, several of the recombinant bacteriophage were expected to have genomic DNA inserts from locations other than region 31E. To identify genomic DNA inserts with sequence similarity to region 31, the clones could have been tested by *in situ* hybridization. Instead, *P* containing clones were identified by cross-hybridization with an overlapping series of cosmids containing sequences from region 31.

Su(var)216 maps very close to *da* in genetic recombination experiments (0.003 cM ; Sinclair, unpublished); therefore, cosmid clones were obtained for the *da* region. One set of cosmid clones, the *JT* series, was provided by C. Cronmiller. The second set, *c1* to *c10*, was isolated using a cDNA (*adm 134G6*; Carlson, 1982) which maps by *in situ* hybridization to region 31E. The two sets of cosmids overlap and span approximately 60 kb of DNA around *da* (only a small portion of

which has been mapped). Two overlapping cosmids that include *da* and the regions immediately proximal and distal to the *da* locus are shown in Figure 14. Recombinant clones from the *Su(var)216*^{P} library were divided into groups based on cross-hybridization of the genomic DNA inserts, then a representative clone from each group was radiolabelled and hybridized to Southern blots of *JT35* and *c7*. Four independently isolated, 17 kb recombinant phage cross-hybridized to the cosmid clones.

A 4.2 kb *EcoRI* fragment containing the *P* element was isolated from one of the recombinant phage clones (λ#6.1) and inserted into pUC19 to create pRP4.2. Hybridization of the 4.2 kb fragment to Southern blots of *JT35* and *c7* cosmid DNA indicated that the *P* element is inserted in a unique 3.8 kb "wild-type" *EcoRI* fragment (Figure 15). In Figure 15 Panel B, a *P* element probe hybridizes to pRP4.2 but not to the 3.8 kb *EcoR1* fragment in *JT35* or *c7* fragment. Hybridization of the *P* element probe to a large fragment in each of the cosmid lanes is caused by *P* element sequences in the vectors *CosPer* (*JT35*) and *CosPneo* (*c7*). Figure 15 Panel C demonstrates that the 3.8 *EcoR1* fragment hybridizes to pRP4.2. The 3.8 kb *EcoRI* was subcloned from *JT35* to create pR3.8.

Additional hybridization experiments and comparison of the restriction endonuclease maps of the two subclones positioned the *P* element within 3 kb of the *da* gene. However, genetic complementation data (Chapter 1) clearly demonstrate

Figure 14. A restriction map of DNA flanking the P element in the 31E region. (Symbols: B, BglII; R, EcoRI; H, HindIII; Hc, HincII; P, PstI; S, SalI; St, SstII; X, XhoI)

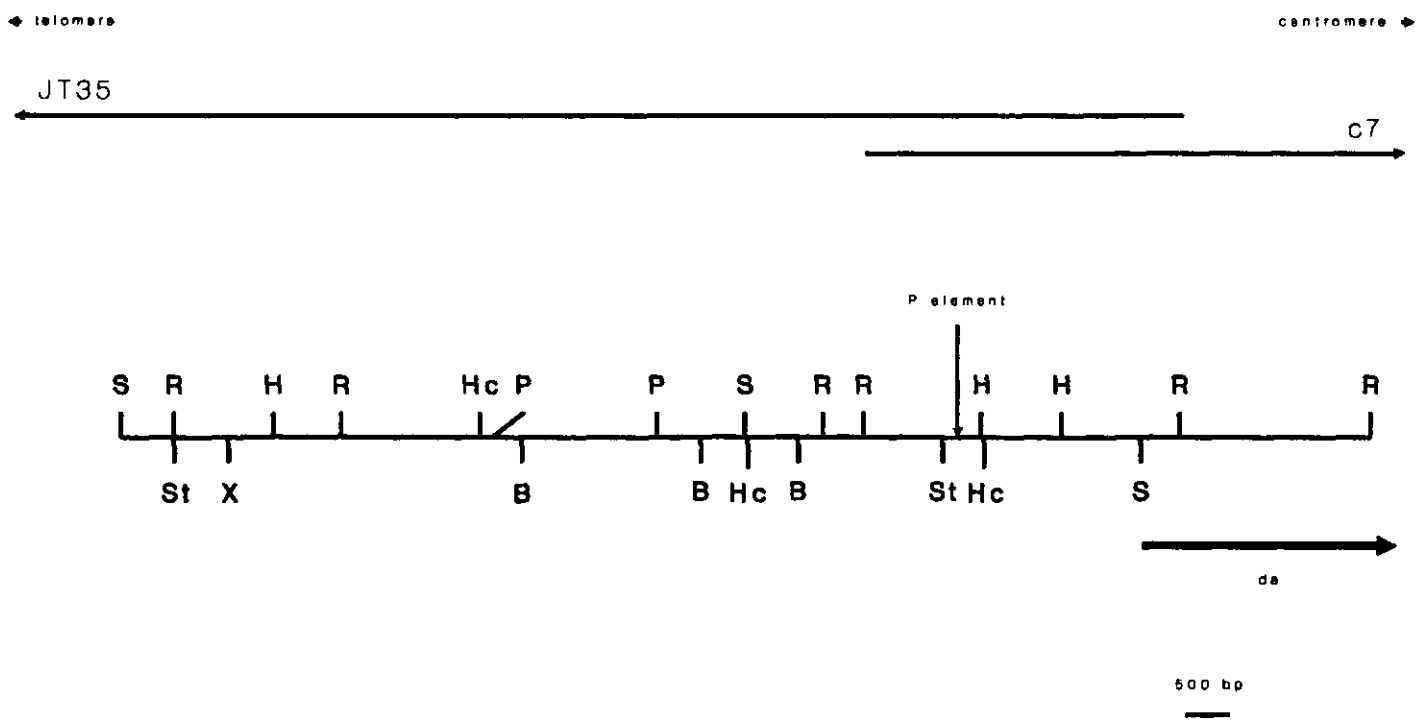
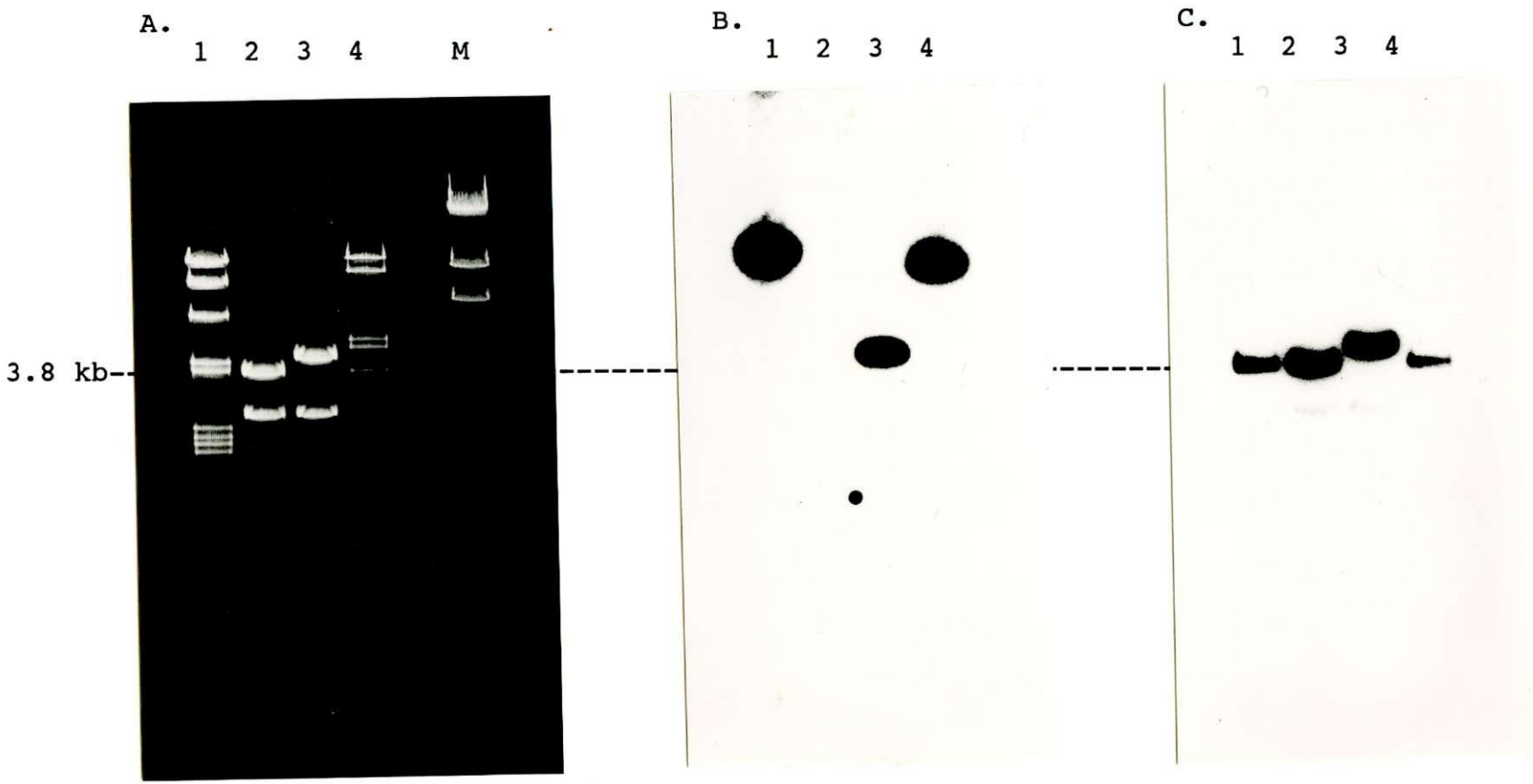


Figure 15. Subclones from the 31 region. Panel A. JT35 (lane 1), pR3.8 (lane 2), pRP4.2 (lane 3), and c7 (lane 4) digested with *EcoRI*. Panel B. A Southern blot of the gel in Panel A probed with a *P* element. Panel C. The same blot re-probed with the 3.8 kb *EcoRI* insert from pR3.8. The marker (lane M) is bacteriophage λ DNA cut with *HindIII*.



that the *P* element does not disrupt the *da* gene (data not shown).

Disregarding the presence of a *P* element, the genomic insert in pRP4.2 is slightly different from that in pR3.8 (Figure 16). Digestion of the two plasmids with *EcoRI* and *SstII* revealed that pRP4.2 lacks approximately 180 bp of DNA that is present in pR3.8. Sequence analysis of pRP4.2, pR3.8, and overlapping DNA fragments from the *P* element-mutated chromosome (see Chapter 4) indicates that the *b pr cn* chromosome has an extra *EcoRI* site, caused by a single basepair transition, located between the two sites in pR3.8. This extra site served as one of the cloning sites for pRP4.2.

Restriction endonuclease mapping and cross-hybridization experiments positioned the *P* element insertion of pRP4.2 in a fragment with homology to a 1.5 kb *HindIII/EcoRI* fragment from pR3.8 (Figures 16-18). Comparison of the size of the wild-type fragment with the transposon-containing fragment suggests the element is approximately 600 bp long. This interpretation is consistent with the observation that the *P* element is missing several internal restriction endonuclease sites that are normally present in full length (2.9 kb) *P* elements (Figure 16).

A single *HindIII* site not present in pR3.8 was present in pRP4.2. When pRP4.1 was digested with *HindIII* and probed with radiolabelled *P* element DNA, only DNA fragments from one side of the enzyme recognition site hybridized (Figure 18). There

Figure 16. Restriction map of pR3.8, pRP4.2, and a wildtype P element. The thick line on the pRP4.2 map represents P element DNA. (Symbols: R, EcoRI; H, HindIII; P, PstI; S, SalI; St, SstII; X, XhoI)

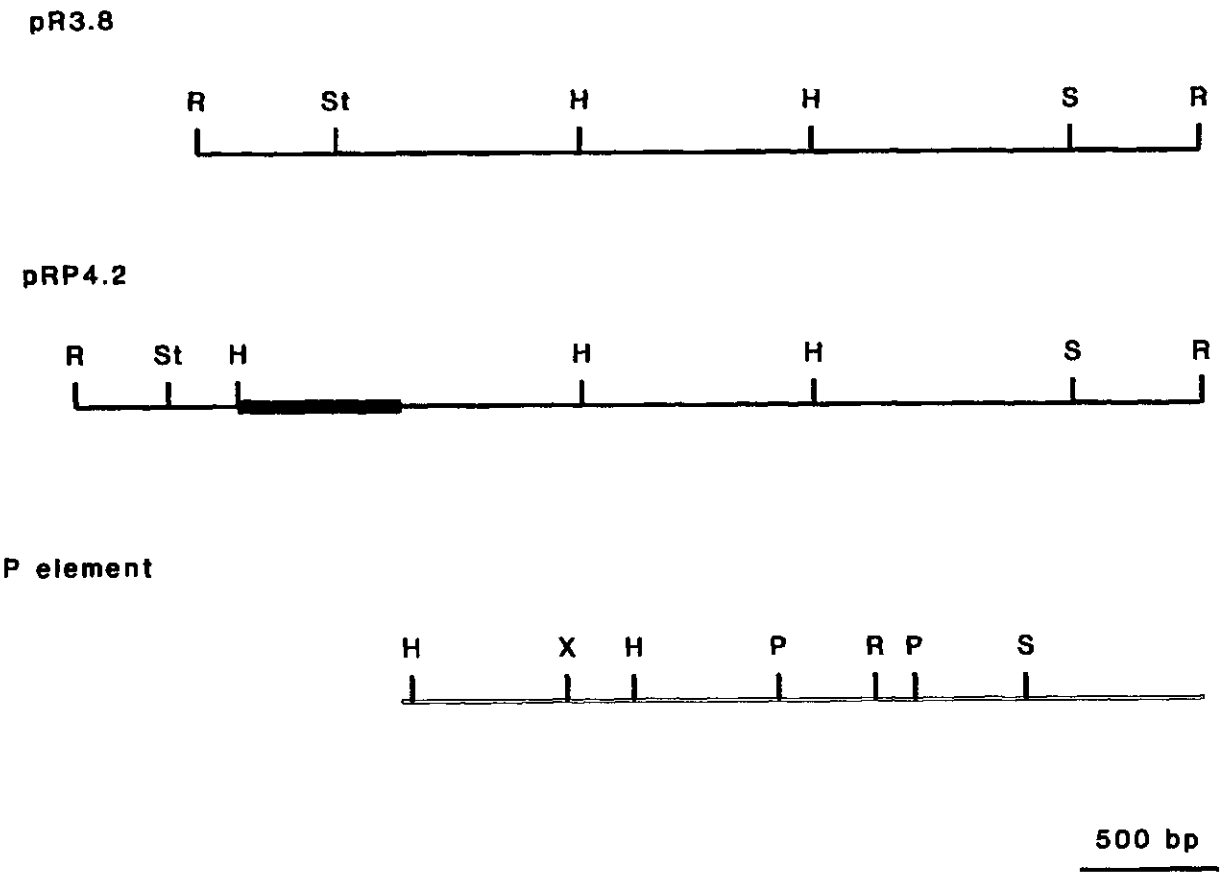


Figure 17. pR3.8 and pRP4.2 probed with a 1.5 kb *EcoRI/HindIII* fragment from pR3.8 (see Figure 16).

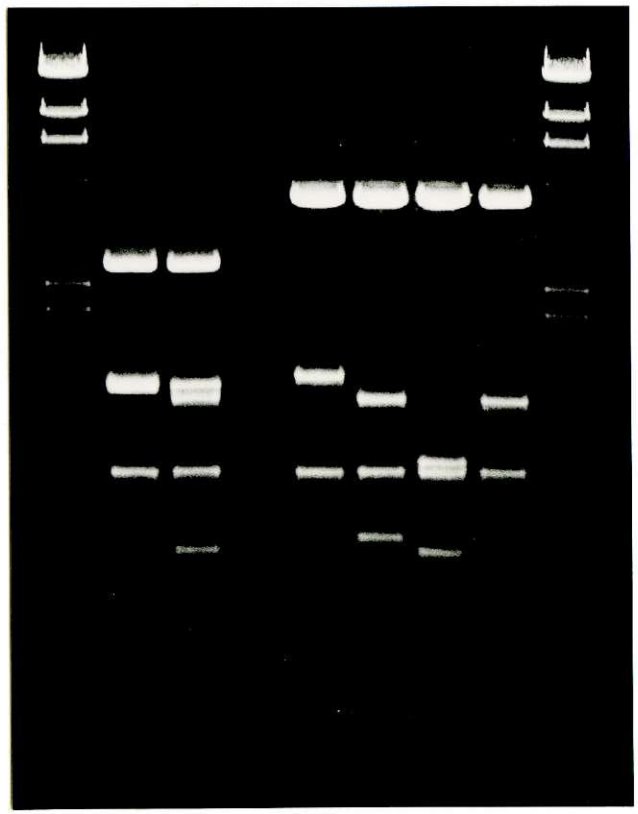
Lanes:	1. pR3.8	*EcoRI/HindIII*
	2. pRP4.2	*EcoRI/HindIII*
	3. pR3.8	*HindIII*
	4. pRP4.2	*HindIII*
	5. pR3.6	*HindIII/SstII*
	6. pRP4.2	*HindIII/SstII*
	M λ	*HindIII*


A.
M 1 2 3 4 5 6 M
1.5 kb
0.9 kb
B.
1 2 3 4 5 6

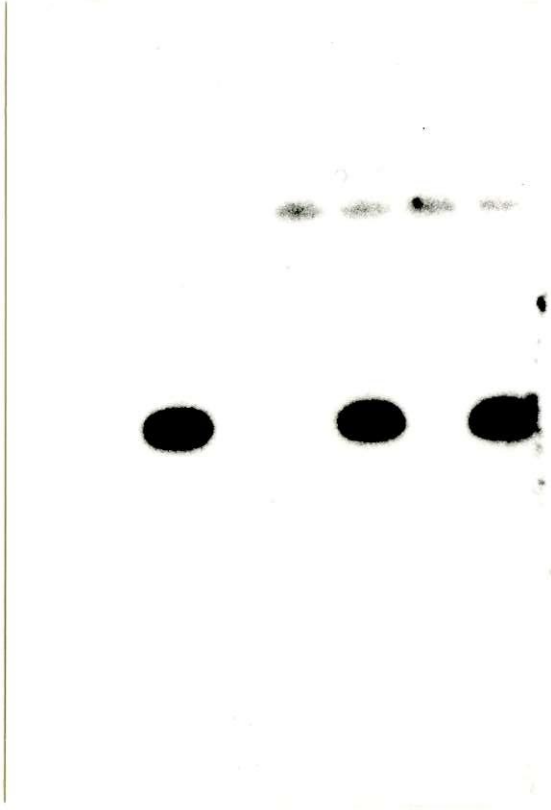
Figure 18. pR3.8 and pRP4.2 probed with a 500 bp *HindIII/EcoRI P* element fragment from the construct HBΔ89 (Simon *et al.*, 1985)

Lanes:	1. pR3.8	*EcoRI/HindIII*
	2. pRP4.2	*EcoRI/HindIII*
	3. pR3.8	*HindIII*
	4. pRP4.2	*HindIII*
	5. pR3.6	*HindIII/SstII*
	6. pRP4.2	*HindIII/SstII*
	M λ	*HindIII*

A.



B.



are two *HindIII* sites in full length *P* elements (Figure 16): one is located 38 bp from one end of the element, and the other is approximately 840 bp distant. Therefore, the hybridization data suggest that the conserved *HindIII* site in pRP4.2 is the one closest to the end of the *P* element.

The precise location and size of the *P* element insert was determined by sequencing. A 1.4 kb *HindIII* fragment that cross-hybridized to the *P* element was subcloned and then sequenced on a single DNA strand along with 300 bp of DNA from the adjacent 850 bp *HindIII/EcoRI* fragment (see Figure 16). The sequence of the *P* element, inferred from a comparison of the genomic DNA with a cloned *P* element (O'Hare and Rubin, 1983), appears in Appendix 4. The *P* element in pRP4.2 is 609 bp long and is located approximately 950 bp from the nearest wild-type *HindIII* site and 200 bp from the adjacent *SstII* site. The centre of the element is deleted with respect to a full length *P* element, but it retains the 31 bp perfect inverse terminal repeats required for transposition. The same 8 bp of genomic DNA are reiterated on both sides of the element: a characteristic sequence duplication caused by *P* element insertion (O'Hare and Rubin, 1983).

The open reading frames in intact *P* elements all extend 5′ to 3′ away from the *HindIII* site that is retained in pRP4.2. *In vivo* transcription from the *P* element in 31E is therefore predicted to be oriented towards *da*. Since the transcription initiation and termination sites are still

present in the truncated transposon, a mRNA of less than 600 bp is predicted. Because of the presence of additional elements of unknown size in the *OK#1* strain, I have not searched for this transcript.

If the lethal phenotype of *Su(var)216^{P}* is associated with the *P* element in region 31E, then revertants of *Su(var)216^{P}* should be altered at the site of transposon insertion. *Su(var)216^{P}* revertants *R28* and *R34* were tested for the loss of the *P* element *HindIII* endonuclease recognition site. Figure 19 demonstrates the absence of the *HindIII* site when *R28* genomic DNA was digested with *EcoRI/HindIII* and probed with a flanking 350 bp *SstII/EcoRI* fragment from pRP4.2. Mobility differences between the balancer and the *b pr cn* chromosome fragments are caused by the previously described *EcoRI* polymorphism. Identical results were obtained for *R34*. Subsequent cloning and sequencing experiments (data not shown) have confirmed that the *P* element is entirely absent from both revertants. Furthermore, no flanking wild-type sequences were deleted when the element excised. Taken together, the cloning and reversion studies support the hypothesis that the *P* element in pRP4.2 causes the *Su(var)216P* phenotype *in vivo*.

Transcripts Originating Near the Cloned *P* element

Cronmiller *et al.* (1988) observed three transcripts that hybridized to a 4.7 kb *SalI* fragment immediately adjacent to *da*. The *P* element resides within this fragment, so smaller

internal fragments were used to map the transcription units further and to obtain cDNA clones for each transcript.

To search for putative transcription units that might be disrupted by the *Su(var)216^{P} P* element insert, Northern blots of early embryo mRNA (0-6 h) were probed with DNA fragments adjacent to the transposon. In this study only two transcripts were detected when Northern blots were probed with fragments from this region (Figure 20). Restriction endonuclease fragments to the left of the site of *P* element insertion hybridized to an 800 nt transcript, while sequences to the right hybridized to an 1100 nt transcript. A wild-type 1.0 kb *SstII/HindIII* fragment which encompasses the site of *P* element insertion hybridized to both mRNAs. Neither transcript is altered in size in the mutant strains *Su(var)216* and *Su(var)216^{P}* (I.P. Whitehead, personal communication).

To locate the transcription units adjacent to the *P* element more precisely, cDNA clones were isolated from a third larval instar imaginal disc cDNA library constructed in *λgt10*. This method detected clones representing three transcription units within the 4.7 kb *SalI* fragment. Initially, the library was probed with two fragments adjacent to the *P* element. One was a 1.5 kb *EcoRI/HindIII* fragment, which hybridized to both the 800 nt and 1100 nt transcripts. The second was a 1.0 kb *HindIII* fragment that hybridized only to the 800 nt message. Twelve 1200 bp and eight 800 nt cDNAs were isolated. Restriction maps of the two types of cDNA appear in Figure 21.

Figure 20. Embryonic transcripts that hybridize to pR3.8. Northern blots of poly(A^+) RNA (5 µg/lane) were probed with (A) a 1.4 kb *BglII/SstII* fragment, (B) a 450 bp *SstII/HindIII* fragment, and (C) a 900 bp *HindIII* fragment (see Figure 16). Transcript sizes were inferred using high and low molecular weight RNA ladders (BRL) as size standards.

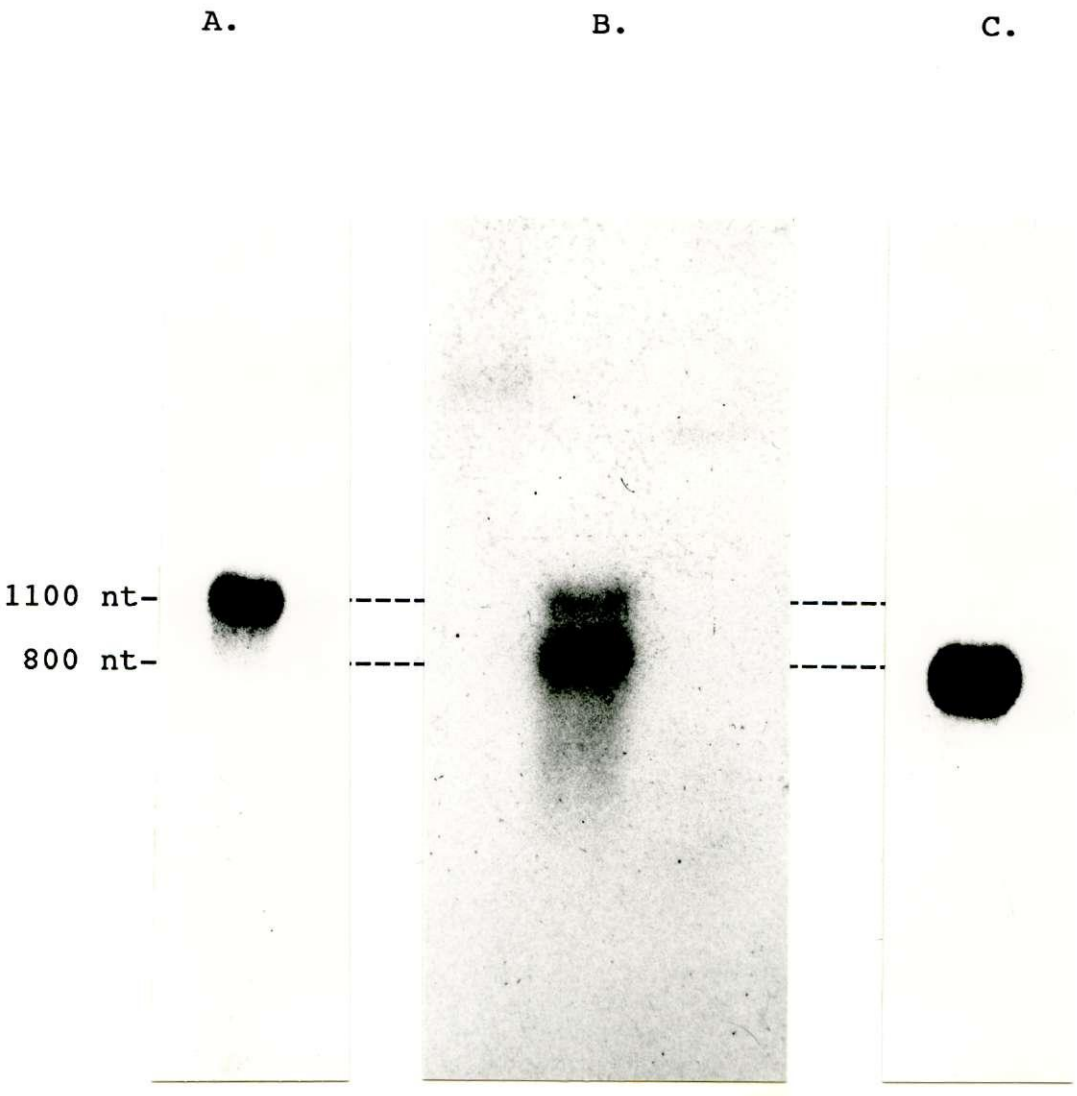
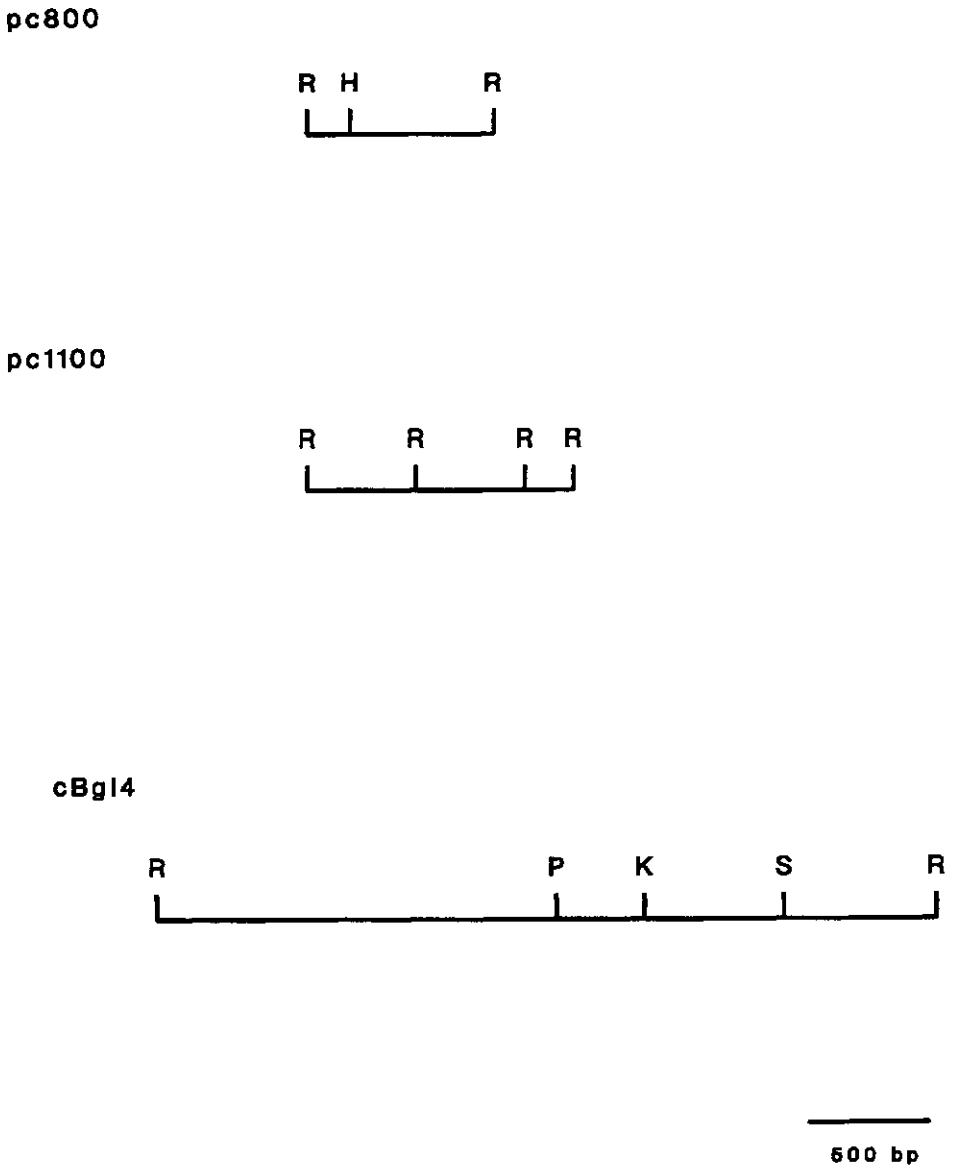


Figure 21. Restriction maps of the three cDNA clones that hybridize to pR3.8. (Symbols: R, EcoRI; H, HindIII; K, KpnI; K, KpnI; P, PstI; S, SalI)



Copy DNAs of similar size hybridized to each other, but the two classes of cDNA did not cross-hybridize. The same two classes of cDNA were also present in a library made from embryonic RNA. One representative clone from each size class was hybridized to a Northern blot of early embryo mRNA. Each clone hybridized to a transcription unit similar in size to the cDNA probe, suggesting that the 800 and 1200 bp cDNAs are nearly full length.

A representative 800 nt cDNA , pc800, hybridized only to sequences on the side of the *P* element nearest to *da* (Figure 22). The 1.1 kb species, represented by pc1100, hybridized to sequences on the opposite side of the *P* element (Figure 23). In addition to hybridizing to the 3.8 kb *EcoRI* fragment, the larger cDNA also hybridized to the adjacent 0.5 kb and 5.8 kb *EcoRI* fragments (Figure 23).

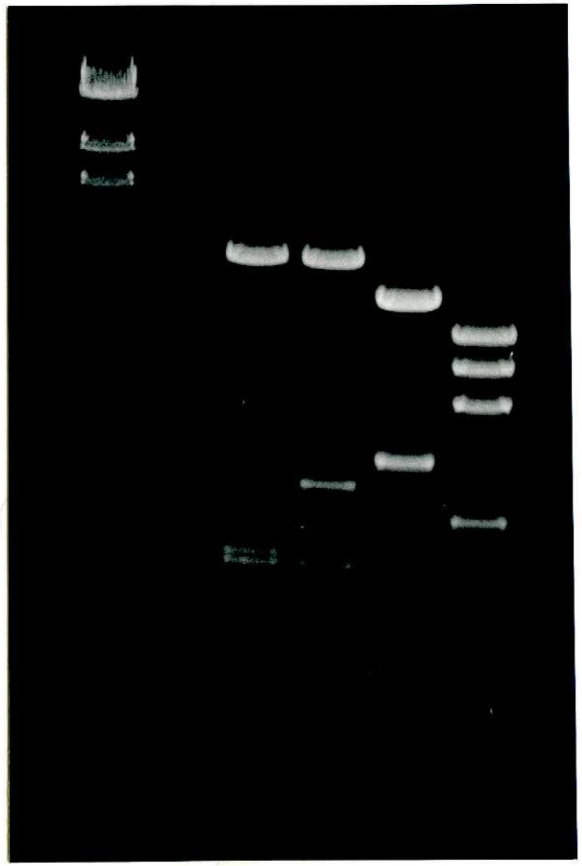
To search for the third transcript near *da* (Cronmiller *et al.*, 1988) that was not detected on Northern blots, the imaginal disc library was screened with a subclone from the 4.7 kb *SalI* fragment that failed to hybridize to either pc1100 or pc800. Using a 600 bp *SalI/BglII* fragment centromere distal to the sites of hybridization for pc1100 and pc800, a set of three homologous clones was identified. These clones hybridize to several restriction endonuclease fragments within the larger 5.8 kb *EcoRI* fragment, but they do not hybridize to pc1100. The longest cDNA recovered (cBgl4) is 3.2 kb (Figure 21). This is significantly larger than the 1900, 1100, and 800

Figure 22. pc800 hybridized to subcloned DNA from the 31E region. pHc2.7 is a 2.7 kb *HincII* subclone that spans the site of *P* element insertion (see Figure 14). pR5.8 is an *EcoRI* subclone that overlaps pHc2.7 (see Figure 14).

Lanes:	1.	pR3.8	*HindIII/SstII*
	2.	pRP4.2	*HindIII/SstII*
	3.	pHc2.7	*EcoRI/BglII*
	4.	pR5.8	*EcoRI/BglII*
	M	λ	*HindIII*

A.

M 1 2 3 4



2.0 kb-

0.56 kb-

124

B.

M 1 2 3 4

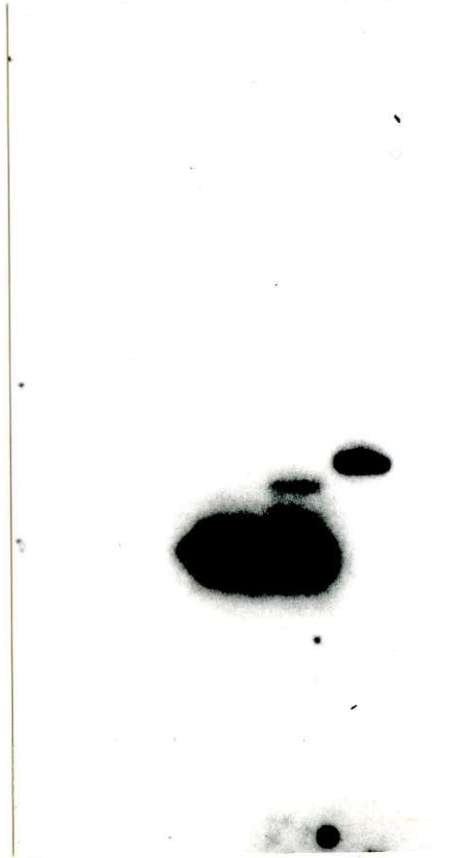


Figure 23. pc1100 hybridized to subcloned DNA from the 31E region. pHc2.7 is a 2.7 kb *HincII* subclone that spans the site of *P* element insertion (see Figure 14). pR5.8 is an *EcoRI* subclone that overlaps pHc2.7 (see Figure 14).

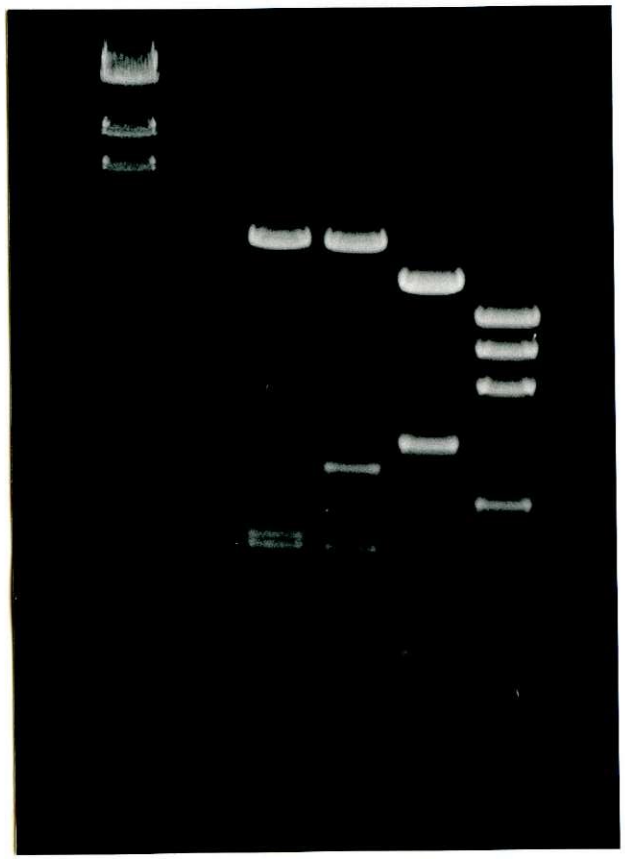
Lanes:	1. pR3.8	*HindIII/SstII*
	2. pRP4.2	*HindIII/SstII*
	3. pHc2.7	*EcoRI/BglII*
	4. pR5.8	*EcoRI/BglII*
	5. λ	*HindIII*

A.

M 1 2 3 4

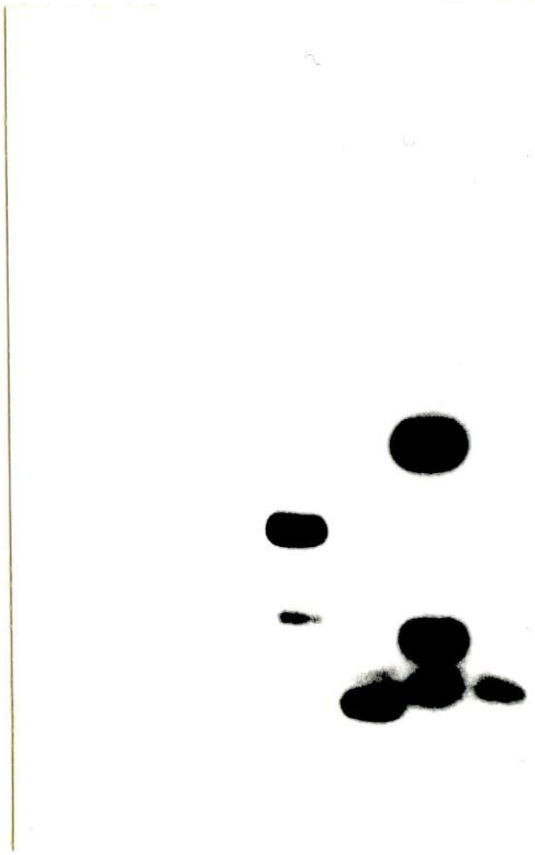
2.0 kb-

0.56 kb-



B.

M 1 2 3 4



nt transcripts observed by Cronmiller *et al.* (1988). Perhaps our cDNA represents an unprocessed message or an imaginal disc-specific transcript. In any event, this 3.2 kb cDNA helps to define the probable extent of the regulatory sequences associated with the 1100 nucleotide transcription unit. Thus, both candidates for the *Su(var)216* gene (represented by pc1100 and pc800) have been positioned between flanking transcription units.

Alignment of Genomic, cDNA, and *P* element Sequences

Since both the pc1100 and pc800 cDNA clones hybridized to sequences adjacent to the site of *P* element insertion, the pattern of hybridization alone did not resolve which transcription unit encoded *Su(var)216*^{P}. To address this question further, the DNA sequences of the pc800 and pc1100 were determined. These sequences were then compared with the DNA adjacent to the site of *P* element insertion.

The cDNA pc800 was sequenced on a single strand. Subsequent analyses demonstrated that this transcript is not encoded by *Su(var)216*^{P}. The sequence of the 800 bp cDNA is presented in Appendix 4, along with the genomic sequence of the gene it represents. The cDNA pc1100 was digested with *EcoRI*, and three fragments, 200, 450, and 450 bp long, were subcloned into pUC19 and then sequenced inward from the polylinker on both strands. The orientation and order of the *EcoRI* fragments was determined by comparing the cDNA sequence

with the sequence of cloned genomic DNA (see below) and by sequencing across the *EcoRI* recognition sites in the parent phage clone using synthetic primers (see Chapter 4).

Comparison of the cDNA sequences with the genomic DNA flanking the *P* element revealed that the genes represented by pc800 and pc1100 are divergently transcribed. The orientation of the genes was inferred from the following properties of the cDNAs relative to the genomic sequence: (1) the location of the poly(A)$^+$ tail, (2) a long open reading frame, and (3) the orientation of the splice sites, and (4) the position of the polyadenylation signal. These features are discussed in detail in a subsequent section. The site of *P* element insertion is 2 bp upstream of the 5′ end of the 1100 bp cDNA and approximately 560 bp from the 5′ end of the 800 bp cDNA (see Figure 26, Appendix 4). These data suggest that the *P* element most likely affects the expression of the larger transcription unit. The proof of this assertion is presented in Chapter 4.

The Cloned *P* element is Adjacent to *cdc2Dm*

The sequence and conceptual translation of pc1100 is presented in Figure 24. Excluding the poly(A) tail, the cDNA is 1035 nucleotides long and contains 32 nucleotides of 5′ flanking sequence, an open reading frame of 891 nucleotides, and a 3′ flanking region of 106 nucleotides. A potential polyadenylation signal (AATAAA; Proudfoot and Brownlee, 1976) is located 88 nucleotides beyond the ochre stop codon of the

Figure 24 . The nucleotide and deduced protein sequence of the product of the *cdc2Dm* locus. Amino acids that are invariant among kinases are indicated in bold-face type; highly conserved residues are in italics. Thin lines highlight conserved kinase sequences; a thick line shows the location of the PSTAIR sequence.

```
                                                GGTGGCTTGCAAAGAAATAGCTTAATAAATT

                                                                              15
Met Glu Asp Phe Glu Lys Ile Glu Lys Ile Gly Glu Gly Thr Tyr
ATG GAG GAT TTT GAG AAA ATT GAG AAG ATT GGC GAG GGC ACA TAT

                                                                              30
Gly Val Val Tyr Lys Gly Arg Asn Arg Leu Thr Gly Gln Ile Val
GGC GTG GTG TAT AAA GGT CGC AAT CGC CTG ACG GGC CAA ATT GTG

                                                                              45
Ala Met Lys Lys Ile Arg Leu Glu Ser Asp Asp Glu Gly Val Pro
GCA ATG AAG AAA ATC CGC TTG GAG TCC GAC GAC GAA GGC GTT CCA

                                                                              60
Ser Thr Ala Ile Arg Glu Ile Ser Leu Leu Lys Glu Leu Lys His
TCA ACC GCG ATC AGA GAA ATT TCG TTG CTT AAG GAG TTG AAA CAT

                                                                              75
Glu Asn Ile Val Cys Leu Glu Asp Val Leu Met Glu Glu Asn Arg
GAA AAC ATT GTC TGT TTG GAG GAT GTT TTG ATG GAG GAG AAC CGC

                                                                              90
Ile Tyr Leu Ile Phe Glu Phe Leu Ser Met Asp Leu Lys Lys Tyr
ATA TAC TTC ATC TTT GAA TTC CTA TCG ATG GAC CTC AAG AAA TAC

                                                                             105
Met Asp Ser Leu Pro Val Asp Lys His Met Glu Ser Glu Ser Val
ATG GAT TCG CTG CCA GTT GAT AAG CAC ATG GAG AGT GAA TTG GTC

                                                                             120
Arg Ser Tyr Leu Tyr Gln Ile Thr Ser Ala Ile Leu Phe Cys His
CGT AGC TAT TTG TAC CAA ATA ACT AGC GCC ATT CTT TTC TGC CAT

                                                                             135
Arg Arg Arg Val Leu His Arg Asp Leu Lys Pro Gln Asn Leu Leu
CGT CGG CGA GTA CTT CAC CGT GAT CTT AAG CCG CAG AAC TTA CTA
```

```
                                                                  150
Ile Asp Lys Ser Gly Leu Ile Lys Val Ala Asp Phe Gly Leu Gly
ATC GAC AAG AGT GGC CTC ATA AAA GTC GCC GAC TTT GGA CTT GGC

                                                                  165
Arg Ser Phe Gly Ile Pro Val Arg Ile Tyr Thr His Glu Ile Val
CGA TCC TTT GGC ATT CCG GTG CGC ATT TAT ACG CAC GAG ATT GTT

                                                                  180
Thr Leu Trp Tyr Arg Ala Pro Glu Val Leu Leu Gly Ser Pro Arg
ACC TTG TGG TAC AGA GCG CCG GAG GTG CTA CTG GGT TCA CCC CGG

                                                                  195
Tyr Ser Cys Pro Val Asp Ile Trp Ser Ile Gly Cys Ile Phe Ala
TAT TCC TGT CCC GTC GAT ATC TGG TCC ATT GGA TGC ATA TTC GCG

                                                                  210
Glu Met Ala Thr Arg Lys Pro Leu Phe Gln Gly Asp Ser Glu Ile
GAG ATG GCA ACG AGA AAG CCG CTA TTC CAG GGT GAC TCG GAA ATT

                                                                  225
Asp Gln Leu Phe Arg Met Phe Arg Ile Leu Lys Thr Pro Thr Glu
GAC CAG TTG TTT AGA ATG TTT AGA ATT CTG AAA ACA CCT ACC GAA

                                                                  240
Asp Ile Trp Pro Gly Val Thr Ser Leu Pro Asp Tyr Lys Asn Thr
GAC ATT TGG CCG GGC GTT ACT TCG CTA CCC GAC TAT AAG AAC ACG

                                                                  255
Phe Pro Cys Trp Ser Thr Asn Gln Leu Thr Asn Gln Leu Lys Asn
TTC CCC TGC TGG TCC ACG AAC CAA TTG ACC AAT CAG TTA AAG AAT

                                                                  270
Leu Asp Ala Asn Gly Ile Asp Leu Ile Gln Lys Met Leu Ile Tyr
CTC GAT GCG AAT GGT ATT GAT CTC ATA CAA AAG ATG TTA ATC TAC

                                                                  285
Asp Pro Val His Arg Ile Ser Ala Lys Asp Ile Leu Glu His Pro
GAT CCA GTT CAT CGC ATT TCC GCC AAG GAC ATT TTG GAG CAT CCC


Tyr Phe Asn Gly Phe Gln Ser Gly Leu Val Arg Asn Oc
TAT TTC AAT GGT TTT CAA TCG GGC TTA GTT CGA AAT TAACGTTCGGT

ATTCTCGTTTGACTTTAACTAAGAATTTTAAAACAAGAGATCTTGGTATCTAA

TCTAAAGCAAAATAGCCGTAAATAAAACTAAGGGTGTAAAAC[poly(A)]
```

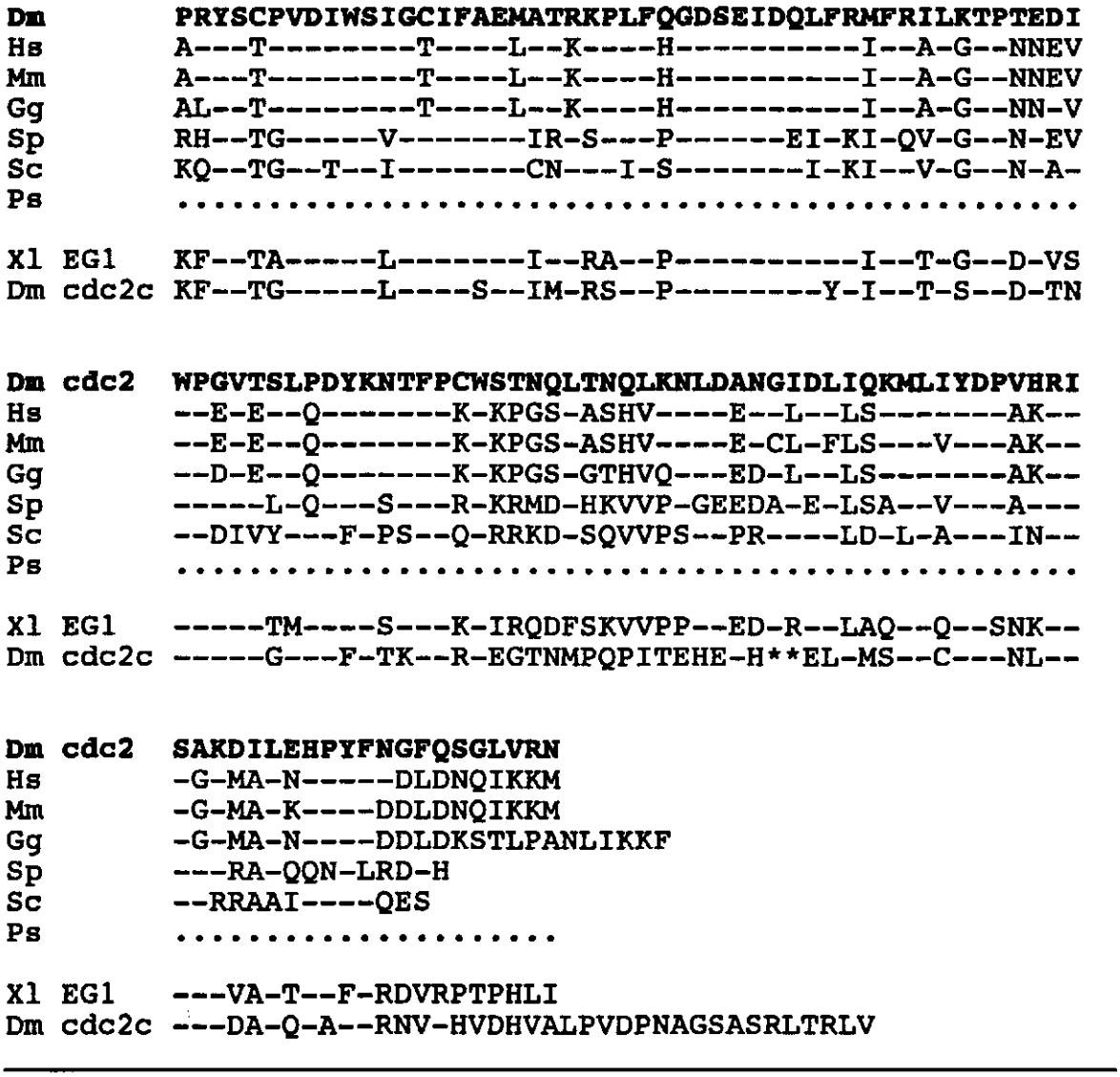
open reading frame.

The open reading frame in pc1100 codes for a 297 amino acid polypeptide (M_r 34,442). Comparison of the predicted protein sequence with sequences in the EMBL/Genbank data base suggests that pc1100 encodes a kinase. The conceptually translated protein contains the sequences Arg^{127}-Asp-Leu^{129}, Asp^{146}-Phe-Gly^{148}, Ala^{171}-Pro-Glu^{173}, and Gly^{11}-Glu-Gly-Thr-Tyr-Gly^{16}. The first three sequences are typical of protein kinase catalytic domains (Hanks *et al.*, 1988; Hunter, 1987); the last sequence matches a consensus motif (Gly-X-Gly-X-X-Gly) found in protein kinases and other nucleotide binding proteins (Hanks *et al.*, 1988). Furthermore, when the predicted protein is aligned with known kinase sequences, it contains 9 amino acid residues which are invariant amongst kinases (Gly^{16}, Lys^{33}, Glu^{51}, Asp^{128},Asn^{133}, Asp^{146}, Gly^{148}, Glu^{173}, and Arg^{275}) plus 5 other residues which are highly conserved (Hanks *et al.*, 1988). A variant of the consensus Asp-Leu-Lys-Pro-Glu-Asn, Asp^{128}-Leu-Lys-Pro-Gln-Asn^{133}, suggests that the putative kinase has serine/threonine specificity (Hanks *et al.*, 1988).

Sequence comparisons indicate that the deduced protein is a member of the CDC28-cdc2$^+$ kinase subfamily, and is most similar to the highly conserved CDC2 proteins. It contains a 16 residue PSTAIR sequence (EGVPSTAIREISLLKE; Figure 24, Figure 25) identical to those found in all homologues of the *S. pombe cdc2*$^+$ gene product (Norbury and Nurse, 1989). There is 72% sequence similarity between the *Drosophila* protein and

Figure 25. Comparison of cdc2Dm with other members of the CDC2 protein family. Symbols: - sequence identity; * gap to conserve alignment; ... incomplete sequence.

```
Dm[1]          M**E**DFEKIEKIGEGTYGVVYKGRNR**LTGQ*IVAMKKIRLESDD
Hs[2]          -**-**-YT----------------HK**T---*V----------EE
Mm[3]          -**-**-YI----------------H-**V---*----------EE
Gg[4]          -**-**-YT----------------HK**T---*V----------EE
Sp[5]          -**-**NYKRL-------------A-HK**-S-**----------DES
Sc[6]          -SG-LANYKRL--V----------ALDLRPGQ--RV--L-------E-
Ps[7]          ....................----ALDLRVTNET*-*-L------QE-

Xl[8] EG1      -**-**N-Q-V-------------A---**E--E*---L-----DTET
Dm[9] cdc2c    -TTILDN-QRA---------I---A-TN**S---*D--L------GET


Dm             EGVPSTAIREISLLKELKHE****NIVCLEDVLMEE*NRIYLIFEFLS
Hs             ----------------R-P****---S-Q----QDS*-L--------
Mm             ----------------R-P****---S-Q----QDS*-L--------
Gg             ----------------H-P****-----Q----QDA*-L--------
Sp             ----------------VND-NNRS-C-R-L-I-HA-S*KL--V----D
Sc             ----------------DD****---R-Y-IVHSDAHKL--V----D
Ps             ----------------MQHR****---R-Q--VHS-K*-L--V--Y-D

Xl EG1         ----------------N-P****---K-L--IHTEN*KL--V----N
Dm cdc2c       ---------------N---P****-V-Q-F--VISG*-NL-M---YLN


Dm             MDLKKYMDSLPVDKHM**ESELVRSYLYQITSAILFCHRRRVLHRDLK
Hs             ------L--I-PGQY-**D-S--K------LQG-V---S---------
Mm             ------L--I-PGQF-**D-ST-K---H--MQG-V---S---------
Gg             ------L-TI-SGQYL**DRSR-K------LQG-V---S---------
Sp             --------RISETGATSLDPR--QKFT--LVNGVN---S--II-----
Sc             L---R--EGI-K-QPLGAD**I-KKFMM-LCKG-AY--SH-I------
Ps             L----H---S-EFVK***DPRQ-KMF---MLCG-AY--SH---------

Xl EG1         D----F--GSNISG*ISL*A*--K---F-LLQGLA---SH---------
Dm cdc2c       -----L--K**KKDVF**T-Q-IK--MH--LD-VA---TN-I-------


Dm             PQNLLIDKSGL*IKVADFGLGRSFGIPVRIYTHEIVTLWYRAPEVLLGS
Hs             -------DK-T*--L-----A-A----I-V----V------S-------
Mm             -------DK-T*--L-----A-A----I-V----V------S-------
Gg             -------DK-V*--L-----A-A------V----V------S-------
Sp             --------E-N*L-L-----A----V-L-N-------------------
Sc             ------N-D-N*L-LG----A-A--V-L-A-----------------G
Ps             -------RRTNCV-L-----A-A------TF---V..............

Xl EG1         ------NSDGA*--L-----A-A--VT--TF---V---------I---C
Dm cdc2c       -----V-TA-K*--L-----A-A-NV-M-A----V---------I---T
```



---

[1]*Drosophila melanogaster*, [2]*Homo sapiens* (Lee and Nurse, 1987), [3]*Mus musculus*, [4] *Gallus gallus* (Krek and Nigg, 1989), [5]*Schizosaccharomyces pombe* (Hindley and Phear, 1984), [6]*Saccharomyces cerevisiae* (Lorincz and Reed, 1986), and [7]*Pisum sativa* (Feiler and Jacobs, 1990) CDC2 homologues.
[8]*Xenopus laevis* CDC2-related protein
[9]*Drosophila melanogaster* CDC2-related protein

the human *CDC2* gene product, approximately 70% similarity with the mouse and chicken CDC2 proteins, and 68% similarity with the *Schizosaccharomyces pombe cdc2* gene product (see Figure 25). The most closely related protein in *Drosophila* is the *cdc2c* gene product (Lehner and O'Farrell, 1990) which shares 58% sequence identity with the deduced protein product. These similarities suggest that the putative kinase gene is the *Drosophila* homologue of the fission yeast *cdc2* gene. Since this work was completed, Lehner and O'Farrell (1990b) and Jimenez *et al.* (1990) have independently confirmed this speculation by rescuing the phenotype of a yeast *cdc2* mutant with a cloned *cdc2Dm* gene.

cdc2Dm Transcription

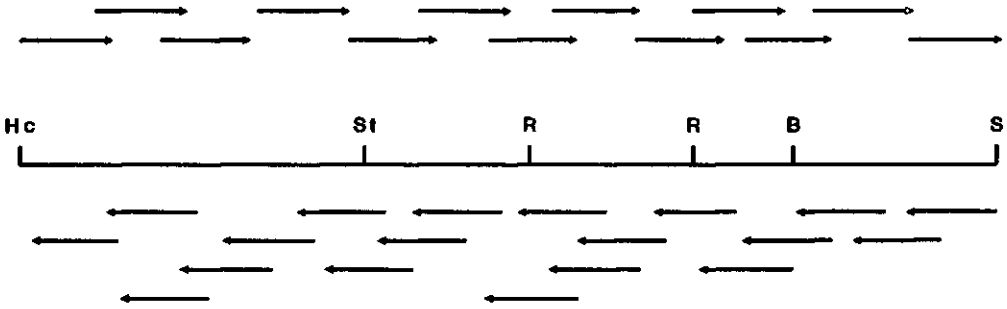
Northern blots of mRNA from different developmental stages were probed with the *cdc2Dm* cDNA (data not shown). An 1100 nt transcript is present early in development (0-9 hours), but is rare in larvae, pupae and adult males. Some transcription is evident in adult females, presumably because of the maternal contribution of *cdc2Dm* transcript in developing eggs.

cdc2Dm Gene Structure

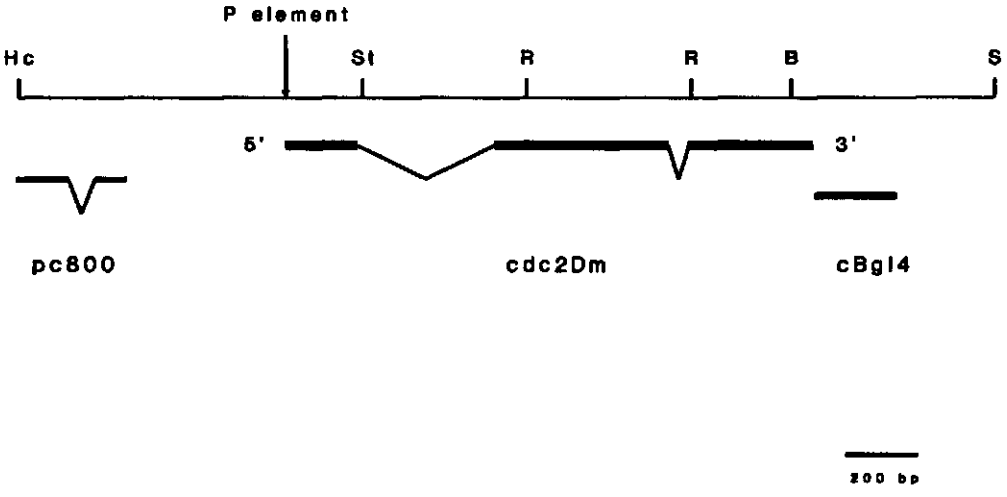
To deduce the structure of the *cdc2Dm* transcription unit, a 2.9 kb *HincII/SalI* fragment of genomic DNA was sequenced (Figure 27). The fragment contains sequences homologous to

Figure 26. The sequencing strategy for cdc2Dm. A. Extent and direction of sequencing the directed deletions is shown by arrows. B. The locations of cdc2Dm (pc1100) and flanking transcription units. Thick lines below the restriction map represent exons. Thinner lines represent introns. (Symbols: B, BglII; R, EcoRI; Hc, HincII; St, SstII)

A.



B.



cDNAs that hybridize to opposite sides of the *cdc2Dm* transcription unit; therefore, the entire regulatory region of *cdc2Dm* has probably been sequenced. The complete sequence appears in Appendix 4.

A comparison of the cDNA and genomic sequences indicates that the *cdc2Dm* gene has two introns (see Figure 26 and Appendix 4). Intron 1 is 385 bp long, and intron 2 is 57 bp long. Both introns have typical GT/AG splice junctions. Intron 1 interrupts the codon that specifies Arg^{50} within the PSTAIR sequence of the *cdc2Dm* protein. Intron 2 interrupts the codon that specifies Arg^{218}.

Many regulatory elements that influence transcription by polymerase II are located just 5′ to the coding region. If cBgl4, the clone that hybridizes immediately 3′ to *cdc2Dm*, represents a *bona fide* transcription unit, it seems unlikely that *cdc2Dm* possesses 3′ regulatory sequences because the polyadenylation site of cBgl4 is only 14 bp from the 3′ end of *cdc2Dm* (Figure 26 and Appendix 4). Since the translation initiation sites of *cdc2Dm* and the gene represented by pc800 are separated by less than 550 bp (Figure 26 and Appendix 4), this region was searched for regulatory motifs. A TATA box-like sequence (AATAAA) is located 115 bp 5′ to the translational start site of the *cdc2Dm* gene (Figure 27). Twenty-two basepairs downstream from this TATA-like sequence is a sequence (ATCGTTT) similar to the consensus insect cap site ATCAG/TTC/T (Hultmark *et al.*, 1986). However, at position

Figure 27. Potential upstream regulatory sequences of *cdc2Dm*. Symbols: * TATA-like sequences; ++ a cap site sequence; =, - reiterated motifs.

```
GAAACAACAA AAATGAAGTA AAACAGTTGC GGTATTCCAA TTACATTTTT

TAAATTAATT TCTTTAGTAC CGTACTACTG GTACTCACCT TCAAAAGATA

TAAAATAGAA ATTAATTGCA CCAAAAACTC ATAAGTTAAT TAATAGTATA

TTAGCAGAAA CGTTTGTCTC CGAACTCAAA CAAAGTGATG TCTTAATTAA

          **********
TTGAAATCAC TATAAAAAAA AGCGTGGAAT CAATAAGTTG CCTGAATATT

                                 ***********          +++++++
GAGTTTCATT CCCACATTCC AAATGAATAA ATGTAGCTAG CTTAGCATCG
+++++
TTTAAACTGT CTGGTAATAC TAGAGCATAT ACGTCAAAAA CGCGCTAATT

TAAAAGTCGG TGGCTTGCAA AGAAATAGCT TAATAAATT ATG GAG GAT
       ... .......... .......... ......... Met Glu Asp

TTT GAG AAA ATT GAG AAG ATT GGC GAG GGC ACA TAT GGC
Phe Glu Lys Ile Glu Lys Ile Gly Glu Gly Thr Tyr Gly
```

175 upstream of the translation start site there exists a sequence (TATAAA) which is a better match with the canonical TATA-box. Therefore, it will be necessary to determine the initiation site for transcription in order to infer the location of the true TATA sequence.

Three re-iterated sequence motifs occur in the region immediately 5′ to *cdc2Dm*. The sequence TGAATA is repeated twice (at positions -117 and -148; Figure 27), and so is the sequence CATTCC (positions -126 and -134). The sequence AATTAATT/A occurs four times in the region between the translation initiation sites of the two genes. The significance of these short motifs, if any, is not currently known.

Sequencing the 5′ End of a *cdc2*-like Gene from *D. virilis*

A detailed mapping of upstream transcriptional control elements associated with *cdc2Dm* was beyond the scope of this work. Nonetheless, the central role of this gene in controlling cell cycle and initiation of chromosome condensation, as well as the relatively small region between *cdc2Dm* and its 5′ neighbour (less than 600 bp), makes such studies intriguing. To obtain preliminary data for future studies on the control of *cdc2Dm* expression, a gene with sequence similarity to *cdc2Dm* was cloned from *Drosophila virilis*. The sequence 5′ to this gene was then compared to that of *D. melanogaster* to identify conserved regulatory

motifs.

A *D. virilis* genomic DNA library constructed in *EMBL3* was screened with a *cdc2Dm* cDNA using conditions that permit complementary DNA strands to anneal even if their nucleotide sequences differ by as much as 28%. Ten recombinant bacteriophage were recovered, all of which share essentially identical restriction endonuclease digestion fragments. A single fragment which cross-hybridized to the first 180 bp of pc1100 was subcloned. The restriction map of the cloned fragment is completely different from that of the region surrounding *cdc2Dm* (Figure 28). The clone was sequenced in one direction only to identify the 5′ end of the *D. virilis* gene (see Figure 27). The sequence encoding the first 217 amino acids of the *cdc2*-like gene was determined along with 300 bp 5′ to the initiation codon (Figure 29, Figure 30). To ensure the accuracy of the 5′ sequence, this region was also sequenced on the opposite DNA strand using synthesized DNA primers.

Several lines of evidence suggest that the cloned and partially sequenced gene is the *Drosophila virilis cdc2* gene. First, the *D. virilis* nucleotide sequence is 75.5% identical to that of the *cdc2Dm* coding region (Figure 29). Second, the inferred splice sites for the first and second introns are located at the same points in the DNA sequence. Third, the predicted amino acid sequence of the *D. virilis* protein differs from the *cdc2Dm* sequence by only 9 residues (Figure

Figure 28. Restriction map of a subclone of D. virilis DNA with homology to the first and second exons of cdc2Dm. (Symbols: B, BglII; R, EcoRI; K, KpnI; S, SalI; St, SstII)

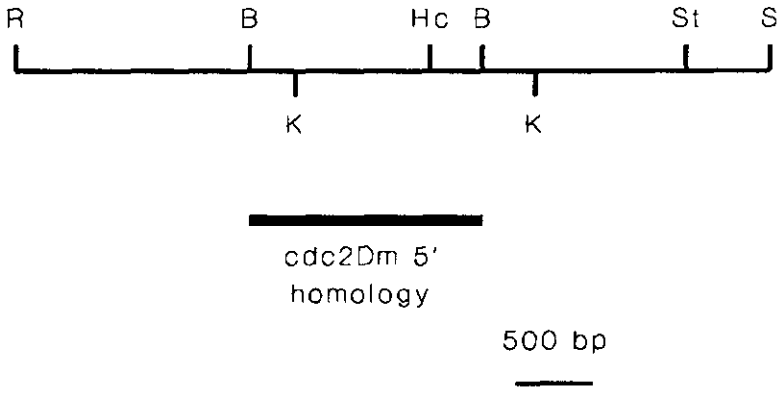


Figure 29. Sequence comparison of *cdc2Dm* (above) and a similar gene in *D. virilis* (below).

```
                                                                  15
Met Glu Asp Phe Glu Lys Ile Glu Lys Ile Gly Glu Gly Thr Tyr
ATG GAG GAT TTT GAG AAA ATT GAG AAG ATT GGC GAG GGC ACA TAT
--A --C --C --A --- --- --A --- --- --A --A --T --T --- ---

                                                                  30
Gly Val Val Tyr Lys Gly Arg Asn Arg Leu Thr Gly Gln Ile Val
GGC GTG GTG TAT AAG GGT CGC AAC CGC CTG ACG GGC CAA ATT GTG
--T --- --- --- --C --- --- --T T-A --T --T --- --- --C --C

                                                                  45
Ala Met Lys Lys Ile Arg Leu Glu Ser Asp Asp Glu Gly Val Pro
GCA ATG AAG AAA ATC CGC TTG GAG TCC GAC GAC GAA GGC GTT CCA
--A --- --T --A --- --- --T --T --- --G --T --G --- --T --T

                                                                  60
Ser Thr Ala Ile Arg Glu Ile Ser Leu Leu Lys Glu Leu Lys His
TCA ACC GCG ATC AGA GAA ATT TCG TTG CTT AAG GAG TTG AAA CAT
--T --T --- --A --- --- --- --A --A --A --A --A --A --- ---

                                                                  75
Glu Asn Ile Val Cys Leu Glu Asp Val Leu Met Glu Glu Asn Arg
GAA AAC ATT GTC TGT TTG GAG GAT GTT TTG ATG GAG GAG AAC CGC
CC- --T --- --A --C --A --A --- --G --A --- --- --A --- --T
Pro

                                                                  90
Ile Tyr Leu Ile Phe Glu Phe Leu Ser Met Asp Leu Lys Lys Tyr
ATA TAC TTG ATC TTT GAA TTC CTA TCG ATG GAC CTC AAG AAA TAC
--- --- C-A --A --- --- --T T-- --T --- --- --T --A --G --T

                                                                 105
Met Asp Ser Leu Pro Val Asp Lys His Met Glu Ser Glu Leu Val
ATG GAT TCG CTG CCA GTT GAT AAG CAC ATG GAG AGT GAA TTG GTC
--- --- --C T-A --- CCG --A --- --T C-- --T --C C-- C-A --T
                    Pro Glu         Leu Asp     Gln
```

```
                                                                    120
Arg Ser Tyr Leu Tyr Gln Ile Thr Ser Ala Ile Leu Phe Cys His
CGT AGC TAT TTG TAC CAA ATA ACT AGC GCC ATT CTT TTC TGC CAT
A-G --- --C --- --T --- --- --A -AT --T --- T-A --T --T ---
                                 Asn

                                                                    135
Arg Arg Arg Val Leu His Arg Asp Leu Lys Pro Gln Asn Leu Leu
CGT CGG CGA GTA CTT CAC CGT GAT CTT AAG CCG CAG AAC TTA CTA
--A --C --G --C --- --- --- --- T-A --A --- --A --T C-T --T

                                                                    150
Ile Asp Lys Ser Gly Leu Ile Lys Val Ala Asp Phe Gly Leu Gly
ATC GAC AAG AGT GGC CTC ATA AAA GTC GCC GAC TTT GGA CTT GGC
--T --T --A -A- --T A-T --T --- --T --A --T --- --- --C ---
            Asn     Ile

                                                                    165
Arg Ser Phe Gly Ile Pro Val Arg Ile Tyr Thr His Glu Ile Val
CGA TCC TTT GGC ATT CCG GTG CGC ATT TAT ACG CAC GAG ATT GTT
A-G --T --G --T --A --A --A --- --- --- --T --T --A --A ---

                                                                    180
Thr Leu Trp Tyr Arg Ala Pro Glu Val Leu Leu Gly Ser Pro Arg
ACC TTG TGG TAC AGA GCG CCG GAG GTG CTA CTG GGT TCA CCC CGG
--T C-- --- --T C-G --T --A --A --A T-- T-- --C --T --A A-A

                                                                    195
Tyr Ser Cys Pro Val Asp Ile Trp Ser Ile Gly Cys Ile Phe Ala
TAT TCC TGT CCC GTC GAT ATC TGG TCC ATT GGA TGC ATA TTC GCG
--C --T --C --A --T --C --- --- --- --- --G --- --T --T --A

                                                                    210
Glu Met Ala Thr Arg Lys Pro Leu Phe Gln Gly Asp Ser Glu Ile
GAG ATG GCA ACG AGA AAG CCG CTA TTC CAG GGT GAC TCG GAA ATT
--A --- --T --A --G --A --A T-- --T --A --- --T --- --- ---

Asp Gln Leu Phe Arg Met Phe Ar
GAC CAG TTG TTT AGA ATG TTT AG
--T --A --A --C C-- --- --- --
```

Figure 30. DNA sequence of the 5′ flanking region of a *cdc2*-like gene from *Drosophila virilis*. Underlined sequences indicate the location of DNA primers.

```
                                                                    50
GATTTAAAAA  TGCATACATA  CATATATTAA  GAAAACGCAT  CAGCCGTGAC

                                                                   100
GTTGTGGCCT  TAGCGAGATG  CGGCTTCATA  CTGAACGTTC  GGCTTTTGAG

                                                                   150
GCCGGCGTTC  GGCTGCCACC  CTCAGCAGTC  TTACTCTGTC  TGGGCACAGT

                                                                   200
AAAATGGTCG  CAGGTACCAT  TGGGGTCGCC  TCGTTTCGAT  TTTTCTTGTT

                                                                   250
TGCATTAAAA  TTCCCATTTG  GTATGTCTAT  ACATTGTATG  GTTAGTGGTA

                                                                   300
ATAGTGTATG  TTTATTTTCA  GTGTTTCAAT  TATCGGTGCT  TATAAATATA

                                                                   350
CGATTGAAAT  TTTCATAAAT  ATATTTAATA  AGCTACCGTG  GTATTCTAAA

                                                                   400
AACTGTGGCA  ACCTTAAAAA  AAAAACCTGC  ATTTTTTCGG  AAACTACCCG

                                                                   458
GGAGTAAAATGGAAGACTTCGAAAAAATTGAAAAGATTGGAGAAGGTACTTATGGCGT
       MetGluAspPheGluLysIleGluLysIleGlyGluGlyThrTyrGly
```

29). Each substitution, with the exception of Glu^{97}, has an identical amino acid at the same position in at least one other member of the *CDC2* protein family. The *cdc2Dm* Asp^{97} and *D. virilis* Glu^{97} residues are similar in charge. Finally, cBgl4, the cDNA that hybridizes to DNA adjacent to *cdc2Dm* also hybridizes to a DNA fragment adjacent to the *D. virilis* gene (data not shown).

Unfortunately, a comparison of the 5' region of the *D. virilis* and *D. melanogaster* clones reveals no strong sequence similarities. In particular the reiterated sequence motifs adjacent to the *cdc2Dm* gene are not found near the *D. virilis* sequence (Figure 30).

DISCUSSION

This chapter described the cloning and sequencing of a gene with strong sequence similarity to the *cell-division-cycle-2* (*cdc2*) gene of humans and *S. pombe*. Recently, two research groups have reported the isolation of cDNA clones from *Drosophila melanogaster* which, when placed in an expression vector, are able to rescue cell cycle progression in yeast defective for the *cdc2* product (Jimenez *et al.*, 1990; Lehner and O'Farrell, 1990). These cDNA clones hybridize to 31E on *Drosophila* polytene chromosomes and are identical in sequence to the gene adjacent to the *Su(var)216*-associated *P* element. The cDNA sequence reported by Jimenez *et al.* (1990) extends 23 bp further 5' than our cDNA. This suggests that the *P* element in *Su(var)216*^{P} is within the transcribed portion of *cdc2Dm*, although this was not apparent on Northern blots of adult mRNA (I.P. Whitehead, personal communication).

We are currently re-investigating the expression of *Su(var)216*^{P} because the mutant is not completely lethal as a homozygote (see Chapter 3). This suggests that some *Su(var)216*^{P} mRNA may be transcribed and this mRNA could be used as a molecular marker to test for the perdurance of the *cdc2Dm* mRNA. In *Drosophila*, some maternally inherited messenger RNAs are degraded at the mid-blastula transition when transcription is initiated from the zygotic genome. Jimenez *et al.* (1990) have suggested that *cdc2Dm* mRNA may be

excluded from the developing zygote after the mid-blastula transition. If a *P* element can be detected in a mutant *cdc2Dm* transcript, then it may be possible to ask whether maternally derived *cdc2Dm* mRNA localizes to post-mid-blastula transition embryos that do not inherit the *Su(var)216*^{P} allele from either parent.

The *cdc2Dm* gene is simple in its organization. It has two introns, unlike the *S. pombe* gene which has four introns (Hindley and Phear, 1984), and the *S. cerevisiae* gene which has none (Lorincz and Reed, 1986). The sites of the introns within the coding sequence of *cdc2Dm*, are entirely different from those in *S. pombe*.

Homologues of *cdc2* have been found in a variety of organisms, and it seems likely that the product of the *cdc2* gene is required for normal mitotic progression in all eukaryotes (see Nurse, 1990). The highest level of *cdc2Dm* mRNA is detected in the early embryo when cell division is most frequent. During later stages far less mRNA is detectable. Fine-grained analyses indicate that *cdc2Dm* mRNA is expressed in the dividing nervous system of larvae, but not in other mitotically inactive tissues (Jimenez *et al.*, 1990). In adults, *cdc2Dm* mRNA is rare in males but somewhat more abundant in females, probably because they sequester *cdc2Dm* mRNA in their eggs (Jimenez *et al.*, 1990). The presence of *cdc2Dm* cDNA clones in an imaginal disc cDNA library suggests, not surprisingly, that *cdc2Dm* is also expressed in this

proliferating tissue.

Observation of *cdc2Dm* mRNA in whole-mount embryos suggests that *cdc2Dm* may be transcriptionally regulated at division 14 (Jimenez *et al.*, 1990). At this stage, the early synchronous cell divisions of the embryo give way to a spatially and temporally restricted sequence of cell divisions (Foe, 1989). At a gross level, *cdc2Dm* transcription increases in the regions that are about to divide. Several genes that display spatially restricted patterns of transcription have complex upstream regulatory sequences that permit the binding of diverse transcription factors. In at least one case, *engrailed*, these sequence elements have been conserved through evolution (Kassis *et al.*, 1989). As a prelude to promoter deletion studies we looked for 5′ sequence conservation in a *Drosophila virilis* homologue of *cdc2Dm*.

When *cdc2Dm* and a similar sequence from *Drosophila virilis* were aligned to test for sequence similarity 5′ to the translational start site of the genes, no strong similarity was found. *Drosophila melanogaster*, and *Drosophila virilis* diverged approximately 60 million years ago (Beverley and Wilson, 1984), and this evolutionary distance should be sufficient to distinguish between functional and nonfunctional sequences (estimated unconstrained divergence time, 1% per million years: Hayashida and Miyata, 1983; Perler *et al.*, 1980). This suggests that the three re-iterated sequences 5′ to the translational start site of *cdc2Dm* might not be

important for transcriptional regulation. However, this interpretation is subject to qualification. It is possible that the cloned gene from *D. virilis* is inactive, or is a gene with slightly different functions from *cdc2Dm*. If this were the case, then the regulatory regions associated with the *D. virilis* and *D. melanogaster* genes might be quite different. Further experiments are necessary to determine the significance of the re-iterated sequences adjacent to *cdc2Dm*.

In yeast, the *cdc2* gene product functions in the G_1 to S and G_2 to M phase transitions of the cell cycle. Thus, it is perhaps surprising that a *P* element in *cdc2Dm* is associated with suppression of PEV. However, $p34^{cdc2}$ kinase activity has been implicated in the control of chromatin condensation during mitosis. In the macroplasmodium of *Physarum polycephalum*, histone H1 phosphorylation is strongly correlated with chromatin condensation (Bradbury *et al.*, 1973, 1974). Phosphorylation of histones is probably brought about by $p34^{cdc2}$, since $p34^{cdc2}$ phosphorylates the same sites on histone H1 *in vitro* as are phosphorylated *in vivo* (Langan *et al.*, 1989). Furthermore, in *Drosophila melanogaster*, alterations in the availability of histones for DNA binding, either by altering the amount of template or altering histone modifications, appears to suppress PEV (Moore *et al.*, 1979; Mottus *et al.*, 1980).

The *cdc2* gene product may also act as a regulator of gene expression via its effects on chromatin. In the macronucleus

of *Tetrahymena*, a growth-associated histone H1 kinase activity has been observed with strong enzymatic and physical properties to *cdc2* H1 kinase in other eukaryotes. The macronucleus undergoes division without mitosis; therefore, this activity must serve some function other than simply being part of a mitosis-associated chromatin condensation pathway. Since phosphorylation of histones in *Tetrahymena* can be induced by physiological stresses, p34 may play a role.

Consistent with either a gross or more subtle role for p34 kinase in chromatin condensation is the observation that the recognition site for phosphorylation by *cdc2* kinase is found most frequently in DNA binding proteins (Suzuki, 1989). The structure of phosphorylation sites on known $p34^{cdc2}$ substrates is predicted to form a beta-turn that could bind DNA in the minor groove. Phosphorylation of these sites would disrupt the structure, thereby preventing DNA binding. Such a mechanism could relieve constraints on chromatin at the time of mitosis, thereby permitting some other mechanism to condense the chromatin (Moreno and Nurse, 1990), perhaps at the level of the nuclear scaffold. The same loosening of protein-DNA interactions might facilitate the passage of polymerase during S phase, or it could be exploited by the cell to regulate gene expression transiently in restricted chromatin domains (Roth *et al.*, 1991; Roth *et al.*, 1988). Thus, a Su(var)-like phenotype might result if the specificity or regulation of the *cdc2Dm* encoded kinase were subtly

altered.

These possibilities made the detailed investigation of *cdc2Dm* particularly desirable. The analysis of *cdc2* mutants both with respect to the cell cycle and the suppression of position-effect variegation are reported in the following chapter.

CHAPTER 4

INTRODUCTION

Drosophila melanogaster is one of many eukaryotes in which a homologue of the fission yeast *cdc2*$^{+}$ gene has been identified. In yeast p34^{cdc2} kinase is required for the initiation of DNA synthesis (G_1-S) and for the proper execution of mitosis (G_2-M). The two temporal requirements are genetically separable (Booher and Beach, 1987), and at each point p34 differs in terms of its activity (Moreno *et al.*, 1989), its phosphorylation state (Gould and Nurse, 1989), and its association with other proteins (Booher *et al.*, 1989).

Two lines of evidence suggest that homologues of *cdc2*$^{+}$ have similar functions in cell cycle regulation throughout the animal kingdom. First, the human *cdc2*$^{+}$ gene can substitute for the fission yeast gene in both its G_1-S and G_2-M roles (Lee and Nurse, 1987). Although the converse experiment has not been performed, p34 stimulates DNA replication in extracts from mammalian G_1 cells (D'Urso *et al.*, 1990) and *Xenopus* eggs (Blow and Nurse, 1990). Second, the kinase encoded by the *cdc2* homolog of *Xenopus laevis* is a component of M-phase promoting factor (MPF) (Gautier *et al.*, 1988; Dunphy *et al.*, 1988) and a component of a starfish M-phase-specific histone H1 kinase (Arion *et al.*, 1988; Labbé *et al.*, 1988). Thus *cdc2* gene activity is associated with the control of cell cycle events in a wide variety of taxa.

Entry into mitosis is believed to be brought about by the

stimulation of $p34^{cdc2}$ kinase activity, which rises to a peak at M-phase (reviewed in Nurse, 1990). $p34^{cdc2}$ phosphorylates other proteins to initiate such major M-phase events as chromosome condensation, cytoskeletal reorganization, nuclear envelope breakdown, and changes in cell shape. In *S. pombe*, the activation of $p34^{cdc2}$ requires a cyclin-like protein, $p56^{cdc13}$, and the *cdc25*$^{+}$ gene product. This activation is inhibited by the *wee1*$^{+}$ gene product, which is a putative serine/threonine kinase. The balance of these independent activation/inhibition pathways modulates p34 function, resulting in the advancement or delay of mitosis.

Genes with sequence similarity to *cdc13*, *cdc25*, and *wee1* have been found in several eukaryotes (Hadwiger *et al.*, 1989; Surana *et al.*, 1991; Igarashi *et al.*, 1991; Sadhu *et al.*, 1990; Lehner and O'Farrell, 1989; Edgar and O'Farrell, 1989) suggesting that they too are part of a universal regulatory mechanism.

In fission yeast, the onset of mitosis can also be altered by direct mutation of *cdc2*. Loss of function mutations delay or prevent mitosis, while dominant altered-function mutations advance the onset of mitosis.

In *Drosophila melanogaster*, genetic studies on the regulation of entry into S phase and mitosis are only beginning (see Glover, 1991), but several regulatory genes have been isolated. *String*, the homolog of the yeast *cdc25* gene, has been cloned. During early embryogenesis,

overexpression of *string*$^{+}$ advances the onset of mitosis, suggesting that the *string* product is a mitotic activator (Edgar and O'Farrell, 1989). Two genes with sequence similarity to the *cdc13* gene product have also been cloned (Lehner and O'Farrell, 1989). The product of one such gene (cyclin A) is essential for cell cycle progression in the developing nervous system (Lehner and O'Farrell, 1990a).

In addition to the work described in the previous chapter, two other groups have cloned *cdc2Dm*. The sequence of a cDNA and the wild-type distribution of *cdc2Dm* mRNA has been determined (Jimenez *et al.*, 1990; Lehner and O'Farrell, 1990), but no mutants have yet been reported. This chapter describes the preliminary genetic and molecular analysis of ten *cdc2Dm* mutations, with special reference to *Su(var)216*.

MATERIALS AND METHODS

Cuticle Preparations

Embryos were collected overnight on a petri dish spread with yeast paste (yeast mixed with 5% (w/v) acetic acid and 5% ethanol). The dish was maintained at 25^0 for 24-48 hours to allow wild-type embryos to hatch. Mutant embryos that failed to hatch were collected for examination. The surface of the petri plate was washed with water, and the embryos were collected with a sieve made of nitex netting. The embryos were thoroughly washed; then they were placed in 50% bleach for 3 min. The bleach was removed by washing several times with a solution of 0.8% NaCl, 0.1% Triton X-100. The embryos were drained by placing absorbent paper under the sieve; then they were transferred to a tube containing 700 µl of PEMFA (100mM Pipes (pH 6.5), 2 mM EGTA, 5 mM $MgSO_4$, 1% formaldehyde) and 700 µl of heptane. The tube was rotated for 30 min at room temperature. The lower phase was removed, 700 µl of methanol was added, and the mixture was vortexed for 15 s. The embryos were allowed to sink. The overlying liquid was removed, and the embryos were washed 3 times with 700 µl of methanol. The methanol was replaced with acetic acid: glycerol (4:1), and the tube was incubated at 60^0 for at least 1 hour. The solution was diluted 1:1 with Hoyer's mountant, transferred to a microscope slide, overlaid with a coverslip, and left on a warming tray for 24-48 hours before viewing under a microscope.

Primers

For sequencing, eight primers were designed that spanned the *cdc2* gene. The primer sequences were: BB1 ACGTTTGTCCTCCGAACTC; BB2, GTCTGGTAATACTAGAGC; BB3, TTCCATCAACCGCGATCA; BB4, ACAATCCTATATGCGTAC; BB5, ATGGAGGAGAACCGCATA; BB6, GTCGCCGACTTTGGACT; BB7,TGTTTAGGTAACCACAGG; BB8, CGATCCAGTTCATCGCAT. The first primer was identical to a genomic DNA sequence located approximately 250 bp upstream from the translation start site of the gene. The remainder were identical to sequences spaced at approximately 180 bp intervals along the genomic DNA. The eighth primer hybridized to a sequence in the third exon of *cdc2*, approximately 180 bp from the 3′ end of the gene. Thus, the entire coding region of *cdc2Dm* could be sequenced with the primers.

The primers were synthesized in the U.B.C. Oligonucleotide Synthesis Laboratory.

Primer purification: A Sep-pak C_{18} column (Millipore, #51910) was prepared by washing first with 10 ml of 100% acetonitrile (HPLC grade), then with 10 ml of water. The DNA primer, resuspended in 1.5 ml of 0.5 M ammonium acetate, was passed over the column. The column, with DNA bound, was washed with water; then the oligonucleotide was eluted from the column in 3 ml of 40% acetonitrile. The DNA was pelleted by lyophilizing the acetonitrile solution for approximately 4 hours. The pellet was resuspended in 300 mM sodium acetate and

precipitated with two volumes of 95% ethanol. After centrifugation, the pellet was vacuum dried, and resuspended ready for use.

Cloning using the polymerase chain reaction

Mutant alleles of *cdc2* were cloned by amplifying the gene-encoding sequences from total genomic DNA using the polymerase chain reaction. The amplified DNA was subcloned and individual clones were sequenced to determine the site of the mutation.

Five fruitflies, all heterozygous for a mutant allele, were homogenized in 200 µl of buffer (100 mM Tris-HCl pH 8.0, 50 mM NaCl, 50 mM EDTA, 1% SDS, 0.15 mM spermine, 0.5 mM spermidine) with 100 µg/ml proteinase K. The mixture was incubated at 65^0 for 1 hour. Thirty µl of 8 M potassium acetate were added and the mixture was left on ice for 1 hour. The sample was centrifuged (10,000 rpm for 10 min) and the pellet was discarded. Genomic DNA was precipitated from the supernatant by the addition of 2 volumes of cold 95% ethanol. Following centrifugation, the pellet was resuspended in 300 mM sodium acetate. RNA was hydrolyzed with RNAse at 100 µg/ml. The sample was mixed with an equal volume of phenol/chloroform (1:1) and centrifuged. DNA was re-precipitated from the aqueous phase by the addition of 2 volumes of cold 95% ethanol. The DNA was pelleted by centrifugation, vacuum dried, and re-suspended in water.

To amplify *cdc2Dm* sequences from genomic DNA, two primers were chosen from opposite sides of the transcribed region. Primer 1 (GGCTGCAGGTAGCTAGCTTAGCATCG) had a GG clamp, a *PstI* restriction endonuclease recognition site, and 18 bp of sequence identical to that found 107 bp upstream of the *cdc2* translation start site. Primer 2 (GGGGATCCGCTAGCAGTGCTCTCTAT) had a GG clamp, a *BamHI* restriction endonuclease site, and 18 bp of sequence identical to that found approximately 150 bp beyond the 3′ terminus of the *cdc2* gene. The primers were synthesized in the U.B.C. Oligonucleotide Synthesis Laboratory.

Polymerase chain reactions were performed using the GeneAmp PCR reagent kit (Cetus Corp.). Three independent reactions were performed for each mutant. Each 99.5 µl PCR reaction mix contained 10 µl of 10X reaction buffer (100 mM Tris-HCl (pH 8.3, at 25^0), 500 mM KCl, 15 mM $MgCl_2$, 0.1% (w/v) gelatin); 2 µl each of 10 mM dATP, dGTP, dCTP, and dTTP; 30 pmol each of primers #1 and #2; and 100 ng of *Drosophila* genomic DNA. The reaction mix was overlaid with paraffin oil (Mallinckrodt, #6357), then placed at 100^0 for three minutes to denature any contaminating proteases. *AmpliTaq* DNA polymerase (0.5 µl; 2.5 Units) was added to the heated solution. DNA amplification reactions were performed immediately in a SingleBlock System thermal cycler (Ericomp, Inc.).

Twenty-six cycles of amplification were performed. The

first 25 were as follows: double stranded DNA was melted at 94°; the primers were annealed to the template at 56°; and the polymerase extended the primers at 72°. Each step in the cycle proceeded for 30 s. The twenty-sixth cycle consisted of an identical melting step, followed by a 2 min annealing reaction and a 5 min primer extension reaction.

The annealing temperature was determined empirically, by increasing the annealing temperature until only a single band of DNA amplification products was visible on agarose gels.

The solution containing the amplified gene product was extracted once each with equal volumes of phenol, phenol/chloroform, and chloroform. The DNA was precipitated with ethanol and resuspended in 100 µl TE. Yields were 70 to 100 µg of amplified product.

The amplified DNA was cloned into a plasmid vector (pUC19) using methods that are described in detail in Chapter 3. One µg of amplified DNA was digested with the restriction endonucleases *PstI* and *BamHI*. The DNA was electrophoresed for several hours on a 1% agarose gel, then a portion of the gel containing the DNA band was excised with a razor-blade. The DNA was purified using glass beads and resuspended in water. Approximately 500 ng of amplified DNA was mixed with 50 ng of *PstI/BamHI*-digested pUC19. The DNA ligation procedure and subsequent transformation of a bacterial host were done as described in Chapter 3.

DNA Sequence Analysis

Sequencing reactions were performed as described in Chapter 2, except that internal *cdc2Dm* primers were used. Each gel-cloned mutant was sequenced only once. For PCR amplified products, five different clones were sequenced to ensure that the mutation present *in vivo* could be distinguished from random mutations that arose during the amplification reaction. The five clones were chosen from amongst the products of three independent PCR reactions.

RESULTS

Alleles of *cdc2*

Eight hemizygous lethal mutations which map to region 31 fail to complement the lethality associated with the *Su(var)216* chromosome (Chapter 1, see Table 14). These are: *B47*, *D57*, *E10*, *E1-4*, *E1-9*, *E1-23*, *E1-24*, and *216P*. To test for allelism with *cdc2Dm*, mutant strains were mated with flies bearing *cdc2.12*, an X-linked construct containing the $cdc2Dm^{+}$ gene, which was generated by transformation (C. Lehner, personal communication). *cdc2.12/cdc2.12; Df(2L)J27/CyO* females were crossed to *Su(var)216/CyO*, *216P/CyO* and *D57/CyO* males (Table 11). In each case, the ectopically placed wild-type function of *cdc2.12* rescued the lethality of *mutant/Df(2L)J27* progeny of both sexes. Similar results were obtained in an analogous test using the *4.1* strain (Table 11), which contains a $cdc2Dm^{+}$ gene construct inserted on the third chromosome (C. Lehner, personal communication). Thus, *Su(var)216*, *216P* and *D57* can be rescued by a single wildtype allele of *cdc2Dm*.

The other five putative *cdc2Dm* mutants were crossed to *cdc2.12/cdc2.12;* $cdc2^{Su(var)216}$*/CyO* flies and the progeny were examined for the presence or absence of $cdc2^{Su(var)216}$*/mutant* survivors. (This test for allelism was used instead of the previous one because the *Df(2L)J27* stock was nearly infertile in the presence of either *cdc2.12* or *4.1*.) When the construct-bearing suppressor stock was crossed to each of the remaining

Table 11. Phenotypic rescue of three mutant alleles of *cdc2Dm*.

Cross	Males		Females	
	Cy	Cy$^+$	Cy	Cy$^+$
Df(2L)J27/CyO ♂ X *216P/CyO*[a] ♀	195	0	208	0
Df(2L)J27/CyO; 4.1/4.1[b] ♂ X *216P/CyO* ♀	87	36	99	48
cdc2.12/cdc2.12; Df(2L)J27/CyO ♀ X *216P/CyO* ♂	73	25	82	36
Df(2L)J27/CyO ♂ X *Su(var)216/CyO* ♀	178	0	181	0
Df(2L)J27/CyO; 4.1/4.1 ♂ X *Su(var)216/CyO* ♀	112	48	121	54
cdc2.12/cdc2.12; Df(2L)J27/CyO ♀ X *Su(var)216/CyO* ♂	86	36	83	41
Df(2L)J27/CyO ♂ X *D57/CyO* ♀	160	0	176	0
Df(2L)J27/CyO; 4.1/4.1 ♂ X *D57/CyO* ♀	63	28	51	19
cdc2.12/cdc2.12; Df(2L)J27/CyO ♀ X *D57/CyO* ♂	86	36	83	41

[a]Flies bearing the chromosome *Su(var)216P b Tft*, denoted *216P*, are homozygous (females) or hemizygous (males) for *In(1)w*^{m4}.

[b]Fly strains carrying either of the two gene constructs *cdc2.12* or *4.1* are homozygous (females) or hemizygous (males) for *w*^{67}, a null allele of the *white* gene.

mutants, the viability of the mutant *trans*-heterozygotes was restored (Table 12). Although the lethal phenotype was alleviated, the majority of crosses showed a marked reduction in the total number of male progeny. The reason for this reduction is unclear, since crosses involving a deficiency do not have a similar effect (Table 11).

One additional putative allele of *cdc2Dm*, *E20*, is viable and fertile in combination with $cdc2^{Su(var)216}$ but fails to complement $cdc2^{D57}$ and $cdc2^{E10}$ (Table 13). *Trans*-heterozygous $cdc2^{D57}/cdc2^{E20}$ females are infecund at 25^0 and both males and females are inviable at 29^0. Each of these phenotypes is rescued by a single copy of *cdc2.12*. Heterozygous $cdc2^{E10}/cdc2^{E20}$ mutants are essentially lethal at 25^0 unless they also carry the $cdc2.12^+$ construct. Surprisingly, the $cdc2.12^+$ construct has little effect on the viability of either $cdc2^{E10}/cdc2^{E20}$ or $cdc2^{E20}/Df(2L)JR16$ progeny at 29^0. These effects might be caused by second site mutations in the stocks or they may reflect unusual properties of $cdc2^{E20}$. Some $cdc2^{E20}/CyO$ heterozygotes have mildly etched tergites. Although this may be a consequence of a second site mutation, other *cdc2Dm* mutants have a similar but more severe recessive phenotype (see below). Taken together these observations suggest that $cdc2^{E20}$ may be semi-dominant.

Table 12. Phenotypic rescue of heteroallelic combinations of *cdc2Dm* mutations.

Cross	Males		Females	
	Cy	Cy^+	Cy	Cy^+
Su(var)216/CyO ♀ X *B47/CyO* ♂	98	0	110	0
cdc2.12/cdc2.12; Su(var)216/CyO ♀ X *B47/CyO* ♂	42	9	136	44
Su(var)216/CyO ♀ X *D57/CyO* ♂	86	0	93	0
cdc2.12/cdc2.12; Su(var)216/CyO ♀ X *D57/CyO* ♂	118	40	172	53
Su(var)216/CyO ♀ X *E10/CyO* ♂	148	0	165	0
cdc2.12/cdc2.12; Su(var)216/CyO ♀ X *E10/CyO* ♂	81	64	134	53
Su(var)216/CyO ♀ X *E1-4/CyO* ♂	166	39	115	46
cdc2.12/cdc2.12; Su(var)216/CyO ♀ X *E1-4/CyO* ♂	9	2	52	28
Su(var)216/CyO ♀ X *E1-9/CyO* ♂	65	0	66	0
cdc2.12/cdc2.12; Su(var)216/CyO ♀ X *E1-9/CyO* ♂	33	20	60	39
Su(var)216/CyO ♀ X *E1-23/CyO* ♂	103	0	82	0
cdc2.12/cdc2.12; Su(var)216/CyO ♀ X *E1-23/CyO* ♂	26	19	59	35
Su(var)216/CyO ♀ X *E1-24/CyO* ♂	127	0	142	1
cdc2.12/cdc2.12; Su(var)216/CyO ♀ X *E1-24/CyO* ♂	39	24	58	28

Table 13. Complementation crosses with *cdc2*^{E20}

Cross	No. of *Cy* versus *Cy*$^{+}$ progeny	
	25^{0}	29^{0}
E20/CyO X *Su(var)216/CyO*	635:281 (0.307)[a]	251:92 (0.268)
E20/CyO X *B47/CyO*	603:201 (0.250)	1719:51 (0.029)
E20/CyO X *D57/CyO*	1808:437 (0.195)	1827:16 (0.009)
E20/CyO X *E10/CyO*	894:53 (0.056)	989:2 (0.002)
E20/CyO X *E1-4/CyO*	730:281 (0.278)	891:254 (0.222)
E20/CyO X *E1-9/CyO*	438:195 (0.308)	634:173 (0.214)
E20/CyO X *E1-23/CyO*	843:419 (0.332)	438:194 (0.307)
E20/CyO X *E1-24/CyO*	1170:649 (0.357)	n.d.[b]
E20/CyO X *216^{P}/CyO*	668:333 (0.333)	833:325 (0.281)
E20/CyO X *Df(2L)JR16/CyO*	422:119 (0.220)	330:4 (0.012)
cdc2.12/cdc2.12; E20/+ X *Df(2L)JR16/CyO*	n.d.	250:38 (0.152)
cdc2.12/cdc2.12; E20/+ X *D57/CyO*	93:53 (0.363)	211:88 (0.294)
cdc2.12/cdc2.12; E20/+ X *E10/CyO*	361:106 (0.227)	120:4 (0.032)
E20/CyO X *cn bw/cn bw* [c]	-	479:497 (0.509)
cn bw/cn bw X *Df(2L)JR16/CyO*	-	188:168 (0.472)

[a] The proportion of straight-winged flies (straight-winged progeny/ straight-winged + curly winged progeny)

[b] not done

[c] *cn bw* was the chromosome on which *E20*, *D57*, *B47*, and *E10* were induced.

Adult Phenotypes

The *cdc*^{216P} and *cdc2*^{E20} mutations are both weak alleles of *cdc2Dm* with respect to viability. At 22^0, rare *cdc2*^{216P}/*cdc2*^{216P} homozygotes eclose (less than 1 homozygote per 300 heterozygotes). These flies have damaged, duplicated or missing machrochaetes, gnarled leg joints, and little or no abdominal cuticle. They do not survive for more than a few days, hence they have not been tested for fertility. Hemizygous *cdc2*^{216P} progeny do not survive to adulthood.

At 22^0, homozygous *cdc2*^{E20} mutants comprise approximately 8% of the adult flies in our *cdc2*^{E20}/*CyO* stock. These flies have mildly etched sternites and tergites but do not display any of the other *cdc2*^{216P}/*cdc2*^{216P} phenotypes. Male homozygotes are sterile and female homozygotes are infecund. A similar phenotype is observed for hemizygous *cdc2*^{E20} males, but hemizygous females are infrequently fertile (1 in 10). It is possible, though unlikely, that these females may actually be *cdc2*^{E20}/*CyO* heterozygotes with poor *Cy* expression. A *cdc2.12/cdc2.12; cdc2*^{E20}/*CyO* stock has been established, but the frequency of homozygous *cdc2*^{E20}/*cdc2*^{E20} progeny remains low, suggesting that the mutant chromosome carries at least one additional lesion that affects homozygous viability.

Occasional *cdc2*$^{E1-4}$ and *cdc2*$^{E1-24}$ hemizygotes survive to adulthood when reared at 22^0 (see Table 15). These have severely etched sternites and tergites but are fertile. The same phenotype is observed amongst almost all progeny-bearing

heteroallelic combinations of *cdc2Dm* mutations (see Table 14), especially those involving $cdc2^{E1-4}$. Thus, a recessive abdominal phenotype is associated with most mutant alleles of *cdc2Dm*.

One heteroallelic combination that expresses only very weak abdominal etching is $cdc2^{D57}/cdc2^{E20}$. At moderate temperatures (18^0, 22^0 and 25^0), $cdc2^{D57}/cdc2^{E20}$ females are infecund and males are fertile. The same phenotype is observed regardless of the parental source of each mutant allele. The ovaries of 10 infecund $cdc2^{D57}/cdc2^{E20}$ females raised at 25^0 were dissected and examined. In each case no ovarioles were visible within the ovary (data not shown); however, the possibility that some germline proliferation has occurred cannot be excluded.

Lethal Phases

Excluding $cdc2^{E20}$, all of the *cdc2Dm* mutants are essentially lethal as hemizygotes; however, the pattern of complementation between different mutant alleles (Table 14) suggests that several of the mutant gene products still retain some activity. These partially functional products may provide clues as to the function of the wild-type gene product, if the flies that carry them die at recognizable stages of development (*e.g.* gastrulation) or when specific tissues are being elaborated (*e.g.* imaginal disc growth).

Table 14. Complementation matrix for alleles of *cdc2Dm*. Ratios indicate the number of curly to straight-winged flies amongst progeny from the cross $cdc2^{x}/CyO$ X $cdc2^{y}/CyO$

	Su(var) 216	*B47*	*D57*	*E10*	*E1-4*
Su(var) 216	-	1716:0	177:0	1220:0	136:50
B47	460:0	-	283:0	516:0	114:29
D57	258:0	308:0	-	631:0	102:0
E10	248:0	775:0	523:0	-	562:44
E1-4	439:115	156:10	359:0	186:0	-
E1-9	131:0	342:0	161:0	150:0	375:77
E1-23	730:0	1021:0	857:0	831:0	636:14
E1-24	509:23	1496:0	1368:0	1405:0	173:24
216P	551:1	1508:0	407:0	892:0	607:267

	E1-9	*E1-23*	*E1-24*	*216P*
Su(var) 216	217:0	215:0	1577:0	215:10
B47	211:0	279:0	290:0	125:0
D57	140:0	107:0	842:0	253:0
E10	226:0	194:0	416:0	389:0
E1-4	319:0	207:4	451:21	432:102
E1-9	-	346:0	357:1	132:0
E1-23	933:0	-	641:0	199:0
E1-24	1514:7	492:0	-	1366:344
216P	799:1	387:20	721:179	-

To begin a more detailed analysis of the roles of *cdc2Dm* in a genetically tractable metazoan, the lethal phase for each *cdc2Dm* mutant was determined (Table 15). Each cross was designed so that all progeny classes but one, the *mutant/deficiency* class, potentially survived and were phenotypically distinct. In pilot studies, five of the mutants, $cdc2^{Su(var)216}$, $cdc2^{E10}$, $cdc2^{E1-4}$, $cdc2^{E1-9}$, and $cdc2^{E1-24}$ died most frequently as pupae, regardless of the parental source of the mutation-bearing chromosome (Table 15). In two cases, $cdc2^{E1-23}$ and $cdc2^{B47}$, progeny that inherited the mutation from their fathers died most frequently as larvae, while in the reciprocal cross they died most frequently as pupae. However, the most dramatic results were obtained for $cdc2^{D57}$.

In three independent experiments, hemizygous $cdc2^{D57}/Df(2L)JR16$ progeny of $cdc2^{D57}/CyO$ mothers died as embryos, whereas hemizygous progeny that inherited $cdc2^{D57}$ from their fathers died as larvae. Thus, the $cdc2^{D57}$ allele displays a maternal effect.

To confirm the embryonic lethality of $cdc2^{D57}$, the $cdc2^{D57}$ and *Df(2L)JR16* second chromosomes were re-balanced over a multiply inverted chromosome marked with *Black-cells* (*Bc*). In flies bearing the *Bc*-balancer, dark pigment grains are visible underneath the cuticle. When $cdc2^{D57}/Bc$ mothers were crossed to *Df(2L)JR16/Bc* males, no non-*Bc* larvae were observed amongst 484 progeny. A similar result was obtained in the cross $cdc2^{D57}/Bc$ X $cdc2^{D57}/Bc$.

Table 15. Lethal phases of *cdc2Dm* mutants raised at 22°C.

Male X *Df(2L)JR16 /CyO*	% Embryonic lethality	% Larval lethality	% Pupal lethality	% Adult viability
Su(var)216 /+	3 (10/408)	9 (35/408)	25 (105/408)	0
B47/+	3 (10/433)	19 (81/433)	13 (53/433)	0
D57/+	2 (6/383)	28 (107/383)	5 (19/383)	0
E10/+	2 (6/502)	5 (24/502)	24 (117/502)	0
E1-4/+	5 (17/394)	8 (31/394)	25 (97/394)	2 (4/249)
E1-9/+	4 (13/424)	12 (49/424)	25 (105/424)	0
E1-23/+	2 (7/340)	25 (83/340)	9 (29/340)	0
E1-24/+	3 (13/446)	13 (54/446)	21 (91/446)	6 (16/288)

Female X *Df(2L)JR16 /+*	% Embryonic Lethality	% Larval Lethality	% Pupal Lethality	% Adult Viability
Su(var)216 /CyO	5 (21/461)	14 (61/461)	19 (85/461)	0
B47/CyO	1 (4/399)	11 (42/399)	22 (87/399)	0
D57/CyO	31 (127/414)	6 (25/414)	12 (47/414)	0
E10/CyO	5 (17/406)	10 (37/406)	23 (91/406)	0
E1-4/CyO	10 (44/453)	17 (75/453)	30 (135/453)	1 (1/199)
E1-9/CyO	5 (21/479)	10 (45/479)	26 (121/479)	0
E1-23/CyO	2 (5/440)	12 (49/440)	22 (93/440)	0
E1-24/CyO	4 (16/449)	2 (9/449)	31 (139/449)	4 (10/285)

While *cdc2*^{D57} hemizygotes die most frequently as embryos or larvae, the majority of *cdc2*^{E10} hemizygotes die as pupae (Table 15). This is surprising since *cdc2*^{D57}/*cdc2*^{E20} heterozygotes are inviable only at 29^{0}, while *cdc2*^{E10}/*cdc2*^{E20} mutants are strongly inviable at lower temperatures. Furthermore, *cdc2*^{D57}/*cdc2*^{E20} females are always infecund, whereas *cdc2*^{E10}/*cdc2*^{E20} females are fecund. The different phenotypes of *cdc2*^{D57} and *cdc2*^{E10} hemizygotes suggest that the two mutants are qualitatively different. It is curious in this regard that progeny from the cross *cdc2*^{D57}/*CyO* X *cdc2*^{E10}/*CyO* die most frequently as embryos, even if *cdc2*^{E10} is maternally inherited (data not shown). In contrast, progeny from the cross *cdc2*^{D57}/*CyO* X *Df(2L)JR16* die as larvae. These observations suggest that *cdc2*^{E10} may have stronger effects on viability than a null mutation. It is possible that both *cdc2*^{D57} and *cdc2*^{E10} have additional recessive lethal mutations on the second chromosome. However, it seems unlikely that both would share the same second site lethal mutation. Hence the odd pattern of lethality remains intriguing.

Embryonic and Larval Phenotypes

If *cdc2Dm* exerts its influence predominantly through its effects on cell cycle progression, then the terminal phenotypes of *cdc2Dm* mutants might be expected to exhibit gross defects in cell proliferation. In this respect, two periods of development are of particular interest: the

embryonic and pupal stages. During embryogenesis the tissues of the larva proliferate. Most of these tissues (with the exception of a few tissues such as the nervous system) are histolysed at the pupal stage. The majority of adult tissues originate from specific foci of proliferating cells within the larvae which only differentiate at the pupal stage during metamorphosis. To test for potential disruption of cell proliferation in larval and adult precursor cells, the embryonic phenotype of *cdc2*^{D57} hemizygotes was investigated along with the pupal lethality of *cdc2*^{E10}*/Df(2L)JR16* hemizygotes and *cdc2*^{E10}*/cdc2*^{B47} heterozygotes.

Since *Df(2L)J27* deletes *cdc2Dm* and *Df(2L)J27/Df(2L)J27* homozygotes survive until after the embryonic epidermis secretes a cuticle (at approximately 15-18 hours in the 22 hour embryonic development at 25^0), the cuticles of hemizygous *cdc2*^{D57} females were examined for pattern defects. Sixty-eight cuticles were observed from embryos which failed to hatch when *cdc2*^{D57}*/+* females were mated to *Df(2L)J27/CyO* males. Only nine embryos had mild head or cuticle defects. In every other case the cuticle appeared normal with no obvious holes or missing structural landmarks. Thus, the strongest of our alleles does not grossly disrupt this aspect of development. This is in contrast to homozygous *Df(2L)J27/Df(2L)J27* embryos which have misshapen heads and holes in their cuticles (Nüsslein-Volhard *et al.*, 1984).

A high proportion of mutants bearing heteroallelic

combinations of *cdc2* mutations arrest at the pupal stage. Many of these appear to cease development predominantly as pharate adults. Dissection of the pupal cases of these mutants again reveals severe sternite and tergite etching. The precursors of these tissues divide very rapidly during the early pupal stage and therefore this phenotype is not surprising for a mutant with a potential defect in cell cycle regulation.

In addition to developmental arrest late in the pupal period, some combinations of mutant *cdc2Dm* alleles die somewhat earlier without any overt differentiation. One such combination, $cdc2^{E10}/cdc2^{B47}$, arrests at Stage P3 (bubble prepupa) to P4 (Bainbridge and Bownes, 1981). At the latter stage, the imaginal discs of the larvae usually evert to elaborate the structures of the adult fly; however, no discs are visible in the $cdc2^{E10}/cdc2^{B47}$ larvae. A similar phenotype is observed for *cdc2*^{E10}*/Df(2L)JR16* larvae. These observations suggest that *cdc2Dm* mutations prevent the proliferation of at least some tissues that give rise to adult structures. These tissues normally undergo rapid proliferation during the larval period.

In addition to mitosis, *cdc2* has been implicated in progression into S phase. In *Drosophila*, S phase is uncoupled from mitosis during the polytenization of many larval tissues (including the salivary glands, which reach their maximum size in third instar larvae). If the *cdc2Dm* mutants affect S phase, then the extent of polytenization might be reduced. Therefore,

I examined larval salivary glands in *cdc2^{E10}/Df(2L)JR16* hemizygous progeny from the cross *cdc2^{E10}/Bc* ♂ X *Df(2L)JR16/Bc* ♀. The salivary glands dissected from wandering third instar *cdc2^{E10}/Df(2L)JR16* larvae were normal in size, and polytene chromosomes were visible in squash preparations. Similar results were obtained with *cdc2^{E1-23}*. Thus, *cdc2^{E10}* and *cdc2^{E1-23}* do not appear to affect polytenization, although I cannot exclude the possibility that maternally inherited *cdc2* mRNA rescues progression into S phase.

The Relationship Between *Su(var)216* and *cdc2Dm*

Several observations suggest that *cdc2* homologues may play a role in the compaction and modification of chromatin, properties also hypothesized for suppressors of PEV. In numerous systems, the activity of p34^{cdc2} kinase is strongly correlated with chromosome condensation during mitosis (see Nurse, 1990; Moreno and Nurse, 1990). p34^{cdc2} phosphorylates histones, which are the principal proteins of chromatin. Also, chromatin modification by p34^{cdc2}-like activity has been observed in the absence of mitosis (Roth *et al.*, 1991). Furthermore, suppressors of PEV affect chromatin condensation (*e.g.* Dorn *et al.* 1986), and their effects may be brought about by histone modification (Dorn *et al.*, 1986; Mottus *et al.*, 1980). Taken together these observations raise the possibility that *cdc2Dm* mutants might suppress PEV by altering the normal pattern of chromatin modification.

While the *cdc2Dm* locus is a good candidate for a *Su(var)* gene, it was essential to demonstrate that the *Su(var)* mutation is within the *cdc2Dm* locus. Sequence analysis of *Su(var)216* (see below) demonstrates that a single point mutation causes an alteration in the *cdc2Dm* protein. So the *Su(var)216* chromosome clearly has a mutation in *cdc2Dm*. It remained to be determined, however, whether *cdc2* mutations suppress PEV.

Amongst 10 mutants, only the *Su(var)216* allele strongly suppresses PEV. When w^{m4}/w^{m4}; *Su(var)216/CyO* males are outcrossed to w^{m4}/w^{m4} females, both male and female progeny have approximately 80% red pigment compared with 10% in w^{m4}/w^{m4} controls (Sinclair *et al.*, 1991). Scored visually, no other *cdc2Dm* mutant suppresses PEV when compared to controls. Furthermore, a single copy of $cdc2Dm^{+}$ inserted on the X chromosome rescues the lethal phenotype of *Su(var)216/Su(var)216*, but has no effect on the Su(var) phenotype (data not shown). This result may not be too surprising since the amount of functional p34 expressed in the construct-bearing strain is not known (euchromatic position effects are well documented in *Drosophila*). These observations can be explained if $cdc2^{Su(var)216}$ is hypothesized to be a rare gain-of-function mutation, and the wild-type gene, unlike many other *Su(var)* loci, is not dose sensitive. However, other circumstantial evidence is less easy to explain.

Hemizygous *Su(var)216/Df(2L)J2* males do not survive to

adulthood, while females display reduced viability and are infecund (Sinclair *et al.*, 1991). The same phenotypes prevail in the presence of either one or two copies of the X linked *cdc2Dm*$^{+}$ gene construct, *cdc2.12* (Table 16). However, when *Su(var)216* was made heterozygous with *Df(2L)J77*, *Df(2L)J27*, *Df(2L)G2*, or *Df(2L)J106* in the presence of *cdc2.12* (*cdc2.12/+; Su(var)216/deletion*), *mutant/deficiency* progeny were male viable and female fertile (Table 16). The mutant phenotype of *cdc2.12/+; Su(var)216/Df(2L)J2* is unlikely to be caused by a combination of poor *cdc2.12* expression exacerbated by deletion for many loci, since *cdc2*^{E10}*/Df(2L)J2* progeny survive and are fertile in the presence of an ectopic copy of *cdc2Dm*$^{+}$. An alternate explanation is that the *Su(var)216* strain carries a *cdc2Dm* mutation and a second site male-lethal, female-sterile mutation which maps very near to *cdc2Dm* and was not removed by the recombination replacement protocols.

The finding that *cdc2.12/+; Su(var)216/Su(var)216* females are viable (Table 13) but still infecund with a wings-held-out phenotype also supports the second-site hypothesis. Since some *Su(var)216/Su(var)216* males are viable in the presence of *cdc2.12*, the second site mutation appears to be quite weak with respect to its effects on viability.

The possibility of a closely linked second site mutation led us to examine the interactions between *Su(var)216* and the other *Su(var)* loci hypothesized to map in region 31. Even in the presence of *cdc2.12*, recessive interactions are observed

Table 16. Mapping a second-site mutation on the *cdc2*$^{Su(var)216}$ chromosome.

Cross	Males		Females	
	Cy	Cy$^+$	Cy	Cy$^+$
cdc2.12/cdc2.12; Su(var)216/CyO ♀ X *Df(2L)J2/CyO* ♂	240	0	351	99
cdc2.12/cdc2.12; Su(var)216/CyO ♀ X *cdc2.12/Y Df(2L)J2/CyO* ♂	95	0	50	39
cdc2.12/cdc2.12; Df(2L)J2/CyO ♀ X *Su(var)216/CyO* ♂	42	0	42	20
cdc2.12/cdc2.12; Su(var)216/CyO ♀ X *Df(2L)J27/CyO* ♂	60	31	91	42
cdc2.12/cdc2.12; Su(var)216/CyO ♀ X *Df(2L)J106/CyO* ♂	30	8	38	12
cdc2.12/cdc2.12; Su(var)216/CyO ♀ X *Df(2L)J77/CyO* ♂	227	99	183	81
cdc2.12/cdc2.12; Su(var)216/CyO ♀ X *Df(2L)G2* ♂	74	47	218	73
R1/CyO ♀ X *Df(2L)J2/CyO* ♂	217	0	275	108
R1/CyO ♀ X *Df(2L)G2/CyO* ♂	153	76	170	74
cdc2.12/cdc2.12; Su(var)216/CyO ♀ X *Su(var)216* ♂	46	10	92	86
Su(var)216/CyO ♀ X *R1/CyO* ♂	31	1	38	13
cdc2.12/cdc2.12; Su(var)216/CyO ♀ X *Su(var)214/CyO*	60	1	150	48

between *Su(var)216* and members of the *Suvar(2)1* complementation group (Table 16). Transheterozygotes of genotype *cdc2.12/+; Su(var)216/Su(var)214* are female infecund and essentially male lethal, just as they are in the absence of *cdc2.12*. Similarly, transheterozygous *Su(var)216/Su(var)207* and *Su(var)216/Su(var)210* females are infecund, with or without *cdc2.12*. In each case, the *trans*-heterozygotes have a wings-held-out phenotype and a red/brown eye colour similar to that of heteroallelic combinations of *Suvar(2)1*. Thus, *cdc2.12* fails to alleviate any of the hypothesized interactions between *Su(var)216* and *Suvar(2)1*.

Initially, the phenotype of *Su(var)216/Suvar(2)1* heterozygotes was interpreted as an interaction between the mutant products of two separate loci (Brock, 1989); however, the second mutation on the *Su(var)216* chromosome might simply be an allele of *Suvar(2)1*. To test this hypothesis, a colleague (I.P. Whitehead) attempted to separate the suppression phenotype from the lethal phenotype of $cdc2^{Su(var)216}$. In a large scale recombination experiment (>100,000 flies), five recombinants were isolated which mapped to the right of *J* and which were viable with *Df(2L)G2* but which still suppressed PEV. One recombinant, *R1*, behaved in a manner similar to the putative second site mutation on the *Su(var)216* chromosome. Homozygotes were viable but female sterile, and *R1/Df(2L)J2* progeny were male lethal and female infecund (Table 16). The remaining recombinant strains have similar

phenotypes (I.P. Whitehead and G. Stromatich, personnal communications).

Taken together, these findings suggest that the *Su(var)216* chromosome suppresses PEV because of a mutation that is tightly linked to *cdc2Dm*. They do not, however, formally exclude the possibility that $cdc2^{Su(var)216}$ also acts as a suppressor of PEV.

The Sequences of *cdc2Dm* Mutant Alleles

Regardless of whether or not altered expression of cdc2Dm protein acts as a suppressor of PEV or not, CDC2 proteins play a central role in the control of cell division. Since we have a large collection of *cdc2Dm* mutants, I conducted studies to correlate specific mutations with mutant phenotypes. To further characterize *cdc2Dm*, 6 mutant alleles were cloned. Two of the mutations, $cdc2^{E1-9}$ and $cdc2^{E1-23}$ were subcloned into pUC19 after a *HindIII/SalI* fragment containing the entire gene was excised from size fractionated genomic DNA. The remainder of the mutations were cloned by amplification of the *cdc2Dm* gene using the polymerase chain reaction, followed by subcloning of the product DNA. In both cases, the DNA isolated contained two different alleles of *cdc2Dm*. Wildtype and mutant chromosomes were differentiated based on the presence or absence of an *EcoRI* site in Intron 1 of *cdc2Dm*. Mutant alleles induced on either the multiply marked *b pr cn* or *b lt rl* chromosomes have an extra *EcoRI* restriction endonuclease site,

while mutants isolated on the *cn bw* chromosome do not. By mating mutant bearing flies to the appropriately marked second chromosome it was possible to generate heterozygous progeny for both the Intron 1 polymorphism and a recessive lethal *cdc2Dm* mutation. Thus, it was possible to identify cloned mutant alleles from wildtype alleles.

Each of the cloned *cdc2Dm* alleles has a single basepair missense mutation caused by a nucleotide transition within the coding region of the gene. Conceptual translation reveals that the resultant amino acid substitutions are not clustered, but are distributed throughout the predicted protein (Figure 32).

The amino acid substitution Gly to Asp in cdc2DmE1-4 is within the PSTAIR sequence, a motif that is absolutely conserved amongst functional homologues of *cdc2*. In both cdc2DmSu(var)216 and cdc2DmD57, the unique amino acid substitution is within the central catalytic core of the enzyme (see Hanks *et al.*, 1988). The two respective alterations are only three residues apart. However, the amino acid substitution in cdc2DmD57 (Arg) replaces Gly^{148}, a residue which is invariant not only within the CDC2 family of proteins, but amongst all kinases (Hanks *et al.*, 1988). In contrast, Ala^{145}, the amino acid that is replaced by Val in cdc2DmSu(var)216, is not invariant amongst all kinases. In fact, an alternative residue is found at this site in the homologous protein of *S. cerevisiae*, although in this case, the substitution is conservative.

Figure 31. Mutations in *cdc2Dm*. Underlining in the protein sequence indicates amino acids conserved amongst all CDC2 proteins.

Mutant	Mutation	Amino Acid Substitution		
		#	Alteration	Domain[a]
E1-4	GGC to GAC	43	G to D	2/3
cdc2$^{Su(var)216}$	GCC to GTC	145	A to V	8
D57	GGA to AGA	148	G to R	7
E1-24	GAG to AAG	196	E to K	9
E1-23	GGT to GAT	206	G to D	9
E1-9	CCC to TCC	242	P to S	11

```
                                                          D          55
MEDFEKIEKIGEGTYGVVYKGRNRLTGQIVAMKKIRLESDDEGVPSTAIREISLL

                                                                    110
KELKHENIVCLEDVLMEENRIYLIFEFLSMDLKKYMDSLPVDKHMESELVRSYLY

                                                    V  R            165
QITSAILFCHRRRVLHRDLKPQNLLIDKSGLIKVADFGLGRSFGIPVRIYTHEIV

                                               K         D          220
TLWYRAPEVLLGSPRYSCPVDIWSIGCIFAEMATRKPLFQGDSEIDQLFRMFRIL

                                  S                                 275
KTPTEDIWPGVTSLPDYKNTFPCWSTNQLTNQLKNLDANGIDLIQKMLIYDPVHR

                             297
ISAKDILEHPYFNGFQSGLVRN
```

[a] The domain number refers to discrete regions of sequence homology amongst kinases (Hanks *et al.*, 1988).

The three remaining mutations, *cdc2*$^{E1-24}$, *cdc2*$^{E1-23}$, and *cdc2*$^{E1-9}$, all cause amino acid substitutions at residues that are highly conserved within the CDC2 protein family. Of this group, *cdc2*$^{E1-9}$ is most interesting, because it causes an amino acid substitution in a region that is either absent or poorly conserved in other kinases (Hanks *et al.*, 1988). Hence it may help to determine the specific properties of cdc2-like kinases.

Recently, the three dimensional structure of the catalytic subunit of cyclic AMP-dependent protein kinase (cAPK) has been determined by X-ray crystallography (Knighton *et al.*, 1991). Since CDC2 proteins are similar in sequence to cAPK and several biochemical properties and features of kinases are conserved (see Hanks *et al.*, 1988), the structure of cdc2Dm may be similar to that of cAPK. Alignment of the two sequences suggests that amino acid substitutions in cdc2DmE1-4, cdc2DmD57, cdc2E1-23, and cdc2DmE1-9 all are likely to be within randomly coiled regions. Similar secondary structures for cdc2Dm are predicted using the algorithm of Chou and Fassman (1974). This algorithm also predicts that the alteration in cdc2DmE1-9 is in a region of random coil. Several amino acid residues adjacent to and including Pro^{242} have no counterparts in cAPK. Other members of the kinase family have sequence insertions at the same position in the cAPK sequence (*e.g.* CDC7 260(93)261; see Knighton *et al.*, 1991). Based on their crystallographic data, Knighton *et al.*

(1991) speculated that additional amino acids in this portion of the sequence are located on the outside of the protein.

DISCUSSION

Ten recessive lethal *cdc2Dm* alleles have been described in this analysis. Lehner and O'Farrel (1990b) isolated a second *cdc2*-like gene, *cdc2c*, from *Drosophila* using the polymerase chain reaction. However, this gene shares only 58% amino acid sequence identity with the *cdc2Dm* gene product and fails to complement *cdc2* defects in fission yeast. Our data demonstrate that *cdc2c* cannot rescue the lethal phenotype of *cdc2Dm in vivo*.

Nine of the ten *cdc2Dm* mutations do not suppress PEV, and the tenth is on a chromosome which has a tightly linked *Su(var)* mutation. Although the $cdc2^{Su(var)216}$ allele has not been recovered without the adjacent *Su(var)* mutation, there is no evidence that the *cdc2Dm* allele is a suppressor of PEV.

Amongst the ten mutations of *cdc2Dm* which have been isolated, $cdc2^{D57}$ is the strongest allele with respect to lethality. Hemizygous mutant progeny which inherit $cdc2^{D57}$ from their mothers die as embryos, while those that inherit it from their fathers die as larvae. This difference in lethal phase suggests that despite being a strong allele, the gene product still retains some activity and that it antagonizes the function of the wild-type gene product. In *Drosophila*, the concentration of gene product is normally proportional to the number of copies of the gene. Since *cdc2* mRNA and p34 protein are sequestered in the egg (Lehner and O'Farrell, 1990b;

Jimenez *et al.*, 1990), *cdc2^{D57}/CyO* mothers should sequester 50% each of mutant and wildtype product. In contrast *Df(2L)JR16/CyO* mothers sequester only wild-type product, but half as much as diploid flies. Given that *cdc2^{D57}/Df(2L)JR16* progeny of *cdc2^{D57}* mothers die earlier than progeny in the reciprocal cross, the *cdc2^{D57}* product may titrate some factor required by wild-type *cdc2Dm* for embryonic development. The nucleotide alteration in *cdc2^{D57}* is consistent with this interpretation. The mutation causes an amino acid substitution in a site conserved throughout all kinases (Hanks *et al.*, 1988). Biochemical analyses suggest that this amino acid site is involved in ATP binding which is required for proper kinase function (see Hanks *et al.*, 1988). Thus, it is possible that the kinase activity of p34 is inactive, yet its ability to interact with other regulatory elements is still intact.

Although the morphology of hemizygous *cdc2^{D57}* embryos has yet to be investigated, the normal appearance of embryonic cuticles suggests that hemizygotes are not grossly defective in cell proliferation during early embryogenesis. In *Drosophila*, the first thirteen rounds of cell division are driven by maternally inherited product (Edgar *et al.*, 1986); however, from division 14 onward, the zygotic genome is active. In the case of *string*, the *Drosophila* homologue of *cdc25*, maternally endowed message is degraded at the transition to zygotic transcriptional control, and failure to produce a wild-type product in the zygote results in mitotic

arrest in G2 of interphase 14, despite normal morphogenetic movements in the early embryo (Edgar and O'Farrell, 1989). The result is a severely deformed embryo that secretes an abnormal cuticle. *Pimples* homozygotes, which arrest at division 15 (C. Lehner, personal communication), also have an abnormal cuticle (Nüsslein-Volhard *et al.*, 1984). Since hemizygous $cdc2^{D57}$ flies do not have defective cuticles, the mutant does not appear to cause cell cycle arrest at the mid-blastula transition. An alternative possibility to widespread cell cycle arrest is that cell division is affected in a critical part of the nervous system. Unlike the cells of other tissue types that only undergo 3-4 divisions following the mid-blastula transition, the nervous system of wild-type flies continues to divide throughout embryogenesis and the larval stages. Such speculation awaits a more detailed analysis of the mutants (which is underway in collaboration with the laboratories of C. Lehner (Max Planck Institute) and P. O'Farrell (UCSF)).

If $cdc2^{D57}$ is not completely inactive, then the *cdc2Dm* null phenotype remains unknown. The maternal dowry of *cdc2* mRNA and protein might be sufficient to permit the completion of embryogenesis, or $cdc2^{-}$ mutants might arrest at the mid-blastula transition, like *string* mutants. Two observations suggest that zygotic *cdc2Dm* activity might be essential for embryogenesis. First, observations of wild-type embryos suggests that *cdc2* transcription is activated in each region of the embryo as that region begins to undergo mitosis

(Jimenez *et al.*, 1990). Second, maternal *cdc2* product is largely excluded from cells of the developing embryo at the mid-blastula transition. This pattern of activity could represent a redundant control mechanism; however, only a few specific maternal transcripts, *string* for example (Edgar and O'Farrell, 1989), have been shown to be degraded at the time zygotic transcription is activated. Thus, it is also possible that zygotic regulation of *cdc2Dm* transcription is a requirement for normal development.

A genetic argument for or against the need for zygotically expressed *cdc2Dm* during embryogenesis will require a true null allele for the locus. The *P* element induced mutation $cdc2^{216P}$ should be particularly useful for isolating an amorphic allele of *cdc2Dm*, since the element can be mobilized in dysgenic crosses. Small intragenic deletions that inactivate the gene could be selected by testing for lethality in *trans* with $cdc2^{E20}$ or $cdc2^{E1-4}$.

The phenotypes of several less severe mutant alleles of *cdc2Dm* are consistent with defects in cell proliferation, although other explanations are not excluded. The absence of obvious imaginal discs and polytene chromosomes in $cdc2^{B47}/cdc2^{E10}$ heterozygotes suggests that both cell division and DNA replication are affected. These observations will, of course, require more detailed documentation. The absence of imaginal disc tissue might be investigated using a disc-specific antibody, and the extent of DNA endoreduplication

could be assayed using quantitative dot-blots.

The severe sternite and tergite etching associated with hemizygous *cdc2Dm* alleles may also reflect a failure of cells to proliferate. Tergites and sternites are cuticular structures secreted by the underling epidermis which covers the abdomen of the adult fly. Most adult tissues are derived from imaginal discs that divide throughout the larval stages; however, the abdominal epidermis is derived from histoblasts which are quiescent during larval development. At pupariation, histoblast cells proliferate rapidly for several divisions (cell cycle times are 2-3 hours compared with 10-12 hours for imaginal disc cells; Madhavan and Madhavan, 1980) before their rate of division slows. Since there are only approximately 6-20 histoblast cells in mature third-instar larvae (Roseland and Schneiderman, 1979) which must divide to cover each abdominal segment, rapid division is necessary to generate enough cells. Cuticle etching might, therefore, be a consequence of cell death, or it might reflect a reduction in the total number of epidermal cells at the time of eclosion. Fewer epidermal cells might be present if the rate of cell division were slowed because of the increased time required to accumulate sufficient functional *cdc2* product to initiate mitosis. Since the imaginal discs divide at a slower rate over a longer period of time, aspects of adult morphogenesis dependent on these cells might be less susceptible to reduced *cdc2* levels and this would explain the lack of pattern defects

in other cuticular structures in mutant flies.

Two of the mutants reported here have amino acid substitutions at the same site as previously reported alterations in the homologous residue from other species. The amino acid substitution in cdc2DmE1-4 (G^{43} to D^{43}) alters the 16 amino acid EGVPSTAIREISLLKE sequence that is absolutely conserved across the CDC2 family of proteins. The *S. pombe* mutant *cdc2-M35* has a glutamate residue at the same position. In fission yeast, the *cdc2-M35* protein product is temperature sensitive, but even at the permissive temperature, mutant haploid cells are elongated and undergo division at a cell size approximately 70% larger than wild-type (Nurse and Thuriaux, 1980). The essentially lethal phenotype of the *Drosophila* mutant confirms the importance of this residue to the *cdc2* product of multicellular organisms. Since some hemizygous flies do survive to adulthood, this suggests that the gene product is at least partially functional, as in the yeast *cdc2-M35* mutant.

Several lines of evidence suggest that the PSTAIR region may be the site of interaction with some regulator of *cdc2* kinase function. First, microinjection of the PSTAIR peptide induces meiotic maturation in starfish oocytes (Labbé *et al.*, 1989), but this phenomenon is prevented if the sequence is truncated or if amino acid substitutions are introduced (Picard *et al.*, 1990). Second, microinjected PSTAIR peptide accelerates nuclear envelope breakdown and chromosome

condensation in *Xenopus* egg extracts compared to MPF alone (Gautier *et al.*, 1988). Finally, microinjection of this peptide into both starfish and *Xenopus* oocytes triggers a specific increase in the concentration of intracellular free Ca^{2+} (Picard *et al.*, 1990) . Only the intact, unmutated form of the PSTAIR peptide has this effect. It might, therefore, be possible to genetically select for mutations in *Drosophila* which suppress the lethality of mutations in $cdc2^{E1-4}$.

In a similar vein, it would be intriguing to test the effects of overexpression of the *Drosophila* cyclin genes on $cdc2^{E1-4}$. Cyclins form part of the functional *cdc2* holoenzyme and some researchers have suggested that the PSTAIR sequence may be important for this interaction (see Draetta, 1990). If so, excess cylin protein may be able to rescue the $cdc2^{E1-4}$ lethal phenotype. Using antibodies to the cyclin proteins it might also be possible to detect differences in the concentration of the *cdc2Dm/cyclin* complex in the mutant relative to wildtype flies.

The $cdc2^{E1-9}$ replaces proline with serine, while a mutation in the homologous codon of *S. cerevisiae* replaces proline with leucine (Lörincz and Reed, 1986). The yeast mutant is temperature sensitive and has an aberrant morphology consistent with constitutively low *cdc2* activity. No unusual phenotypes other than lethality were evident in allelic crosses involving the *cdc2Dm* mutant.

In addition to the cyclins, another *S. pombe* protein,

p13^{suc1}, associates with *cdc2*. A similar protein has been identified in humans (Richardson *et al.*, 1990) and p13^{suc1} itself can be used to purify p34^{cdc2} homologues from other species. These findings suggest that the p13^{suc1} protein may also be part of a universal cell cycle mechanism. In yeast *cdc2*, the region between amino acids 177-208 may be the site of interaction with p13^{suc1} because three mutations within this region, *cdc2.33*, *cdc2.56* and *cdc2.L7*, are reduced in their ability to bind p13^{suc1} *in vitro*. The *cdc2^{E1-24}* mutation lies within the homologous region in *cdc2Dm* (amino acids 171-202), and the mutation in *cdc2^{E1-23}* is only 4 amino acids distant. Thus, these mutations may also disrupt the binding of *cdc2Dm* to p13^{suc1}. Potential alterations in the binding of p13^{suc1} could be tested either *in vivo* or *in vitro*.

An additional *cdc2Dm* mutation, *cdc2^{E10}*, also maps within the potential p13^{suc1} binding region inferred from yeast. Only one PCR-cloned product of this mutant has been sequenced in the current study; however, only a single basepair alteration is present in the entire coding region. This mutation causes a substitution for Leu^{176}. The effects of this substitution could also be tested for interactions with p13^{suc1}. If the mutation proved to be a cloning artifact, it should still be possible to test the effect of the mutation *in vivo* or *in vitro* using the conventional methodologies of molecular biology.

My isolation and partial characterization of several

cdc2Dm mutations has laid the foundations for future, more detailed, studies of p34 kinase in the growth and development of *Drosophila*.

APPENDIX 1. Deficiency mapping recessive lethal and sterile mutations in region 31.

Table 1A. Deficiency mapping of complementation groups located between the centromere distal breakpoints of *Df(2L)J2* and *Df(2L)J27*. The crosses were *mutation/CyO* X *Df/CyO*. Numerical ratios indicate the number of *Cy* to straight-winged progeny. Symbols: -, failure to complement; +, full complementation; S, female sterile.

Mutation	*Df(2L)J2*	*Df(2L)J39*	*Df(2L)G2*
Suvar(2)1^{214}	-[1]	+[1]	n.d.
A102	-[1]	+[1]	+
16-165	-1	+[1]	+
1-44	-[1]	+[1]	+
Su(var)207	-[1]	-[1]	+
fs(2)ry4	68:5 (S)	+	+
C98	-[1]	+[1]	+
Su(var)204	-[1]	-[1]	145:44 (S)
F133	-[1]	-[1]	148:0
trk	n.d	n.d.	611:226 (S)
B149	-[1]	-[1]	n.d.
14-140	-[1]	-[1]	327:0

Mutation	*Df(2L)JR1*	*Df(2L)J77*	*Df(2L)J27*
Suvar(2)1	+	+	+
A102	+	+[1]	+[1]
16-165	+	+[1]	+[1]
1-44	+	+[1]	+[1]
Su(var)207	+	+	+
fs(2)ry4	186:36 (S)	+	+
C98	176:0	+[1]	+[1]
F133	483:0	+[1]	+[1]
trk	n.d.	60:22 (S)	+
B149	273:0	-[1]	+[1]
14-140	n.d.	-[1]	+[1]

[1]Brock (1989)

Table 1B: Deficiency mapping of a representative allele from each complementation group that is lethal in *trans* with *Df(2L)J27*. Each cross was *mutant/CyO* X *Df/CyO*. The ratio presented is the number of *Cy* to straight-winged flies (+, full complementation; S, female sterile; n.d., not done).

Mutation	Df(2L)J27	Df(2L)J106	Df(2L)JR3	Df(2L)JR1
24-127	148:0	+	+	101:1
E1-13^{E1-17}	91:0	206:0	+	148:0
RU26	n.d.	n.d.	144:69 (S)	159:41 (S)
E1-6	n.d.	164:0	179:0	91:0
E1-7	68:0	163:0	157:0	43:0
E1-22	169:0	148:1	189:0	103:0
bsk	-	n.d.	268:0	231:0
DG25$^{23-127}$	424:24 (S)	145:0	225:1 (S)	152:0
pim	203:0	166:0	205:0	+
E2-15	103:0	n.d.	135:0	+
da$^{77-11}$	193:0	150:0	325:0	+
mfs48^{E1-10}	140:0	212:0	81:0	+
Su(var) 216	164:0	268:0	138:0	+
E1-1	123:0	277:0	170:0	+
E1-3	168:0	155:0	n.d.	+
E1-12	233:0	170:0	n.d.	+
E1-19	275:1	123:0	53:0	+
E1-28	211:0	60:0	71:0	+
E2-17	70:0	155:0	160:0	+
l(2) 54^{E1-16}	180:0	154:0	185:0	+
err^{C36}	420:68 (S)	160:0	n.d.	n.d.
PI23	(S)	n.d.	(S)	+
PJ50	(S)	n.d.	n.d.	+
E1-8	270:0	238:0	91:0	+
E1-14	229:0	325:0	180:0	+

Mutation	Df(2L)J27	Df(2L)J106	Df(2L)JR3	Df(2L)JR1
E2-9	69:0	n.d.	n.d.	+
77-12	n.d.	n.d.	325:0	+
77-14	n.d.	n.d.	277:0	+
E2-22	159:0	n.d.	54:0	+
29-85	-[1]	-[1]	121:0	+

[1]Brock (1989)

Table 1C. Deficiency mapping of representative mutations that fail to complement *Df(2L)J27*.

Mutation	*Df(2L)JR16*	*Df(2L)JR17*	*Df(2L)J77*	*Df(2L)G2*
24-127	+	+	93:0	203:0
E1-13^{E1-17}	+	+	258:0	170:0
RU26	+	94:25 (S)	n.d.	n.d.
E1-6	+	61:0	161:0	132:0
E1-7	+	147:0	168:0	116:0
E1-22	+	302:0	178:0	174:0
bsk	107:0	223:0	n.d.	n.d.
DG25$^{23-127}$	155:0	123:0	253:0	n.d.
pim	143:0	187:0	159:0	n.d.
E2-15	150:0	304:0	n.d.	n.d.
da$^{77-11}$	139:0	+	232:0	132:0
mfs(48)$^{E1-10}$	113:0	+	186:0	68:0
Su(var)216	115:0	+	240:0	188:0
E1-1	135:0	+	181:0	201:1
E1-3	163:0	+	-	n.d.
E1-12	137:0	+	217:0	n.d.
E1-19	117:0	+	n.d.	146:0
E1-28	140:0	+	271:0	n.d.
E2-17	186:0	+	306:0	174:0
l(2)54^{E1-16}	+	+	187:0	150:0
err^{E1-27}	+	+	420:3	n.d.
PI23	+	+	(S)	n.d.
PJ50	+	+	(S)	n.d.
E1-8	+	+	135:0	n.d.
E1-14	+	+	197:0	n.d.
E2-9	+	+	329:0	n.d.
77-12	+	+	232:0	n.d.
77-14	+	+	267:0	n.d.
E2-22	+	+	171:0	164:0
29-85	+	+	+	78:0

Table 1D. Deficiency mapping of a representative allele from each complementation group that fails to complement *Df(2L)J39* but complements *Df(2L)JR1* and *Df(2L)J27*.

Mutation	*Df(2L)JR3*	Df(2L)J106	Df(2L)G2	Df(2L)J2
E1-5$^{14-195}$	n.d.	-[1]	162:0	n.d.
E1-18	n.d.	75:0	58:0	n.d.
E1-25	n.d.	72:0	n.d	n.d.
E3	n.d.	-[1]	n.d.	-[1]
H95	102:20[3]	+	308:86[3]	-[1]
da1	+	+	+	-[2]
hup	+	+	+	-[2]
wd1	+	+	+	-[2]
A65	+	+	+	-[1]
D121	+	+	+	-[1]
E24	+	+	+	-[1]
F15	+	+	+	-[1]
G1	+	+	+	-[1]

[1]Brock (1989)
[2]D. Sinclair, unpublished
[3]male and female sterile

APPENDIX 2. A summary of hemizygous lethal mutations in region 31, and the screens in which they were induced.

Table 1. Hemizygous lethal mutants in region 31. Screens were performed over the following deficiencies: Screen 1, *Df(2L)J106*; Screen 2, *Df(2L)J27*; Screen 3, *Df(2L)J27*; Screen 4, *Df(2L)J2* (see Brock, 1989); Screen 5, *Df(2L)J2* (Brock, 1989); and Screen 6, *Df(2L)J27* (Harrington, 1990).

Complementation Group	Deficiency Interval	Mutant	Mutagen	Screen
A102	1	*A102*	EMS	5
16-165	1	*16-165*	GAMMA	4
1-44	1	*1-44*	GAMMA	4
C98	2	*C98*	EMS	5
		27-168	GAMMA	4
		C104	EMS	5
F133	3	*F133*	EMS	5
B149	4	*B149*	EMS	5
		2-119	GAMMA	4
		19-153	GAMMA	4
14-140	4	*14-140*	GAMMA	4
24-127	5	*24-127*	GAMMA	4
		E15	EMS	5
		A63	EMS	5
		A141	EMS	5
		C35	EMS	5
		E56	EMS	5
		H30	EMS	5
		E2-1	EMS	2
		E2-12	EMS	2
		E2-13	EMS	2
		E2-32	EMS	2
		E2-42	EMS	2
E1-13	6	*E1-13*	EMS	1
		E1-17	EMS	1
		G2-5	EMS	6
E1-6	7	*E1-6*	EMS	1

Complementation Group	Deficiency Interval	Mutant	Mutagen	Screen
E1-7	7	*E1-7*	EMS	1
E1-22	7	*E1-22*	EMS	1
DG25	8	*23-127*	GAMMA	4
		29-142	GAMMA	4
		25-159	GAMMA	4
		D22	EMS	5
		C93	EMS	5
		E2-5	EMS	2
		E2-21	EMS	2
pim	9	*E1-15*	EMS	1
E2-15	9	*E2-15*	EMS	2
da	10	*F75*	EMS	5
		E2-20	EMS	2
		E2-35	EMS	2
		E2-30	EMS	2
		E2-24	EMS	2
		E1-21	EMS	2
		E1-26	EMS	1
		G2-10	GAMMA	6
		77-11	P ELEMENT	3
mfs48	10	*E2-43*	EMS	2
		77-13	P ELEMENT	3
		E1-12	EMS	1
Su(var)216/cdc2	10	*D57*	EMS	5
		E20	EMS	5
		B47	EMS	5
		E10	EMS	5
		E1-4	EMS	1
		E1-9	EMS	1
		E1-23	EMS	1

Complementation Group	Deficiency Interval	Mutant	Mutagen	Screen
		E1-24	EMS	1
E1-1	10	*E1-1*	EMS	1
		B35	EMS	5
		B26	EMS	5
		13-47	GAMMA	4
		13-83	GAMMA	4
		E2-16	EMS	2
		E2-31	EMS	2
		E2-38	EMS	2
		G2-3	GAMMA	6
		G2-7	GAMMA	6
E1-3	10	*E1-3*	EMS	1
		E113	EMS	5
		G2-	GAMMA	6
E1-12	10	*E1-12*	EMS	1
		E2-29	EMS	2
		E2-33	EMS	2
		G2-9	GAMMA	6
E1-19	10	*E1-19*	EMS	1
		E34	EMS	5
		G78	EMS	5
E1-28	10	*E1-28*	EMS	1
E2-17	10	*E2-17*	EMS	2
		B106	EMS	5
		E2-44	EMS	2
		G2-6	GAMMA	6
1(2)54	11	*H113*	EMS	5
		E2-24	EMS	2
		C70	EMS	5
		G2-4	EMS	6

Complementation Group	Deficiency Interval	Mutant	Mutagen	Screen
		E1-2	EMS	1
		E1-16	EMS	1
err	11	*A76*	EMS	5
		C36	EMS	5
		E1-27	EMS	1
E1-8	11	E1-8	EMS	1
E1-14	11	*E1-14*	EMS	1
E2-9	11	*E2-9*	EMS	2
		E2-23	EMS	2
77-12	11	*77-12*	P ELEMENT	3
77-14	11	*77-14*	P ELEMENT	3
E2-22	11	*E2-22*	EMS	2
29-85	12	*29-85*	EMS	5
		E2-34	EMS	2
		E2-2	EMS	2
		E2-7	EMS	2
E1-5	13	*E1-5*	EMS	1
		E1-11	EMS	1
		A61	EMS	5
		14-195	GAMMA	4
E1-18	13	*E1-18*	EMS	1
E1-25	13	*E1-25*	EMS	1
E3	13	*E3*	EMS	5
H95	14	*H95*	EMS	5
		B100	EMS	5
		E73	EMS	5
A65	15	*A65*	EMS	5
D121	15	*D121*	EMS	5
E24	15	*E24*	EMS	5
F15	15	*F15*	EMS	5

Complementation Group	Deficiency Interval	Mutant	Mutagen	Screen
G1	15	*G1*	EMS	5

APPENDIX 3. Miscellaneous complementation crosses for mutants in region 31.

Table 1. Complementation crosses for mutants in region 31. Ratios represent the proportion of *Cy* to straight-winged progeny recovered from the cross *mutant1/CyO* X *mutant2/CyO*.

Deficiency Interval	Locus	Cross	Progeny
2	*C98*	*C98* X *27-168*	276:0
		C98 X *C104*	264:0
		27-168 X *C104*	65:0
10	*E1-1*	*13-83* X *B26*	227:0
		B26 X *13-47*	107:0
		E2-31 X *G2-3*	163:0
		E2-31 X *G2-7*	143:0
		E2-31 X *E2-16*	446:0
		E2-31 X *E2-38*	79:0
		E2-16 X *E1-1*	403:46
		E2-16 X *E2-38*	410:0
		G2-3 X *E2-38*	142:0
		E1-1 X *G2-7*	160:11
10	*E1-3*	*E1-3* X *E113*	205:0
		G2-4 X *E113*	-[1]
10	*E1-12*	*E1-12* X *E2-29*	191:0
		E1-12 X *E2-33*	207:0
		E1-12 X *G2-9*	-[1]
10	*E1-19*	*E1-19* X *G78*	84:0
		G78 X *E34*	346:0
10	*E2-17*	*E2-17* X *B106*	43:0
		E2-44 X *B106*	190:14
		G2-6 X *B106*	-[1]
10		*G78* X *E34*	120:0
		E1-19 x *G78*	84:0
10	*mfs48*	*E2-43* x *mfs48*	194:0
		E2-43 x *77-13*	490:0
		E2-43 x *E1-10*	89:0

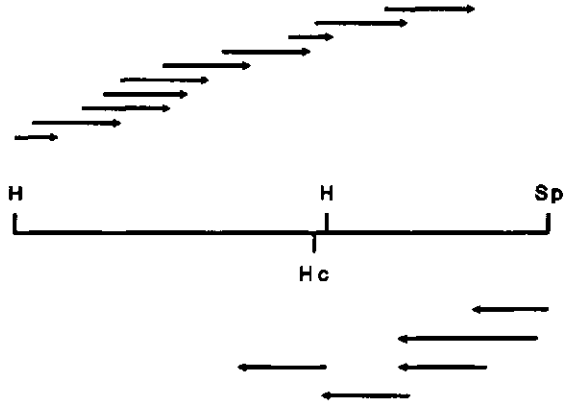
Deficiency Interval	Locus	Cross	Progeny
		E1-10 X *mfs48*	199:0
		77-13 X *mfs48*	1270:398[2]
11	*err*	*RE54* X *A76* (29°)	244:21[3]
		RE54 X *C36*	208:0
		A76 X *C36* (29°)	483:0
		C36 X *E1-27*	166:0
11	*E1-14*	*E1-14* x *33-161*	209:0
11	*E2-9*	*E2-9* x *E2-23*	163:0
11	*77-14*	*77-14* x *E1-8*	70:20
11	*77-12*	*77-12* X *E1-8*	67:15
12	*29-85*	*29-85* x *E2-34*	531:17
		29-85 x *E2-2*	405:2
		29-85 x *E2-7*	400:0
13	*E1-5*	*14-195* x *A65*	57:0
		E1-5 x *14-195*	130:0
		E1-11 x *14-195*	229:0
14	*H95*	*H95* x *B100*	226:18[3]
		H95 x *E73*	387:94[3]
		E73 x *B100*	57:10[3]

[1]Harrington (1990)
[2]data of I.P. Whitehead
[3]female sterile straight-winged progeny

APPENDIX 4. Sequencing data for the region surrounding *cdc2Dm*.

Figure 1. The sequencing strategy for pc800 and the gene it represents. Arrows indicate the direction of sequencing and the extent of the directed deletions. (see Chapter 3 for symbols).

A. Genomic DNA



B. cDNA

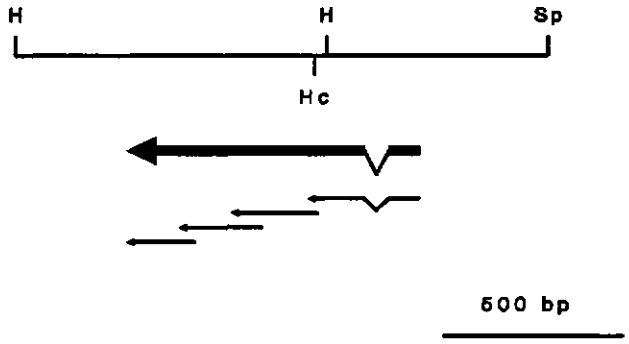


Figure 2. The complete genomic sequence of a region encompassing the genes represented by pc800 (underlined; nt 270-1150), pc1100 (*cdc2Dm*; double underlined; nt 1650-3100),and cBg14 (underlined; 3100-3250).

AAGCTTAAAA CACGACGAAC CAGGTAAATG GGTTGAAGAA ATTTATATAA

AAACTTAGTA TAAGTGTAAT AAGAAAAACT TTGGGTTTCA ATAGCTATAA

CACCATTTGG AAAATCCAAT TTTCAAATCT ACTATAAACA ACGTATAAGG

AAAAATAAAG AAAAAATATT AGAAATGTGT TTTTATAATG GTATCATAGC 200

TTCATACAAT TTTACTAAGG TCCTCGTTTT TAGTCCCCAT AAATCCAGGA

ATTATTTCGA TATTCCGCTC TCTTAAGACA TTCCCCTGAC ACAAATCCTA

ATCATAGATA TTAACAGGTA TATGAAAAGT TTATTGCATT GGAAAAACAG

TTTGTTGTGC ATTTAGCTCC ATCGGTAATG TGCGTAGGCG CGGTTGCTTT 400

CGCACAGCCT GTGCAGATCG TCCTTTCGCT TGATGACCCT TCCCTGGCCA

TGGGCTGCGT CGAGAATCTC CCAGGCCAAC TTTTCCGGCA GGGACACCTT

ACGCTCCTTC TCGCGGGCAG CTTCCAAGAG CCACTTCATG GCCAGAAAAT

ATGACCGCTT CGTGGTGATG GGAACAGGGA CTTGATAGGT GACACCACCA 600

CGTTTTATGG CGGTCACTTG AAGGAGCGGT CGGCAGTTTT CAACCGCTTG

TTTCAGAAGC GTTTCGGGAT TGGTGTTTAT GGTTGTCTTC TCCCCTTTGG

CCAGATTCAT GTGCTCCGTC TGGGTTCGTT TTATTAGCTC CAGCGTCTTG

GACAAAAGCG TTCTGGCCAA GGCACTGTTC CCCTTTTTCG TTATATAATT 800

GATCATTTTA TGTTTTGTAT CGTCGTGAAA GATGGTATCA GACGATTTGT

TGACCGCCGC CTTGATGGGC ACATGGTAAA GCTTGGACAG ATCATTCTTT

TGCTCTAGTT GTTTATATTT TTGTACAATT GGTTCGACAT AGTGTGTGGG

ATACACCGAT ATCAATCGAA GGCAGCTACA ATAAACAAAT GGGTGATCAG 1000

TCATTTGTGT TTTTAGAGAT AAGTAACACT TTAGAGAAGT TTAACTTACC

TGAGCCTTGA AGTTTTCTCT GCAATCCTAC CTAAAAGCGA CATGTTTACA

TCGTCTGCTG TGTCGGCAAA AAATAAAAAA ACTATGTTAT ATATATGTTA

TGCACATTTG CGGCATGCAT ATAGGTGTGT CAAGATATCG ACAAAGAGCT 1200

TAGTAATTTT GAAACAACAA AAATGAAGTA AAACAGTTGC GGTATTCCAA

TTACATTTTT TAAATTAATT TCTTTAGTAC CGTACTACTG GTACTCACCT

TCAAAAGATA TAAAATAGAA ATTAATTGCA CCAAAAACTC ATAAGTTAAT

TAATAGGATA TTAGCAGAAA CGTTTGTCTC CGAACTCAAA CAAAGTGATG 1400

TCTTAATTAA TTGAAATCAC TATAAAAAAA AGCGTGGAAT TTTACAGTAC

ACTAAAATTA ACTTTAAAAA AATTAACAAC ATTTTTAAGA TACAGCAATT

CAATAAGTTG CCTGAATATT GAGTTTCATT CCCACATTCC AAATGAATAA

ATGTAGCTAG CTTAGCATCG TTTAAACTGT CTGGTAATAC TAGAGCATAT 1600

ACGTCAAAAA CGCGCTAATT TAAAAGTCGG TGGCTTGCAA AGAAATAGCT

TAATAAATTA TGGAGGATTT TGAGAAAAGT GAGAAGATTG GCGAGGGCAC

ATATGGCGTG GTGGATAAGG GTCGCAACCG CCTGACGGGC CAAATTGTGG

CAATGAAGAA AATCCGCTTG GAGTCCGACG ACGAAGGCGT TCCATCAACC 1800

GCTATCAGGT AAAATGCCGC GGCTTGGACG CCCAAGCCTC AAAATGTGTC

ATGTTCTTTC GCGACTTTTT TCCATTCAAA TGGCGGATAT GCTTAATTGA

AAGTATGTTC CTTGACATCT TATGGGTTGG TTTGAAGATT TTGCAATATT

GTTTTTAATT TATACTGTGG AAGTCTAGCA TAATTATGTT ACGGCTTATG 2000

TTCATCATAC ATATGTGTGT GTATGTACAA TCCTATATGC GTACTTATGC

TCATGTGTAC ATCATCATAC TTTCTATTTA TGTTTATTAA TTGGAAGCCT

GCGAATACTT TCTGGTTCAA CTGATAGACC AATAACGAAA ATACTTTAAT

CACATGTTTT TTCTATAATG TAGTAATATT TAATATCCAT TACAGAGAAA 2200

TTTCGTTGCT TAAGGAGTTG AAACATGAAA ACATTGTCTG GTTGGAATGG

AGGAGAACCG CATATACTTG ATCTTTGAAT TCCTATCGAT GGACCTCAAG

AAATACATGG ATTCGCTGCC AGTTGATAAG CACATGGAGA GTGAATTGGT

CCGTAGCTAT TTGTACCAAA TAACTAGCGC CATTCTTTTC TGCCATCGTC 2400

GGCGAGTACT TCACCGTGAT CTTAAGCCGC AGAACTTACT AATCGACAAG

AGTGGCCTCA TAAAAGTCGC CGACTTTGGA CTTGGCCGAT CCTTTGGCAT

TCCGGTCTCC ATTTATACGC ACGAGATTGT TACCTTGTGG TACAGAGCGC

CGGAGGTGCT ACTGGGTTCA CCCCGGTATT CCTGTCCCGT CGATATCTGG 2600

TCCATTGGAT GCATATTCGC GGAGATGGCA ACGAGAAAGC CGCTATTCCA

GGGTGACTCG GAAATTGACC AGTTGTTTAG AATGTTTAGG TAACCACAGG

TAATTTACTT TCTATTCCCT GTGATACTCA CACTCATTGA TTGCAGAATT

CTGAAAACAC CTACCGAAGA CATTTGGCCG GGCGTTACTT CGCTACCCGA 2800

CTATAAGAAC ACGTTCCCCT GCTGGTCCAC GAACCAATTG ACCAATCAGT

TAAAGAATCT CGATGCGAAT GGTATTGATC TCATACAAAA GATGTTAATC

TACGATCCAG TTCATCGCAT TTCCGCCAAG GACATTTTGG AGCATCCCTA

TTTCAATGGT TTTCAATCGG GCTTAGTTCG AAATTAACGT TCGGTATTCT 3000

CGTTTGACTT TAACTAAGAA TTTTAAAACA AGAGATCTTG GTATCTAATC

TAAAGCAAAA TAGCCGTAAA TAAAACTAAG GGTGTAAAAC AATCAACGTT

TACTTAATTT ATGTAAGTGT ATTTACAGAT TTACGCCAAA TTACCAGCGC

TCTAACAGAA TAATAAGGCT TCAAGGCTTT AATTATTATA CAAAAAGAAA 3200

GTTAAATATG GAAATCCTCG TTGAAACTAG TCCTATTATA GAGAGCACTG

CTAGCACCGC TGTCATGACG TCTAAAGACG CCAAAGCAGC CGCGCCTCTT

CATCACGTAA GCACCAATGA TGGTCGGCAC CGCGATGAGC AGGACAACCA

AGAGTCCAAC CCATATAAGA CTGCCCGTCG AACTTTCTAT CATATGTTCA 3400

TTCGCCACTC TGAGCAGCGT AACATCGTTT AACTTGTTCG GACCGCCACA

TTTGACATCT TTGGCCAGAA CGGGCGTGGT CTTGTTGATT CGATCTATCA

AGACGTTGAT CAAGAAGTCA TTGGATTCGT CGCAATTCCA CGGGTTGAAT

CTAAGATCCA GAGCCTTAAG TTTATCCCAG CGCACTAGTA GTTCTTTCGG 3600

CAAAGTCGAC

Figure 3. The DNA sequence and conceptual translation of pc800.

```
          TAACATAGTTTTTTTATTTTTTGCCGACACAGCAGACGATGTAAAC

ATGTCGCTTTTAGGTAGGATTGCAGAGAAAACTTCAAGGCTCAGCTGCCTTCGA
MetSerLeuLeuGlyArgIleAlaGluLysThrSerArgLeuSerCysLeuArg

TTGATGTCGGTGTATCCCACACACTATGTCGAACCAATTGTACAAAAATATAAA
LeuMetSerValTyrProThrHisTyrValGluProIleValGlnLysTyrLys

CAACTATAGCAAAAGAATGATCTGTCCAAGCTTTACCATGTGCCCATCAAGGCG
GlnLeuGluGlnLysAsnAspLeuSerLysLeuTyrHisValProIleLysAla

GCGGTCAACAAATCGTCTTATACCATCTTTCACGACGATACAAAACATAAAATG
AlaValAsnLysSerSerAspThrIlePheHisAspAspThrLysHisLysMet

ATCAATTATATAACGAAAAAGGGGAACAGTGCCTTGGCCAGAACGCTTTTGTCC
IleAsnTyrIleThrLysLysLysAsnSerAlaLeuAlaArgThrLeuLeuSer

AAGACGCTGGAGCTAATAAAACGAACCCAGACGGAGCACATGAATCTGGCCAAA
LysThrLeuGluLeuIleLysArgThrGlnThrGluHisMetAsnLeuAlaLys

GGGGAGAAGACAACCATAAACACCAATCCCGAAACGCTTCTGAAACAAGCGGTT
GlyGluLysThrThrIleAsnThrAsnProGluThrLeuLeuLysGlnAlaVal

GAAAACTGCCGACCGCTCCTTCAAGTGACCGCCATAAAACGTGGTGGTGTCACC
GluAsnCysArgProLeuLeuGlnValThrAlaIleLysArgGlyGlyValThr

TATCAAGTCCCTGTTCCCATCACCACGAAGCGGTCATATTTTCTGGCCATGAAG
TyrGlnValProValProIleThrThrLysAsgSerTyrPheLeuAlaMetLys

TGGCTCTTGGAAGCTGCCCGCGAGAAGGAGCGTAAGGTGTCCCTGCCGGAAAAG
TrpLeuLeuGluAlaAlaArgGluLysGluArgLysValSerLeuProGluLys

TTGGCCTGGGAGATTCTCGACGCAGCCCATGGCCAGGGAAGGGTCATCAAGCGA
LeuAlaTrpGluIleLeuAspAlaAlaHisGlyGlnLysArgValIleLysArg

AAGGACGATCTGCACAGGCTGTGCGAAAGCAACCGCGCCTACGCACATTACCGA
LysAspAspLeuHisArgLeuCysGluSerAsnArgAlaTyrAlaHisTyrArg

TGGAGCTAAATGCACAACAAACTGTTTTTCCAATGCAATAAACTTTTCATATAC
TrpSer***

CTGTTAA
```

Figure 4. The sequence of the *P* element associated with *cdc2Dm* in region 31E. The 31 bp inverted repeats are underlined.

GCTAATTTAA AAGTCGGTGG CATGATGAAA TAACATAAGG TGGTCCCGTC

GAAAGCCGAA GCTTACCGAA GTATACACTT AAATTCAGTG CACGTTTGCT

TGTTGAGAGG AAAGGTTGTG TGCGGACGAA AAAACATTAA CCCTTACGTG

GAATAAAAAA AAATGAAATA TTGCAAATTT TGCTGCAAAG CTGTGACTGG

AGTAAAATTA ATTC..... .deletion. ....AATAATAA

TAATTTTGAA ATTACAAATA ATGTAAAGGA AAAATTAATA TTAGCAGCGC

GAAACGTCGA TGTTGATAAA CAAGTAAAAT CTTTTTATTT TAAAATTAGA

ATATATTTTA GAATTAAGTA CTTCAACAAA AAAATTGAAA TTAAAAANCA

AAAACAAAAG TTAATTGGAA ACTCCAAATT ATTAAAAATA AAACTTTAAA

AATAATTTCG TCTAATTAAT AATTCAAACC CCACGGACAT GCTAAGGGTT

AATCAACAAT CATATCGCTG TCTCACTCAG ACTCAATACG ACACTCAGAA

TACTATTCCT TTCACTCGCA CTTATTGCAA GCATACGTTA AGTGGATGTC

TCTTGCCGAC GGGACCACCT TATGTTATTT CATCATGGTC GGTGGCTTGC

REFERENCES

Alfageme, C.R., G.T. Rudkin, and L.H. Cohen (1980). Isolation, properties and cellular distribution of D1, a chromosomal protein of *Drosophila*. Chromosoma 78, 1-31.

Annunziato, A.T., L.-L.Y. Frado, R.L. Seale, and C.L.F. Woodcock (1988). Treatment with sodium butyrate inhibits the complete condensation of interphase chromatin. Chromosoma 96, 132-138.

Arion, D., L. Meijer, L. Brizuela, and D. Beach (1988). *cdc2* is a component of the M phase-specific histone H1 kinase: evidence for identity with MPF. Cell 55, 371-378.

Axton, M.J., V. Dombradi, P.T.W. Cohen, and D.M. Glover (1990). One of the protein phosphatase 1 isoenzymes in Drosophila is essential for mitosis. Cell 63, 33-46.

Bainbridge, S.P., and M. Bownes (1981). Staging the metamorphosis of *Drosophila melanogaster*. J. Embryol. Exp. Morph. 66, 57-80.

Baker, W.K. (1963). Genetic control of pigment differentiation in somatic cells. Am. Zool. 3, 57-69.

Baker, W. K. (1967). A clonal system of differential gene activity in *Drosophila*. Dev. Biol. 16, 1-17.

Baker, W.K. (1968). Position-effect variegation. Adv. Genet. 14, 133-169.

Barigozzi, C., S. Dolfini, M. Fraccaro, C.R. Raimondi, and L. Tiepolo (1966). *In vitro* study of the DNA replication patterns of somatic chromosomes of *Drosophila melanogaster*. Exp. Cell Research 43, 231-234.

Benton, W.D., and R.W. Davis (1977). Screening λ*gt* recombinant clones by hybridization to single plaques *in situ*. Science 196, 180-182.

Beverly, S.M., and A.C. Wilson (1984). Molecular evolution in *Drosophila* and higher Diptera. II. A time scale for fly evolution. J. Mol. Evol. 21, 1-13.

Biessmann, H., P. Kruger, C. Schroper, and E. Spindler (1981). Molecular cloning and preliminary characterization of a *Drosophila melanogaster* gene from a region adjacent to the centromeric beta-heterochromatin. Chromosoma 82, 493-503.

Blow, J.J., and P. Nurse (1990). A *cdc2*-like protein is involved in the initiation of DNA replication in *Xenopus* egg extracts. Cell 62, 855-862.

Boffa, L.C., R.J. Gruss, and V.A. Allfrey (1981). Manifold effects of sodium butyrate on nuclear functions. J. Biol. Chem. 256, 9612-9621.

Booher, R., and D. Beach (1987). Interaction between *cdc13*$^+$ and *cdc2*$^+$ in the control of mitosis in fission yeast; dissociation of the G_1 and G_2 roles of the *cdc2*$^+$ protein kinase. EMBO J. 6, 3441-3447.

Booher, R.N., C.E. Alfa, J.S. Hyams, and D.H. Beach (1989). The fission yeast cdc2/cdc13/suc1 protein kinase: regulation of catalytic activity and nuclear localization. Cell 58, 485-497.

Bradbury, E.M., R.J. Inglis, and H.R. Matthews (1974). Control of cell division by very lysine rich histone (f1) phosphorylation. Nature 247, 257-261.

Bradbury, E.M., R.J. Inglis, H.R. Matthews, and N. Sarner (1973). Phosphorylation of very lysine rich histone in *Physarum polycepharum*: correlation with chromosome condensation. Eur. J. Biochem. 33, 131-139.

Brock, J.-A. K. (1989). A genetic analysis of region 31 on chromosome 2 of *Drosophila melanogaster*. M.Sc. Thesis. University of British Columbia.

Brosseau, G.E. Jr. (1970). V-type position effects for *e*$^+$ and *ro*$^+$ in *Drosophila*. Dros. Inf. Serv. 45, 100.

Bryant, P.J. (1970). Cell lineage relationships in the imaginal wing disc of *Drosophila melanogaster*. *Dev. Biol. 22, 389-411.*

Carlson, J. (1982). Jonah genes. PhD. Thesis, Stanford.

Catcheside, D.G. (1947). The *P*-locus position effect of *Oenothera*. J. Genet. 48, 31-42.

Cattanach, B.M. (1974). Position effect variegation in the mouse. Genet. Res. Camb. 23, 291-306.

Caudy, M., E.H. Grell, C. Dambly-Chaudiere, A. Ghysen, L.Y. Jan, and Y.N. Jan (1988). The maternal sex determination gene *daughterless* has zygotic activity necessary for the formation of peripheral neurons in *Drosophila*. Genes Dev. 2, 843-852.

Caudy, M., H. Vassin, M. Brand, R. Tuma, L.Y. Jan, and Y.N. Jan (1988). *daughterless, a* Drosophila gene essential for both neurogenesis and sex determination, has sequence similarities to *myc* and the *achaete-scute* complex. Cell 55, 1061-1067.

Chou, P.Y., and S.D. Fassman (1974). Prediction of protein conformation. Biochem. 13, 222.

Christman, J.K., N. Weich, B. Schoenbrun, N.K. Schneiderman, and A. Acs (1980). Hypomethylation of DNA during differentiation of Friend erythroleukemia cells. J. Cell Biol. 86, 366-370.

Clark, S.H., and A. Chovnick (1986). Studies of normal and position-affected expression of *rosy* region genes in *Drosophila melanogaster*. Genetics 114, 819-840.

Clark-Adams, C.D., D. Norris, M.A. Osley, J.S. Fassler, and F. Winston (1988). Changes in histone gene dosage alter transcription in yeast. Genes Dev. 2, 150-159.

Cline, T.W. (1989). The affairs of *daughterless* and the promiscuity of developmental regulators. Cell 59, 231-234.

Cohen, J. (1962). Position effect variegation at several closely-linked loci in *Drosophila melanogaster,* Genetics 47, 647-659.

Cronmiller, C., P. Schedl, and T.W. Cline (1988). Molecular characterization of *daughterless*, a *Drosophila* sex determination gene with multiple roles in development. Genes and Development 2, 1666-1676.

Demerec, M., and H. Slizynska (1937). Mottled white 258-18 of *Drosophila melanogaster*. Chromosoma 33, 319-344.

Devlin, R.H., B. Bingham, and B.T. Wakimoto (1990). The organization and expression of the *light* gene, a heterochromatic gene of *Drosophila melanogaster*. Genetics 125, 129-140.

Dimitri, P., and C. Pisano (1989). Position effect variegation in *Drosophila melanogaster*. Relationship between suppression effect and the amount of *Y* chromosome. Genetics 122, 793-800.

Doree, M. (1990). Control of M-phase by maturation-promoting factor. Current Opinion Cell Biol. 2, 269-273.

Dorn, R., S. Heymann, R. Lindigkeit, and G. Reuter (1986). Suppressor mutation of position effect variegation in *Drosophila melanogaster* affecting chromatin properties. Chromosoma 93, 398-403.

Draetta, G. (1990). Cell cycle control in eukaryotes: molecular mechanisms of *cdc2* activation. TIBS 15, 378-383.

Dunphy, W.G., L. Brizuela, D. Beach, and J. Newport (1988). The Xenopus *cdc2* protein is a component of MPF, a cytoplasmic regulator of mitosis. Cell 54, 423-431.

Durrin, L.K., R.K. Mann, P.S. Kayne, and M. Grunstein (1991). Yeast histone H4 N-terminal sequence is required for promoter activation *in vivo*. Cell 65, 1023-1031.

D'Urso, F., R.L. Marraccino, Marshak, D.R., and J.M. Roberts (1990). Cell cycle control of DNA replication by a homolog from human cells of the p34 *cdc2* protein kinase. Science 250, 786-791.

Edgar, B.A., and P.H. O'Farrell (1989). Genetic control of cell division patterns in the Drosophila embryo. Cell 57, 177-187.

Edgar, B.A., and P.H. O'Farrell (1990). The three postblastoderm cell cycles of *Drosophila* embryogenesis are regulated in G2 by *string*. Cell 62, 469-480.

Edgar, B.A., C.P. Kiehle, and G. Schubiger (1986). Cell cycle control by the nucleo-cytoplasmic ratio in early *Drosophila* development. Cell 44, 365-372.

Edgar, B.A., and G. Schubiger (1986). Parameters controlling transcriptional activation during early *Drosophila* development. Cell 44, 871-877.

Eissenberg, J.C. (1989). Position effect variegation in *Drosophila*, towards a genetics of chromatin assembly. Bioessays 11, 14-17.

Eissenberg, J.C., T.C. James, D.M. Foster-Hartnett, T. Hartnett, V. Ngan, and S. Elgin (1990). Mutation in a heterochromatin-specific chromosomal protein is associated with suppression of position-effect variegation in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA 87, 9923-9927.

Engels, W.R., W.K. Benz, C.R. Preston, P.L. Graham, R.W. Phillis, and H.M. Robertson (1987). Somatic effects of *P* element activity in *Drosophila melanogaster*. Genetics 117, 745-757.

Feiler, H.S., and T.W. Jacobs (1990). Cell division in higher plants, a *cdc2* gene, its 34-kDa product, and histone H1 kinase activity in pea. Proc. Natl. Acad. Sci. USA 87, 5397-5401.

Felsenfeld, G., and J.D. McGhee (1986). Structure of the 30 nm chromatin fiber. Cell 44, 375-377.

Feinberg, A.P., and B. Vogelstein (1983). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132, 6-13.

Feinberg, A.P., and B. Vogelstein (1984). A technique for radiolabbeling DNA restriction endonuclease fragments to high specific activity: addendum. Anal Biochem. 137, 266-267.

Foe, V.E. (1989). Mitotic domains reveal early commitment of cells in *Drosophila* embryos. Development 107, 1-22.

Foe, V.E., and B.M. Alberts (1985). Reversible chromosome condensation induced in *Drosophila* embryos by anoxia: visualization of the interphase nuclear organization. J. Cell Biol. 100, 1623-1636.

Frischauf, A.M., H. Leharch, A. Poustka, and N. Murray (1983). Lambda replacement vectors carrying polylinker sequences. J. Mol. Biol. 170, 827-842.

Gall, J.G., E.H. Cohen, and M.L. Polan (1971). Repetitive DNA sequences in *Drosophila*. Chromosoma 33, 319-344.

Garcia-Bellido, A., and J.R. Merriam (1969). Cell linelage of the imaginal disks in *Drosophila* gynandromorphs. J. Exp. Zool. 170, 61-76.

Garcia-Bellido, A., and J.R. Merriam (1971). Parameters of the wing imaginal disc development of *Drosophila melanogaster*. Dev. Biol. 24, 61-87.

Gaunt, S.J., and P.M. Singh (1990). Homeogene expression patterns and chromosomal imprinting. Trends Genet. 6, 208-212.

Gautier, J., C. Norbury, M. Lokha, P. Nurse, and J. Maller (1988). Purified maturation-promoting factor contains the product of a Xenopus homolog of the fission yeast cell cycle control gene *cdc2*. Cell 54, 433-439.

Glover, D.M. (1991). Mitosis in the *Drosophila* embryo - in and out of control. Trends Genet. 7, 125-131.

Grunstein, M., and D. Hogness (1975). Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. USA. 72, 3961-3965.

Gowen, J.W., and E.H. Gay (1934). Chromosome constitution and behavior in ever-sporting and mottling in *Drosophila melanogaster*. Genetics 19, 189-208.

Gowen, J.W., and E.H. Gay (1935). Effect of temperature on sporting eye color in *Drosophila melanogaster*. Science 77, 312.

Gould, K.L., and P. Nurse (1989). Tyrosine phosphorylation of the fission yeast *cdc2*$^{+}$ protein kinase regulates entry into mitosis. Nature 342, 39-45.

Gsell, R. (1971). Untersuchungen zur stabilitat einer *yellow* position-effekt-variegation in imaginalscheiben-kulturen von *Drosophila melanogaster*. Molec. Gen. Genet. 110, 218-237.

Hadwiger, J.A., C. Wittenberg, M.A. de Barros Lopez, H.E. Richardson, and S.I. Reed (1989). A family of cyclin homologs that control G_1 phase in yeast. Proc. Natl. Acad. Sci. USA 86, 6255-6259.

Han, M., and M. Grunstein (1988). Nucleosome loss activates yeast downstream promoters *in vivo*. Cell 55, 1137-1145.

Hanks, S.K., A.M. Quinn, and T. Hunter (1988). The protein kinase family, conserved features and deduced phylogeny of the catalytic domains. Science 241, 42-52.

Harrington, M. (1990). Suppression of position-effect variegation in *Drosophila melanogaster* by antimorphic mutations of heterochromatin protein components. B.Sc. Honours Thesis. University of British Columbia.

Hartmann-Goldstein, I.J. (1967). On the relationship between heterochromatinization and variegation in *Drosophila*, with special reference to temperature-sensitive periods. Genet. Res. 10, 143-159.

Hayashi, S., A. Ruddell, D. Sinclair, and T. Grigliatti (1990). Chromosomal structure is altered by mutations that suppress or enhance position effect variegation. Chromosoma 99, 391-400.

Hayashida, H., and T. Miyata (1983). Unusual evolutionary conservation and frequent DNA segment exchange in class 1 genes of the major histocompatibility complex. Proc. Natl. Acad. Sci. USA 80, 2671-2675.

Healy, M.J., R.J. Russell, and G.L.G. Miklos (1988). Molecular studies on interspersed repetitive and unique sequences in the region of the complementation group uncoordinated on the *X* chromosome of *Drosophila melanogaster*. Mol. Gen. Genet. 213, 63-71.

Heitz, E. (1934). Uber alpha und beta-heterochromatin sowei Konstanz und Bau der Chromeren bei Drosophila. Biol. Zentralbl. 54, 588-609.

Henikoff, S. (1979). Position-effects and variegation enhancers in an autosomal region of *Drosophila melanogaster*. Genetics 93, 106-115.

Henikoff, S. (1981). Position-effect variegation and chromosome structure of a heat shock puff in *Drosophila*. Chromosoma 83, 381-393.

Henikoff, S. (1984). Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene 28, 351-359.

Henikoff, S. (1990). Position-effect variegation after 60 years. Trends Genet. 6, 422-426.

Henikoff, S., and T.D. Dreesen (1989). Trans-inactivation of the *Drosophila brown* gene: evidence for transcriptional repression and somatic pairing dependence. Proc. Natl. Acad. Sci. USA. 86, 6704-6708.

Hess, O. (1970). Genetic function correlated with unfolding of lampbrush loops by the Y chromosome spermatocytes of *Drosophila hydei*. Molec. Gen. Genet. 106, 328-346.

Hessler, A.Y. (1958). V-type position effects at the light locus in *Drosophila melanogaster*. Genetics 43, 395-403.

Hilliker, A.J., R. Appels, and A. Schalet (1980). The genetic analysis of *D. melanogaster* heterochromatin. Cell 21, 607-619.

Hindley, J., and G.A. Phear (1984). Sequence of the cell division gene CDC2 from *Schizosaccharomyces pombe*; patterns of splicing and homology to protein kinases. Gene 31, 129-134.

Hinton, T. (1949). The modification of the expression of a position effect. Am. Nat. 83, 69-94.

Hinton, T., and W. Goldsmith (1950) An analysis of phenotypic reversions at the *brown* locus in *Drosophila*. J. Exp. Zool. 114, 103-114.

Hultmark, D., R. Klemenz, and W.J. Gehring (1986). Translational and transcriptional control elements in the untranslated leader of the heat-shock gene *hsp22*. Cell 44, 429-438.

Hunter, T. (1987). A thousand and one protein kinases. Cell 50, 823-829.

Igarashi, M., A. Nagata, S. Jinno, K. Suto, and H. Okayama (1991). *Wee1*$^{+}$-like gene in human cells. Nature 353, 80-83.

Jackson, D.A. (1991). Structure-function relationships in eukaryotic nuclei. Bioessays 13, 1-10.

James, T.C., and S.C.R. Elgin (1986). Identification of a nonhistone chromosomal protein associated with heterochromatin in *Drosophila melanogaster* and its gene. Mol. Cell. Biol. 6, 3862-3872.

James, T.C., J.C. Eissenberg, C. Craig, V. Dietrich, A. Hobson, and S.C.R. Elgin (1989). Distribution patterns of HP1, a hetrochromatin-associated nonhistone chromosomal protein of *Drosophila*. Eur. J. Cell Biol. 50, 170-180.

Janning, W. (1970). Bestimmung des heterochromatisierungsstadiums beim *white*-positionseffekt mittels rontgeninduzierter mitotischer rekombination in der augenanlage von *Drosophila melanogaster*. Molec. Gen. Genet. 107, 128-149.

Jimenez, J., L. Alphey, P. Nurse, and D.M. Glover (1990). Complementation of fission yeast *cdc2*^{ts} and *cdc25*^{ts} mutants identifies two cell cycle genes from *Drosophila*, a *cdc2* homologue and *string*. EMBO 9, 3565-3571.

Jowett, T. (1986). Preparation of nucleic acids. In, D.B. Roberts (ed.) *Drosophila*, a practical approach. IRL Press, Washington D.C. pp 275-286.

Judd, B.H. (1955). Direct proof of a variegated-type position effect at the *white* locus in *Drosophila melanogaster*. Genetics 196, 739-744.

Jurgens, G. (1985). A group of genes controlling the spatial expression of the bithorax complex in *Drosophila*. Nature 316, 153-155.

Karlik, C.C., J.W. Mahaffey, M.D. Coutu, and E.A. Fyrberg (1984). Organization of contractile protein genes within the 88F subdivision of the *D. melanogaster* third chromosome.

Karpen, G.H., and A.C. Spradling (1990). Reduced DNA polytenization of a minichromosome region undergoing position-effect variegation in *Drosophila*. Cell 63, 97-107.

Kassis, J.A., C. Desplan, D.K. Wright, and P.H. O'Farrell (1989). Evolutionary conservation of homeodomain-binding sites and other sequences upstream and within the major transcription unit of the *Drosophila* segmentation gene *engrailed*. Mol. Cell. Biol. 9, 4304-4311.

Kassis, J.A., M.L. Wong, and P.H. O'Farrell (1985). Electron microscope heteroduplex mapping identifies regions of the engrailed locus that are conserved between *Drosophila melanogaster* and *Drosophila virilis*. Mol. Cell. Biol. 5, 3600-3609.

Kayne, P.S., U.-J. Kim, M. Han, J.R. Mullen, F. Yoshizaki, and M. Grunstein (1988). Extremely conserved histone H4 N terminus is dispensible for growth but essential for repressing the silent mating loci in yeast. Cell 55, 27-39.

Khesin, R.B., and B.A. Bashkirov (1979). Influence of deficiency of the histone gene-containing 38B-40 region on X-chromosome template activity and the white gene position effect variegation in *Drosophila melanogaster*. Mol. Gen. Genet. 162, 323-328.

Kidwell, M.G. (1986). P-M mutagenesis. In, D.B. Roberts (ed.) *Drosophila*, a practical approach. IRL Press, Washington D.C. pp 59-81.

Knighton, D.R., J. Zheng, L.F.T. Eyck, V.A. Ashford, N-H. Xuong, S.S. Taylor, and J.M. Sowadski (1991). Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. Science 253, 407-414.

Kornher, J.S., and S.A. Kauffman (1986). Variegated expression of the *Sgs-4* locus in *Drosophila melanogaster*. Chromosoma 94, 205-216.

Krek, W., and E.A. Nigg (1989). Structure and developmental expression of the chicken *CDC2* kinase. EMBO 8, 3071-3078.

Labbe, J.C., A. Picard, G. Peaucellier, J.C. Cavadore, P. Nurse, and M. Doree (1989). Purification of MPF from starfish, identification as the H1 histone kinase $p34^{cdc2}$ and a possible mechanism for its periodic activation. Cell 57, 253-263.

Lakhotia, S.C. (1974). EM autoradiographic studies on polytene nuclei of *Drosophila melanogaster*. III. Localisation of non-replicating chromatin to the chromocentre heterochromatin. Chromosoma 46, 145-160.

Langan, T.A., J. Gautier, M. Lokha, R. Hollingsworth, S. Moreno, P. Nurse, J. Maller, and R.A. Scalfani (1989). Mammalian growth associated histone H1 kinase, a homolog of *cdc2⁺/CDC28* protein kinases controlling mitotic entry in yeast and frog cells. Mol. Cell. Biol. 9, 3860-3868.

Lasko, P.F., and M.L. Pardue (1988). Studies of the genetic organization of the *vestigial* microregion of *Drosophila melanogaster*. Genetics 120, 495-502.

Lawrence, P.A., S.M. Green, and P. Johnston (1978). Compartmentalization and growth of the *Drosophila* abdomen. J. Embryol. Exp. Morph. 43, 233-245.

Lee, M.G., and P. Nurse (1987). Complementation used to clone a human homolog of the fission yeast cell cycle control gene *cdc2*. Nature 327,680-685.

Lefevre, G. Jr. (1976). A photographic representation and interpretation of the polytene chromosome of *Drosophila melanogaster* salivary glands. pp. 36-61. In, The Genetics and Biology of Drosophila, Vol. 1a, Edited by M. Ashburner and E. Novitski. Academic Press, New York.

Lefevre, G., and W. Watkins (1986). The question of the total gene number in *Drosophila melanogaster*. Genetics 113, 869-895.

Lehner, C.F., and P. O'Farrell (1989). Expression and function of *Drosophila* cyclin A during embryonic cell cycle progression. Cell 56, 957-968.

Lehner, C.F., and P. O'Farrell (1990a). The roles of *Drosophila* cyclins A and B in mitotic control. Cell 61, 535-547.

Lehner, C.F., and P. O'Farrell (1990b). *Drosophila cdc2* homologs, a functional homolog is coexpressed with a cognate variant. EMBO 9, 3573-3581.

Lewis, E.B. (1978). A gene complex controlling segmentation in *Drosophila*. Nature 276, 565-570.

Lewis, E.B., and F. Bacher (1968). Method of feeding ethyl methanesulfonate (EMS) to Drosophila males. Drosophila Inform. Serv. 43, 193.

Lindsley, D.E., L.S.B. Goldstein, and L. Sandler (1980). Male sterility in maternal-effect mutants. Drosophila Inform. Ser. 55, 84-85.

Lindsley, D.L., and E.H. Grell (1968). Genetic variations of *Drosophila melanogaster*. Carnegie Inst. Wash. Publ. 627.

Lindsley, D.L., and G. Zimm (1986). The genome of *Drosophila melanogaster*.Part 1, Genes A-K. Drosophila Inform. Ser. 64, 1-227.

Lindsley, D.L., and G. Zimm (1986). The genome of *Drosophila melanogaster*. Part 2, lethals; maps. Drosophila Inform. Ser. 65, 1-158.

Locke, J., M.A. Kotarski, and K. D. Tartoff (1988). Dosage-dependent modifiers of position effect variegation in *Drosophila* and a mass action model that explains their effect. Genetics 1320, 181-198.

Lorincz, A.T., and S.I. Reed (1986). Sequence analysis of temperature-sensitive mutations in the *Saccharomyces cerevisiae* gene *CDC28*. Mol. Cell Biol. 6, 4099-4103.

Madhavan, M.M., and K. Madhavan (1980). Morphogenesis of the epidermis of adult abdomen of *Drosophila*. J. Embryol. exp. Morph. 60, 1-31.

Mahowald, A.P. (1963). Electron microscopy of the formation of the cellular blastoderm in *Drosophila melanogaster*. Exp. Cell Res. 32, 457-468.

Maniatis, T., E. R. Fritsch, and J. Sambrook (1982). Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Mange, A.P., and L. Sandler (1973). A note on the maternal effect mutants *daughterless*, and *abnormal oocyte* in *Drosophila melanogaster*. Genetics 73, 73-86.

McGhee, J.D., and G. Felsenfeld (1980). Nucleosome structure. Annu. Rev. Biochem. 49, 1115-1156.

Meijer, L., D. Arion, R. Golsteyn, J. Pines, L. Brizuela, T. Hunt, and D. Beach (1989). Cyclin is a component of the sea urchin egg M-phase specific histone H1 kinase. EMBO J. 8, 2275-2282.

Michailidis, J., N.D. Murray, and J.A. Marshall Graves (1988). A correlation between development time and variegated position-effect in *Drosophila melanogaster*. Genet. Res. 52, 119-123.

Miklos, G.L.G., M.J. Healy, P. Pain, A.J. Howells, and R.J. Russell (1984). Molecular and genetic stuies on the euchromatin-heterochromatin transition region of the X chromosome of *Drosophila melanogaster*. I. A cloned entry point near the *uncoordinated (unc)* locus. Chromosoma 89, 218-227.

Moore, G.D., J.D. Procunier, D.P. Cross, and T.A. Grigliatti (1979). Histone gene deficiencies and position effect variegation in *Drosophila*. Nature 282, 312-314.

Moore, G.D., D.A. Sinclair, and T. Grigliatti (1983). Histone gene multiplicity and position-effect variegation in *Drosophila melanogaster*. Genetics 105, 327-344.

Moreno, S., J. Hayles, and P. Nurse (1989). Regulation of *p34^{cdc2}* protein kinase during mitosis. Cell 58, 361-372.

Moreno, S., and P. Nurse (1990). Substrates for p34^{cdc2}: *In vivo* veritas? Cell 61, 549-551.

Morla, A., G. Draetta, D. Beach, and J. Wang (1989). Reversible tyrosine phosphorylation of *cdc2*, dephosphorylation accompanies activation during entry into mitosis. Cell 58, 193-203.

Mottus, R., R. Reeves, and T.A. Grigliatti (1980). Butyrate suppression of position-effect variegation in *Drosophila melanogaster*. Mol. Gen. Genet. 178, 465-469.

Nash, D., and F.C. Janca (1983). Hypomorphic lethal mutations and their implications for the interpretation of lethal complementation studies in Drosophila. Genetics 105, 957-968.

Norbury, C.J., and P. Nurse (1989). Control of the higher eukaryotic cell cycle by p34*cdc2* homologues. Biochimica et Biophysica Acta 989, 85-95.

Noujdin, N.I. (1936). Genetic analysis of certain problems of the physiology of development of *Drosophila melanogaster*. Biol. Zh. (Mosk.) 4, 571-624.

Nurse, P. (1990). Universal control mechanism regulating the onset of M-phase. Nature 344, 503-508.

Nurse, P., and Y. Bissett (1981). Gene required in G1 for commitment to cell cycle and in G2 for control of mitosis in fission yeast. Nature 292, 558-560.

Nurse, P., and P. Thuriaux (1980). Regulatory genes controlling mitosis in the fission yeast *Schizosaccharomyces pombe*. Genetics 96, 627-637.

Nusslein-Volhard, C., E. Wieschaus, and H. Kluding (1984). Mutation affecting the pattern of the larval cuticle in *Drosophila melanogaster*, 1. Zygotic loci on the second chromosome. Wilhelms Roux's Arch. Dev. Biol. 193, 267-282.

O'Farrell, P.H., B.A. Edgar, D. Lakich, and C.F. Lehner (1989). Directing cell division during development. Science 246, 635-640.

O'Hare, K., and G.M. Rubin (1983). Structures of *P* transposable elements and their sites of insertion and excision in the *Drosophila melanogaster* genome. Cell 34, 25-35.

Paro, R., and D.S. Hogness (1991). The polycomb protein shares a homologous domain with a heterochromatin-associated protein of *Drosophila*. Proc. Natl. Acad. Sci. USA 88, 263-267.

Paro, R. (1990). Imprinting a determined state into the chromatin of *Drosophila*. Trends Genet. 6, 416-421.

Perler, F., A. Efstratiadis, P. Lomedico, W. Gilbert, R. Kolodner, and J. Dodgson (1980). The evolution of genes, the chicken preproinsulin gene. Cell 20, 555-566.

Picard, A., J. Cavadore, P. Lory, J. Berenengo, C. Ojeda, and M. Doree (1990). Microinjection of a conserved peptide sequence of $p34^{cdc2}$ induces a Ca^{2+} transient in oocytes. Science 247, 327-329.

Pines, J., and T. Hunter (1989). Isolation of a human cyclin cDNA, evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with $p34^{cdc2}$. Cell 58, 833-846.

Proudfoot, N.J., and G.G. Brownlee (1976). 3' non-coding region sequences in eukaryotic messenger RNA. Nature 263, 211-214.

Reuter, G., R. Dorn, and H.J. Hoffmann (1982). Butyrate sensitive suppressor of position-effect variegation mutations in *Drosophila melanogaster*. Mol. Gen. Genet. 188, 480-485.

Reuter, G., R. Dorn, G. Wustmann, B. Friede, and G. Rauh (1986). Third chromosome suppressor of position-effect variegation loci in *Drosophila melanogaster*. Mol. Gen. Genet. 202, 481-487.

Reuter, G., J. Gausz, H. Gyurkovics, B. Friede, R. Bang, A. Spierer, L.M.C. Hall, and P. Spierer (1987). Modifiers of position-effect variegation in the region from 86-88B of the *Drosophila melanogaster* third chromosome. Mol. Gen. Genet. 210, 429-436.

Reuter, G., M. Giarre, J. Farah, J. Gausz, A. Spierer, and P. Spierer (1990). Dependence of position-effect variegation in *Drosophila* on dose of a gene encoding an unusual zinc-finger protein. Nature 344, 243-244.

Reuter, G., and J. Szidonya (1983). Cytogenetic analysis of variegation suppressors and a dominant temperature-sensitive lethal in region 23-26 of chromosome 2L in *Drosophila melanogaster*. Chromosoma 88, 277-285.

Reuter, G., W. Werner, and H.J. Hoffmann (1982). Mutants affecting position-effect heterochromatization in *Drosophila melanogaster*. Chromosoma 85, 539-551.

Reuter, G., and I. Wolff (1981). Isolation of dominant suppressor mutations for position-effect variegation in *Drosophila melanogaster*. Mol. Gen. Genet. 182, 516-519.

Reuter, G., I. Wolff, and B. Friede (1985). Functional properties of the heterochromatic sequences inducing w^{m4} position-effect variegation in *Drosophila melanogaster*. Chromosoma 93, 132-139.

Richardson, H.E., C.S. Stueland, J. Thomas, P. Russell, and S.I. Reed (1990). Human cDNAs encoding homologs of the small $p34^{cdc28-cdc2}$-associated protein of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Genes Dev. 4, 1332-1344.

Robertson, H.M., C.R. Preston, R.W. Phillis, D.M. Johnson-Schlitz, W.K. Benz, and W.R. Engels (1988). A stable source of *P* element transposase in *Drosophila melanogaster*. Genetics 118, 461-470.

Roseland, C.R., and H.A. Schneiderman (1979). Regulation and metamorphosis of the abdominal histoblasts of *Drosophila melanogaster*. Wilhelm Roux's Archives 186, 235-265.

Roth, S.Y., M.P. Collini, G. Draetta, D. Beach, and C.D. Allis (1991). A cdc2-like kinase phosphorylates histone H1 kinase in the amitotic macronucleus of *Tetrahymena*. EMBO 10, 2069-2075.

Roth, S.Y., A. Dean, and R.T. Simpson (1990). Yeast alpha-2 repressor positions nucleosomes in *TRP1/ARS1* chromatin. Mol. Cell. Biol. 10, 2247-2260.

Roth, S.Y., I.G. Schulman, R. Richman, R.G. Cook, and C.D. Allis (1988). Characterization of phosphorylation sites in histone H1 in the amitotic macronucleus of *Tetrahymena* during different physiological states. J. Cell Biol. 107, 2473-2482.

Rudkin, G.T. (1969) Non-replicationg DNA in *Drosophila*. Genetics (Suppl.) 61, 227-238.

Rushlow, C.A., W. Bender, and A. Chovnick (1984). Studies on the mechanism of heterochromatic position effect at the rosy locus of *Drosophila melanogaster*. Genetics 108, 603-615.

Russell, P., and P. Nurse (1986). *cdc25*$^{+}$ functions as an inducer in the mitotic control of fission yeast. Cell 45, 145-153.

Russell, P., and P. Nurse (1987). Negative regulation of mitosis by *wee1*$^{+}$, a gene encoding a protein kinase homolog. Cell 49, 559-567.

Russell, P., S. Moreno, and S.I. Reed (1989). Conservation of mitotic controls in fission and budding yeasts. Cell 57, 295-303.

Sadhu, K., S.I. Reed, H. Richardson, and P. Russell (1990). Human homolog of fission yeast cdc25 mitotic inducer is predominantly expressed in G2. Proc. Natl. Acad. Sci. USA 87, 5139-5143.

Salas, F., and J.A. Lengyel (1984). New Mutants. Drosophila Inform. Ser. 60, 243-244.

Sandler, L. (1977). Evidence for a set of closely-linked autosomal genes that interact with sex chromosome heterochromatin in *Drosophila melanogaster*. Genetics 86, 567-582.

Schlissel, M.S., and D.D. Brown (1984). The transcriptional regulation of *Xenopus* 5S RNA genes in chromatin, the roles of active stable transcription complexes and histone H1. Cell 37, 903-913.

Schultz, J. (1950). Interrelations of factors affecting hetrochromatin-induced variegation in *Drosophila*. Genetics 35, 134.

Schultz, J. (1956). The relation of heterochromatic chromosome regions to the nucleic acid content of the cell. Cold Spring Harbor Symp. Quant. Biol. 21, 307-327.

Shupbach, T., and E. Wieschaus (1986). Maternal-effect mutations altering the anterior-posterior pattern of the *Drosophila* embryo. Wilhelms Roux's Arch. Dev. Biol. 195, 302-317.

Schupbach, T., and E. Wieschaus (1989). Female sterile mutations on the second chromosome of *Drosophila melanogaster*. I. Maternal effect mutations. Genetics 121, 101-117.

Shuttleworth, J., R. Godfrey, and A. Colman (1990). $p40^{MO15}$, a *cdc2*-related protein kinase involved in negative regulation of meiotic maturation in *Xenopus* oocytes. EMBO 9, 3233-3240.

Simon, J.A., C.A. Sutton, R.B. lobell, R.L. Glaser, and J.T. Lis (1985). Determinants of heat shock-induced chromosome puffing. Cell 40, 805-817.

Sinclair, D.A.R., R.C. Mottus, and T.A. Grigliatti (1983). Genes which suppress position-effect variegation in *Drosophila melanogaster* are clustered. Mol. Gen. Genet. 191, 326-333.

Sinclair, D.A.R., Y.K. Lloyd, and T.A. Grigliatti (1989). Characterization of mutations that enhance position effect variegation in *Drosophila melanogaster*. Mol. Gen. Genet. 216, 328-333.

Sinclair, D.A.R., A.A. Ruddell, J.K. Brock, N.J. Clegg, V.K. Lloyd, and T.A. Grigliatti (1991). A cytogenetic and genetic characterization of a group of closely-linked second chromosome mutations that suppress position-effect variegation in *Drosophila melanogaster*. Genetics, in press.

Singh, P., J.R. Miller, J. Pearce, R. Kothary, R.D. Burton, R. Paro, T.C. James, and S.J. Gaunt (1991). A sequence motif found in a *Drosophila* heterochromatin protein is conserved in animals and plants. Nucl. Acids Res. 19, 789-793.

Slatis, H.M. (1955). Position effects at the *brown* locus om *Drosophila melanogaster*. Genetics 40, 5-23.

Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503-517.

Spierer, P., A. Spierer, W. Bender, and D.S. Hogness (1983). Molecular mapping of genetic and chromomeric units in *Drosophila melanogaster*. J. Mol. Biol. 168, 35-50.

Spofford, J.B. (1976). Position-effect variegation in *Drosophila*. In, Ashburner, M., and E. Novitski (eds) The genetics and biology of *Drosophila*, Vol. 1c. Academic Press, New York, pp 955-1018.

Spofford, J. (1967). Single-locus modification of position-effect variegation in *Drosophila melanogaster*. I. *White* variegation. Genetics 57, 751-766.

Spofford, J. (1969). Single-locus modification of position-effect variegation in *Drosophila melanogaster*. II. Region 3C loci. Genetics 62, 555-571.

Spradling, A.C., and G.H. Karpen (1990) Sixty years of mystery. Genetics 126, 779-784.

Spradling, A.C., and G.M. Rubin (1981). *Drosophila* genome organization: conserved and dynamic aspects. Annu. Rev. Genet. 15, 219-264.

Stern, C., and M. Kodani (1955). Studies on the position effect at the *cubitus interruptus* locus of *Drosophila melanogaster*. Genetics 40, 343-373.

Strausfeld, U., J.C. Labbe, D. Fesquet, J.C. Cavadore, A. Picard, K. Sadhu, P. Russell, and M. Doree (1991). Dephosphorylation and activation of a $p34^{cdc2}$/cyclin B complex *in vitro* by human CDC25 protein. Nature 351, 242-245.

Sullivan, W., J.S. Minden, and B.M. Alberts (1990). *daughterless-abo-like*, a *Drosophila* maternal-effect mutation that exhibits abnormal centrosome separation during the late blastoderm divisions. Development 110, 311-323.

Surana, U., H. Robitsch, C. Price, T. Schuster, I. Fitch, A.B. Futcher, and K. Nasmyth (1991). The role of CDC28 and cyclins during mitosis in the budding yeast S. cerevisiae. Cell 65, 145-161.

Suzuki, M. (1989). SPXX, a frequent sequence motif in gene regulatory proteins. J. Mol. Biol. 207, 61-84.

Szabad, J., G. Reuter, and M-B. Schroeder (1988). The effects of two mutations connected with chromatin function on female germ-line cells of *Drosophila melanogaster*. Mol. Gen. Genet. 211, 56-62.

Szidonya, J., and G. Reuter (1988). Cytogenetic analysis of the *echnoid (ed), dumpy (dp)* and *clot (cl)* region in *Drosophila melanogaster*. Genet. Res., Camb. 51, 197-208.

Tabor, S., and C.C. Richardson (1987). DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. Proc. Natl. Acad. Sci. USA 84, 4767-4771.

Tartof, K.D., C. Bishop, M. Jones, C.A. Hobbs, and J. Locke (1989). Towards an understanding of position effect variegation. Dev. Genet. 10, 162-176.

Tartof, K.D., C. Hobbs, and M. Jones (1984). A structural basis for variegating position effects. Cell 37, 869-878.

Wakimoto, B.T., and M.G. Hearn (1990). The effects of chromosome rearrangements on the expression of heterochromatic genes in chromosome 2L of *Drosophila melanogaster*. Genetics 125, 141-154.

Whitfield, J.M., G.F. Gonzalez, E. Sanchez-Herrero, and D.M. Glover (1989). Transcripts of one of the two *Drosophila* cyclin genes become localized in pole cells during embryogenesis. Nature 338, 337-340.

Whiting, J.H. Jr., J.L. Farmer, and D.E. Jeffery (1987). Improved *in situ* hybridization and detection of biotin-labeled *D. melanogaster* DNA probes hybridized to *D. virilis* salivary gland chromosomes. Drosophila Inform. Ser. 66, 170-171.

Wood, W.B. (1988). The nematode *Caenorhabditis elegans*. pp.1-667. Cold Spring Harbor Laboratory.

Workman, J.L., and R.G. Roeder (1987). Binding of transcription factor TFIIID to the major late promoter during *in vitro* nucleosome assembly potentiates subsequent initiation by RNA polymerase II. Cell 51, 613-622.

Wustmann, G., J. Szidonya, H. Taubert, and G. Reuter (1989). The genetics of position-effect variegation modifying loci in *Drosophila melanogaster*. Mol. Gen. Genet. 217, 520-527.

Yoon, J.S., R.H. Richardson, and M.R. Wheeler (1973). A technique for improving salivary chromosome preparations. Experientia 29, 639-641.

Zhang, P., and R.S. Hawley (1990). The genetic analysis of distributive segregation in *Drosophila melanogaster*. II. Further genetic analysis of the *nod* locus. Genetics 125, 115-127

Zink, B., and R. Paro (1989). *In vivo* binding pattern of a *trans*-regulator of homeotic genes in *Drosophila melanogaster*. Nature 337, 468-471.

Zuckerkandl, E. (1974). Recherches sur les proprietes et l'activite biologique de la chromatine. Biochimie 56, 937-954.