THE GENETIC AND MOLECULAR ANALYSIS OF THE mfs(2)31 LOCUS IN DROSOPHILA MELANOGASTER: A NOVEL SUPPRESSOR OF POSITION-EFFECT VARIEGATION

By

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ABSTRACT

Position-effect variegation (PEV) is the variable inactivation of a euchromatic gene which has been moved, by way of a chromosomal rearrangement, into a heterochromatic environment. The transcriptional repression at the variegating locus is thought to be a consequence of inappropriate packaging of the euchromatin as heterochromatin. Second site mutations which modify the PEV phenotype (Su(var)s and E(var)s), identify loci which encode non-histone chromosomal proteins. Although the majority of mutations which modify PEV exhibit a dominant phenotype, rare recessive su(var) mutations have been reported. This study describes a locus which is identified by one such recessive mutation, mfs(2)31.

A cytogenetic analysis of subdivisions 31D-E was undertaken to determine the precise location of the mfs(2)31 locus and to isolate additional alleles. Five new deficiencies and 123 new lethal mutations were induced, allowing for the partitioning of 31D-E into six cytological subintervals. The mfs(2)31 gene was localized to distal 31E in an interval containing nine lethal complementation groups. Three new mfs(2)31 alleles were recovered, one of which was isolated in a screen for P element insertions.

The new alleles of mfs(2)31 were used for a phenotypic analysis of the locus. Strong alleles that were lethal as homozygotes died in the larval phase, while weaker alleles exhibited the previously described bristle, sterility and su(var) phenotypes. Larval, pupal and adult functions were defined for the
locus. Although no dominant phenotypes were observed, surviving heteroallelic combinations suppressed PEV in a variety of variegating backgrounds. When the weak, $P$-induced, allele was outcrossed in a dysgenic background, all the $mfs(2)31$ phenotypes, including suppression of PEV, were co-reverted. An in situ hybridization to salivary gland polytene chromosomes revealed a $P$ element in distal 31E of this strain.

The $P$ element, located in distal 31E, was cloned from the dysgenic $mfs(2)31$ allele. This element is responsible for the reduced transcription of a 1.2 kb message which is expressed throughout development. Wild-type levels of transcription are restored at this locus in revertants of the $mfs(2)31$ phenotype. The predicted amino acid sequence, as determined from a cDNA analysis, reveals similarities with a mouse microtubule-associated protein and mammalian histone H1.
# TABLE OF CONTENTS

**ABSTRACT**  
ii  

**LIST OF TABLES**  
ix  

**LIST OF FIGURES**  
x  

**LIST OF BIOCHEMICAL ABBREVIATIONS**  
xii  

**LIST OF GENETIC ABBREVIATIONS**  
xiii  

**ACKNOWLEDGEMENTS**  
 xv  

**CHAPTER 1 - LITERATURE REVIEW**  

A. General  
1  

B. Chromatin Structure  
2  

   Histone Proteins  
   2  

   Non-histone Chromosomal Proteins  
   3  

   Heterochromatin and Euchromatin  
   6  

C. Position-effect Variegation  
10  

   The Phenomenon of Position-effect Variegation  
   10  

   Position-effect Variegation and the Chromosomal Breakpoint  
   10  

   The Timing of Position-effect Variegation  
   13  

   Factors Which Modify Position-effect Variegation  
   14  

   The Structural Basis for Position-effect Variegation  
   18  

   Dominant Modifiers of Position-effect Variegation  
   20  

   Cloned Su(var) Loci  
   24  

   Recessive Modifiers of Position-effect Variegation  
   26  

   Summary  
   27
CHAPTER 2 - CYTOGENETIC ANALYSIS OF SUBDIVISIONS 31D-E

INTRODUCTION 29

MATERIALS AND METHODS

Stocks 35
Culture Conditions 37
Cytology 37
Genetic Screens 39
Complementation 43
Mapping by Recombination 43

RESULTS

Cytology 45
Screens 48
Complementation 50
Mapping by Recombination 53

DISCUSSION 57

CHAPTER 3 - GENETIC ANALYSIS OF THE mfs(2)31 LOCUS

INTRODUCTION 62

MATERIALS AND METHODS

Stocks 70
Culture Conditions 71
Complementation 71
Fertility Tests 72
Lethal Phases 72
Pigment Assays 73
Ovary Dissections 74
Thick Sections of Ovaries 74
RESULTS

Complementation of mfs(2)31 With Su(var) Loci

mfs(2)31 inter se Complementation

Fertility of mfs(2)31 Alleles

Studies on mfs(2)31 Oogenesis

Lethal Phases for mfs(2)31

mfs(2)31 Alleles Suppress Position-effect Variegation

mfs^2 has a P Element in 31E

Reversion of mfs^2

DISCUSSION
Gel Electrophoresis 123
DNA Transfer From Agarose Gels to Nylon Membrane 124

RNA Analysis
Isolation of Developmentally Staged Drosophila Cultures 124
  Embryonic Collections 125
  Larval and Pupal Collections 125
Isolation of Poly(A)'RNA 126
Gel Electrophoresis of RNA 127
RNA Transfer From Formaldehyde Gels to Nylon Membranes 128

Hybridization to Nylon Membranes 128
  Gel Purification of Probes 128
  Radiolabelling of Probes 128
  Filter Hybridization 129

Library Construction and Screening
  Construction of a Bacteriophage Library 129
  Screening Bacteriophage Libraries 131
    Preparation of Confluent Plates 131
    Plaque Lifts 131
  Screening Cosmid Libraries 132
  Subcloning Cosmid, Plasmid and Bacteriophage DNA 133
  Mapping DNA 133

DNA Sequencing
  Sequencing Reactions 134
  Polyacrylamide Gel Electrophoresis 135
  Sequencing Strategy 135
RESULTS

Cloning of a P Element From mfs

Mapping λ1

Transcriptional Analysis of λ1 and the mfs(2)31 Locus

Sequencing mfs(2)31

DISCUSSION

LITERATURE CITED
LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cytological limits and origins of chromosomal rearrangements</td>
</tr>
<tr>
<td>2</td>
<td>Origin of previously described mutations in region 31A-32A</td>
</tr>
<tr>
<td>3</td>
<td>Summary of genetic screens</td>
</tr>
<tr>
<td>4</td>
<td>Mapping by recombination</td>
</tr>
<tr>
<td>5</td>
<td>Complementation with dominant Su(var)s</td>
</tr>
<tr>
<td>6</td>
<td>Complementation matrix for mfs(2)31</td>
</tr>
<tr>
<td>7</td>
<td>Fertility of mfs(2)31 hypomorphs</td>
</tr>
<tr>
<td>8</td>
<td>Lethal phases for mfs(2)31 alleles</td>
</tr>
<tr>
<td>9</td>
<td>Pigment assays</td>
</tr>
<tr>
<td>10</td>
<td>Revertant description</td>
</tr>
<tr>
<td>11</td>
<td>Revertant analysis</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Screens for EMS induced lethal mutations</td>
</tr>
<tr>
<td>2</td>
<td>Screen for P element induced lethal mutations</td>
</tr>
<tr>
<td>3</td>
<td>Cytological map for division 31</td>
</tr>
<tr>
<td>4</td>
<td>Cytogenetic map of region 31D-E</td>
</tr>
<tr>
<td>5</td>
<td>Recombination map for complementation groups located in cytogenetic interval #5</td>
</tr>
<tr>
<td>6</td>
<td>Screen for revertants of $mfs(2)31$</td>
</tr>
<tr>
<td>7</td>
<td>Ovarian development in $mfs(2)31$ homozygotes</td>
</tr>
<tr>
<td>8</td>
<td>In situ hybridization to salivary gland polytene chromosomes of a $mfs^2 b pr cn/+) strain</td>
</tr>
<tr>
<td>9</td>
<td>Reversion of $su(var)$ and bristle phenotypes in a hybrid-dysgenesis induced revertant of $mfs^2$.</td>
</tr>
<tr>
<td>10</td>
<td>$mfs^2 b pr cn/CyO$ genomic DNA hybridized with the 900 bp $Hin$ dIII fragment from $p\pi25.1$</td>
</tr>
<tr>
<td>11</td>
<td>Bacteriophage $\lambda$ clones hybridized with the 900 bp $Hin$ dIII fragment from $p\pi25.1$</td>
</tr>
<tr>
<td>12</td>
<td>Localization of $\lambda1$ to subdivision 31E by in situ hybridization</td>
</tr>
<tr>
<td>13</td>
<td>Restriction maps for various fragments from $\lambda1$</td>
</tr>
<tr>
<td>14</td>
<td>Composite restriction map for $\lambda1$</td>
</tr>
<tr>
<td>15</td>
<td>Overlapping subcloned fragments from $\lambda1$ hybridized with JT31 and JT35</td>
</tr>
<tr>
<td>16</td>
<td>Overlapping clones in subdivision 31E</td>
</tr>
<tr>
<td>17</td>
<td>Digested 8.5 kb $Eco$ RI fragment from $\lambda1$ hybridized with $p\pi25.1$</td>
</tr>
<tr>
<td>18</td>
<td>$mfs(2)31$ genomic DNA hybridized with the 4.0 kb $Eco$ RI/Xba I fragment from $\lambda1$</td>
</tr>
<tr>
<td>19</td>
<td>Transcriptional analysis of $\lambda1$</td>
</tr>
<tr>
<td>Page</td>
<td>Description</td>
</tr>
<tr>
<td>------</td>
<td>-------------</td>
</tr>
<tr>
<td>20</td>
<td>Putative mfs(2)31 cDNAs hybridized with pH1.5 and cDNA 6</td>
</tr>
<tr>
<td>21</td>
<td>Poly(A)' RNA from 0-6 hour embryos hybridized with cDNA 6</td>
</tr>
<tr>
<td>22</td>
<td>Poly(A)' RNA from the mfs(2)31 alleles hybridized with cDNA 6</td>
</tr>
<tr>
<td>23</td>
<td>Developmental profile of a putative mfs(2)31 transcript</td>
</tr>
<tr>
<td>24</td>
<td>The complete sequence of cDNA 6</td>
</tr>
<tr>
<td>25</td>
<td>Similarities with the deduced protein sequence of mfs(2)31</td>
</tr>
<tr>
<td>26</td>
<td>The relationship between the P element insert and the 5' end of cDNA 6 in mfs^2.</td>
</tr>
<tr>
<td>27</td>
<td>Partial sequence of the 1.0 Kb HindIII fragment from the plasmid pH1.0</td>
</tr>
<tr>
<td>28</td>
<td>cos50 DNA hybridized with pH1.5</td>
</tr>
<tr>
<td>29</td>
<td>Overlapping fragments from λ1 hybridized with cos50</td>
</tr>
<tr>
<td>30</td>
<td>The complete genomic sequence of the region encompassing the gene represented by cDNA 6</td>
</tr>
<tr>
<td>31</td>
<td>Potential upstream regulatory sequences of mfs(2)31</td>
</tr>
</tbody>
</table>

xi
**LIST OF BIOCHEMICAL ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APS</td>
<td>ammonium persulphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indoyl phosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EMS</td>
<td>ethyl methanesulfonate</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase pairs</td>
</tr>
<tr>
<td>λ-dil</td>
<td>100 mM NaCl, 10 mM Tris-HCl (pH 7.4), 10 mM MgCl₂</td>
</tr>
<tr>
<td>LM</td>
<td>10 g/l tryptone, 5 g/l yeast extract, 2 g/l MgCl₂, 5 g/l NaCl</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-[N-morpholino]propanesulfonic acid</td>
</tr>
<tr>
<td>NBT</td>
<td>nitro-blue tetrazolium</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotides</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethyleneglycol</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque forming units</td>
</tr>
<tr>
<td>PVP</td>
<td>polyvinylpyrrolidene</td>
</tr>
<tr>
<td>SSC</td>
<td>standard saline citrate; 0.15 M NaCl, 0.015 M sodium citrate</td>
</tr>
<tr>
<td>TE</td>
<td>10 mM Tris-HCl (pH 8.0), 1 mM EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-beta-D-galactoside</td>
</tr>
</tbody>
</table>
GENETIC ABBREVIATIONS

STRAINS

OR
Oregon-R; stock derived from wild flies collected in 1925 at Roseburg, Oregon (Lindsley and Grell, 1968).

Canton-S
Canton-Special; stock derived from wild flies collected in Canton, Ohio (Lindsley and Grell, 1968).

CHROMOSOMES

Birm2
A 2nd chromosome from the M' strain, Birmingham. It bears 17 defective P elements (Robertson et al., 1988).

CyO
Curly derivative of Oster; In(2LR)O, Cy dp^{ry} pr cn^{2}; balanced 2nd chromosome (Lindsley and Zimm, 1992).

Δ2-3
P[ry' 2-3](99B); 3rd chromosome with a P insertion which provides transposase activity (Robertson et al., 1988)

TM6
In(3LR)TM6, Hn^{p} ss^{PR} bx^{24e} Ubx^{P15} e; balanced 3rd chromosome (Lindsley and Zimm, 1992).

w^{nd}
In(1)w^{nd}; X chromosome inversion which variegates for the white gene. The euchromatic breakpoint is distal to white and the heterochromatic breakpoint is distal to the ribosomal RNA genes (Hilliker et al., 1980).

w^{nd1b}
In(1)w^{nd1b}; X chromosome inversion which variegates for the white gene. The euchromatic breakpoint is distal to white and the heterochromatic breakpoint is proximal to the ribosomal RNA genes (Hilliker et al., 1980).

w^{nd1j}
In(1)w^{nd1j}; X chromosome inversion which variegates for the white gene. The euchromatic breakpoint is proximal to white and the heterochromatic breakpoint is distal to the ribosomal RNA genes (Tartof et al., 1984).

MARKERS

b
black (2-48.5); black pigment on body (recessive).

Bc
Black cells (2-80.6); black cells appear in 1st instar larvae (dominant); homozygous lethal.

cn
cinnabar (2-57.5); bright red eye color (recessive).

Cy
Curly (2-6.1); wings curled upwards (dominant); homozygous lethal.
$Gla$ Glazed (unknown); reduced gleaming eyes (dominant); homozygous lethal.

$J$ Jammed (2-41.0); narrow, fluid-filled wings (dominant); homozygous lethal.

$pr$ purple (2-54.5); purplish, ruby eye color (recessive).

$ry^{500}$ rosy (3-52.0); reddish-brown eye color (recessive).

$Sb$ Stubble (3-58.22); short, thick bristles (dominant); homozygous lethal.

$Sp$ Sternpleural (2-22.0); sternopleural bristles increased in number (dominant); homozygous lethal.

$Tft$ Tufted (2-102); tufts of bristles on mesothorax (dominant); homozygous lethal.
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CHAPTER 1: LITERATURE REVIEW

A. GENERAL

During a typical cell cycle, a eukaryotic nucleus will transcribe, replicate, repair and mobilize between $10^7$ and $10^9$ bp of DNA. In order to ensure the cell’s survival, these events must be orderly and occur with a high degree of fidelity. To facilitate the execution of these complex processes, DNA is packaged in a hierarchical manner. DNA packaging is dynamic and heterogeneous; chromosomes acquire different states of organization as they proceed through the cell cycle, and often exhibit several states of compaction at any one time. The manipulation of chromosomal architecture is undoubtably the result of complex metabolic processes, yet little is known about the enzymatic activities involved or the substrates upon which they act.

A change in DNA packaging can often be visualized at the microscopic level as an alteration in the morphology and staining properties of chromatin. Chromatin is both a cytological and a biochemical term used to describe DNA and associated proteins, the substrates from which chromosomes are built. Changes in chromatin organization are the result of altered structural relationships between its component parts, both protein and DNA. An understanding of the character and function of the histone and non-histone proteins, which constitute 2/3 of the chromatin mass, would be prerequisite to an understanding of chromosomal and nuclear architecture.
B. CHROMATIN STRUCTURE

Histone proteins

Apart from the DNA itself, the most extensively studied component of chromatin is the histones. These highly conserved and ubiquitous proteins comprise approximately 1/3 the mass of chromatin and have an almost universal role in eukaryotic DNA packaging. At the simplest level of chromatin organization, DNA winds around histone octamers forming a fiber 10 nm in diameter. The octamers are spaced 30 bp apart and consist of a tetramer containing two H3 and two H4 molecules and two H2A-H2B dimers (reviewed by McGhee and Felsenfeld, 1980). The so-called 10 nm fiber is further organized by histone H1 into a more compact structure, 30 nm in diameter. Although the structure of the 30 nm fiber is uncertain, most evidence points to a simple solenoidal conformation with six nucleosomes per helical turn (reviewed by Felsenfeld and McGhee, 1986). This structure is compact, stable under physiological conditions, and presents a considerable barrier, in vitro, to the passage of RNA polymerase. Higher order levels of chromatin structure give DNA a maximum order of compaction greater than 10,000. Although little is known of the nature of these packing structures, most evidence suggests that the chromatin fiber is organized into discreet domains (ie. loops) whose integrity is maintained by proteins other than the histones (reviewed by Jackson, 1991).

In addition to fulfilling an important structural role within chromatin, histones participate directly in the regulation of gene...
expression, either by blocking access to regulatory sequences or by interacting with proteins involved in transcriptional regulation (reviewed by Grunstein, 1990). Such selective and localized activity on the part of a ubiquitous and highly conserved family of proteins is achieved through an array of enzyme-catalyzed post-translational modifications. All core histones can be modified by acetylation, phosphorylation, ADP-ribosylation and ubiquitinization of specific amino acid residues (Wu et al., 1984). Many of these modifications occur in the amino terminal domains which fall outside the core DNA and thus are ideally situated to interact with other DNA sequences and non-histone proteins (Hill and Thomas, 1990). Of these post-translational modifications, acetylation has been most commonly linked to transcriptional regulation (Ip et al., 1988; Hebbes et al., 1988). All four core histones undergo cycles of acetylation and deacetylation which are correlated with changes in transcriptional competence and with the cell cycle (reviewed by Turner, 1991). Although the mechanism of transcriptional regulation associated with histone acetylation remains unclear, functional differences between histones appear to depend as much on which sites are acetylated as on the overall level of acetylation (Johnson et al., 1990; Megee et al., 1990).

Non-histone chromosomal proteins

In addition to the DNA and histones, non-histone chromosomal proteins (NHPs) account for 1/3 of the chromatin mass. Of the NHPs which are known to influence chromatin structure, the moderately
abundant high mobility group (HMG) are the best characterized (reviewed by Johns, 1983). The term HMG is operationally defined by the biochemical properties which allow these proteins to be isolated. Like the histone proteins, the HMGs are highly conserved, they undergo a wide spectrum of post-translational covalent modifications and alterations in their stoichiometry have been correlated with cellular proliferation and differentiation events (Johns, 1983; Weisbrod et al., 1980; Dorbic and Wittig, 1987).

Recently, monoclonal antibodies have become increasingly popular as a tool for the dissection and analysis of nuclear protein fractions. In Drosophila melanogaster, this approach has identified several proteins which are probably involved in chromatin condensation, the best characterized of which are D1, HP1 and BJ1.

D1 is a highly abundant 50 kDa protein which binds to AT rich regions of DNA such as the highly-repetitive satellite sequences (Rodriguez-Alfageme et al., 1980; Levinger and Varshavsky, 1982). It shares many biochemical properties with the HMG proteins and shows limited sequence similarity with HMG I, a satellite DNA binding protein from African Green Monkey (Strauss and Varshavsky, 1984; Ashley et al., 1989). Although the physiological role of D1 remains unclear, since the protein specifically associates with satellite DNA it is plausible that it contributes to the high degree of compaction of these heterochromatic sequences.

HP1 is a 19 kDa protein isolated from a fraction of nuclear
proteins which have tight associations with DNA (James and Elgin, 1986). Immunofluorescence staining of salivary gland polytene chromosomes with an antibody to HP1 shows specific binding to β-heterochromatin, a banded portion of the heterochromatic fourth chromosome, and several discrete regions within euchromatin (James and Elgin, 1986; James et al., 1989). Although the physiological role of HP1 is unknown, the protein sequence does not contain any previously described DNA binding motifs. A detailed description of the HP1 protein and its associated locus are provided elsewhere in this survey (see Cloned Su(var) loci).

BJ1 is a 68 kDa nuclear protein which associates with nucleosomes and is released from chromatin by agents which intercalate with DNA (Frasch, 1991). When hybridized to salivary gland chromosomes, an antibody to BJ1 shows no site preference, associating with all chromosome bands. The BJ1 protein sequence shows strong homologies with the human gene Regulator of Chromatin Condensation (RCC1), a gene which functions in cell cycle regulation, and the Yeast gene SRM1/PRP20, which is involved in mRNA metabolism and maintenance of nuclear structure. It has been proposed that BJ1 binds to chromatin to either establish or maintain a higher order structure required for regulated gene expression (Frasch, 1991).

A third approach to identifying and analyzing NHPs which are involved in chromatin condensation employs genetic assays. Genetic approaches have several advantages over the more traditional biochemical and structural approaches. A sensitive genetic assay
can be used to identify proteins present in very low abundance or with very transient or weak associations with chromatin. In addition, the isolation of mutations in the genes encoding these proteins allows for an analysis of their role in development and can reveal unexpected associations and interactions with other cellular components. One such assay, position-effect variegation (PEV), may prove to be a valuable genetic tool for identifying genes which affect two cytologically differentiable states of chromatin compaction, euchromatin and heterochromatin.

**Heterochromatin and euchromatin**

Eukaryotic chromosomes achieve their greatest level of compaction and appear homogeneous in morphology in late prophase as they prepare for segregation. By telophase, the homogeneity disappears with the majority of the DNA becoming loosely packed and dispersed, a state referred to as euchromatin, while a significant portion of the genome, the heterochromatin, retains a high level of compaction. In *Drosophila melanogaster*, this heterogeneity can be observed in the neural ganglia.

Heterochromatin, the portion of the DNA that remains compacted during interphase, is an almost universal feature of the eukaryotic nucleus. Its distribution is non-random, and is most often associated with centromeres and telomeres, although whole arm and interstitial heterochromatization is not uncommon. Apart from cytological morphology, heterochromatin differs from euchromatin in that it generally replicates later in the cell cycle (Barigozzi et
al., 1966), exhibits lower levels of genetic activity (Hilliker, 1976; Hilliker et al., 1980; Marchant and Holm, 1988) and it undergoes little or no meiotic recombination (Roberts, 1965; Schalet and Lefevre, 1976).

In Drosophila, heterochromatin constitutes nearly 1/2 of the acrocentric X chromosome, the proximal 1/4 of the metacentric second and third chromosomes, all of the Y, and probably most of the small chromosome 4. It is first observed very early in Drosophila development, between stages 10 and 13 of the syncytial blastoderm (Foe and Alberts, 1985), its appearance being correlated with the beginning of transcriptional competence for the zygotic genome (Edgar and Schubiger, 1986).

Drosophila heterochromatin is divided into two classes, alpha (α) and beta (β), which are distinguishable both at a cytological (Heitz, 1934) and a biochemical level (Gall et al., 1971; Spradling and Rubin, 1981; Healy et al., 1988; Devlin et al., 1990). Whereas in most eukaryotic tissues examined, euchromatin appears diffuse and is dispersed throughout the nucleus, a notable exception can be found in the salivary glands of dipterans. Here the chromosomes go through numerous rounds of endoreduplication achieving a ploidy of greater than 1000. The DNA from each round of replication remains associated with the previous copy, thus producing morphologically distinct giant chromosomes which can be readily seen using light microscopy. Each chromosome has a unique banding pattern providing a valuable tool for the Drosophila geneticist (Lefevre, 1974; 1976). It is in salivary gland chromosome preparations that α- and
β-heterochromatin can be cytologically distinguished (Heitz, 1934). α-heterochromatin is the chromatin closest to the centromere. It is underreplicated by a factor of 10 relative to euchromatin in polytene preparations, appearing thin and unbanded (Rudkin, 1969; Gall et al., 1971). The α-heterochromatin from the various Drosophila chromosomes associates during interphase to form the chromocenter. At the biochemical level, α-heterochromatin is rich in highly repetitive satellite DNA with few, if any unique sequences (Gall et al., 1971).

β-heterochromatin is the portion of the Drosophila chromosome which connects α-heterochromatin to euchromatin (Heitz, 1934). It is not underreplicated in the salivary glands but it is very compact, dark staining and unbanded (Rudkin, 1969). The DNA content of β-heterochromatin differs from α-heterochromatin in that it contains more unique sequence and middle-repetitive DNA (Spradling and Rubin, 1981; Healy et al., 1988; Devlin et al., 1990). Unlike α-heterochromatin, β-heterochromatin is known to contain actively transcribed genes (Biessmann et al., 1981; Miklos et al., 1984; Devlin et al., 1990) but at a considerably lower frequency than euchromatin (Hilliker et al., 1980; Marchant and Holm, 1988). There is no known functional basis for distinguishing between α- and β-heterochromatin.

Although long thought to be genetically inert, studies on Drosophila heterochromatin, have revealed an unexpectedly high level of genetic activity (Hilliker et al., 1980; Marchant and Holm, 1988). Utilizing heterochromatic deficiencies, generated by
detaching compound autosomes, complementation maps have been elaborated for essential loci in the centromeric heterochromatin of chromosomes 2 (Hilliker and Holm, 1977) and 3 (Marchant and Holm, 1988). A level of genetic activity approximately 100-fold less (on a per kb basis) than a comparably sized euchromatic segment is observed. Although there is no genetic principle linking the genes located within heterochromatin, these genes can act late in development, well after heterochromatin becomes established as a cytological entity (Hilliker, 1976). The light gene which is located in the centromeric heterochromatin of chromosomal arm 2L is transcribed throughout development suggesting that heterochromatin does not necessarily form a transcriptional block to sequences located within it (Devlin et al., 1990).

It is plausible that NHPs are partially responsible for maintaining the difference in chromatin compaction that exists between euchromatin and heterochromatin. Proteins such as HP1 and D1, which are known to associate specifically with heterochromatin, may perform such a function. It may be possible to identify other proteins involved in this process through genetic assays such as position-effect variegation (PEV). It is notable that the gene which encodes HP1, Su(var)205, was initially identified as a modifier of PEV (Sinclair et al., 1983).
C. POSITION-EFFECT VARIEGATION

The phenomenon of position-effect variegation

Position-effect variegation (PEV) is the variable inactivation of a gene caused by its repositioning in the genome. The phenomenon differs from more classic position effects in that the relocated gene is inactivated in only a subset of the cells in which it is normally expressed. Thus, if the affected gene acts in a cell autonomous manner, the inactivation can often be observed as a mosaic phenotype. First described by Muller (1930) in *Drosophila melanogaster*, PEV has since been observed in vertebrates (Cattanach, 1974), lower eukaryotes (Clutterbuck and Spathas, 1984) and plants (Catcheside, 1938; 1947). Although it has been described in representative species from all kingdoms, the phenomenon has been most extensively studied in the *Drosophilids* (reviewed by Baker, 1968; Spofford, 1976; Eissenberg, 1989; Spradling and Karpen, 1990; Henikoff, 1990; Grigliatti, 1992), a bias which is reflected in the following survey. Unless otherwise indicated, all experiments discussed used *Drosophila melanogaster* as the experimental organism.

Position-effect variegation and the chromosomal breakpoint

The earliest described variegated position effects involve euchromatic genes which have been juxtaposed, by way of chromosomal rearrangements, with broken segments of heterochromatin. Such rearrangements are typical of variegating strains and there is considerable evidence that inactivation is the result of an
interaction of the affected gene with the adjacent heterochromatin. Although the chromosomal breakpoint involved is typically in α-heterochromatin (Tartof et al., 1984), the source of the heterochromatin does not appear to be an important factor (Spofford, 1976). Variegating position effects have been described for virtually all autosomal genes, two exceptions being ebony (Brosseau, 1970) and the bithorax complex (E.B. Lewis, cited in Henikoff, 1990).

Although PEV typically involves an association between a euchromatic gene and a broken segment of heterochromatin, similar effects have been described for heterochromatic genes which are relocated next to euchromatin. The light gene and cubitus interruptus are located in β-heterochromatin of the second and fourth chromosomes respectively and both exhibit variegation when moved into a euchromatic environment (Stern and Kodani, 1955; Hessler, 1958). Interestingly, only breakpoints in centric and distal euchromatin can induce light variegation (Hearn et al., 1991).

There is considerable evidence that PEV is the result of an interaction between the affected gene and the adjacent broken segment of heterochromatin. The first evidence for this interaction came from experiments in which this relationship was disturbed. It has been well documented (Judd, 1955) that PEV only occurs when a gene is located cis to the broken heterochromatin. A possible rare exception to this rule may involve the bw variegating rearrangement (Henikoff, 1981). Complete wild type
function can be restored to a variegating gene if it is removed from the broken segment of heterochromatin by recombination (Judd, 1955). Similarly, wild-type function is restored to a variegating gene if it is removed from the broken segment by a secondary chromosomal rearrangement (Hinton and Goldsmith, 1950; Reuter et al., 1985). Restoration of full gene expression does not require that the gene be returned to its normal euchromatic location, an observation which implies that variegation is not caused by moving a gene from its native environment.

A second observation that implicates the heterochromatin in the variegating phenotype is that more than one gene can variegate in the same rearrangement and that the probability of a gene variegating is a function of its distance from the heterochromatin. This polarity of effect is most evident in the $T(1;4)\text{wm}^{258-21}$ variegating rearrangement (Demerec and Slizynska, 1937; cited in Cohen, 1962), a strain which variegates for the white gene (abnormal eye color) and the roughest gene (disorganized eye facets), both of which are expressed in a cell autonomous manner. The two genes are tightly linked genetically with roughest being closest to the broken heterochromatin. An analysis of the variegated eye reveals that white clones are always smaller than and completely contained within roughest clones. Thus, in no case is the distal white gene inactivated without the more proximal roughest gene also being inactivated. A similar spreading effect has been described for heterochromatic genes in Drosophila hydei (Hess, 1970).
To date, there have been no substantiated exceptions to the polarity of variegation which appears to emanate from the broken piece of heterochromatin. Spreading effects have been described over distances of 80 polytene chromosome bands (1500 kb) from the broken heterochromatin (Schultz, 1950) although typically the distance is much shorter. The extent of spreading appears to be determined by qualities of both the euchromatin and the heterochromatin involved in the variegating rearrangement (Spofford, 1976).

A final observation linking variegation to the broken heterochromatin comes from cytological studies. In polytene chromosome preparations from variegating strains, the euchromatin adjacent to the broken heterochromatin exhibits a morphology reminiscent of β-heterochromatin i.e. compact, dark-staining and unbanded (Hartmann-Goldstein, 1967). In the variegating rearrangement T(1;4)wm258-21, the white gene located at band 3C2 is closer to the broken heterochromatin than the notch gene at band 3C7. The relative visibilities of these two bands in the variegating rearrangement is subject to the same polarity observed at the phenotypic level (Hartmann-Goldstein, 1967). Thus, either both bands are seen, neither are seen or only 3C7 is seen. In no case is 3C2 visible when 3C7 is not.

The timing of position-effect variegation

Clonal analyses of variegating rearrangements have demonstrated that the transcriptional competence of a variegating
gene is determined early in development, and once decided, is propagated clonally (Janning, 1970). These conclusions are based on the observation that variegation is generally characterized by large spot clones which follow the same boundaries as cell lineages. In a number of variegating rearrangements, the timing of the determinative event has been calculated with a moderate degree of precision. The rearrangement In(1)sc<sup>s1</sup> variegates for the rDNA cistrons. Newly hatched larvae with the genotype In(1)sc<sup>s1</sup>/0 contain 14% less rDNA than their wild-type controls (Puckett and Snyder, cited in Spofford, 1976). Variegation of the yellow gene in In(1)sc<sup>8</sup> occurs as early as cellular blastoderm as does roughest variegation in In(1)rst<sup>3</sup>. Variegation prior to early first instar larvae has been detected for the peach gene in Drosophila virilis (in T(Y;5)pe<sup>s1</sup>; Baker, 1967) as well as a number of eye color mutations in Drosophila melanogaster (Baker, 1967; Janning, 1970). Although the bulk of the evidence points to an early determinative event, there are several variegating strains such as In(1)w<sup>ns1b</sup> and In(1)w<sup>nc</sup> which show a very fine grained mosaic phenotype (Tartof, 1984) indicating that in some cases the decision is made late or that an earlier decision can be reversed. Regardless of the time at which variegation is initiated, all of these studies demonstrate the clonal nature of the phenomenon.

Factors which modify position-effect variegation

A number of genetic studies of the PEV phenomenon have described environmental, biochemical and genetic factors which have
a modifying effect on the variegating phenotype. The nature of
these factors has fuelled many theories on the underlying
structural mechanism of variegation. The environmental factor
having the greatest impact on the extent of expression of a
variegating locus is temperature (Gowen and Gay, 1935). Cultures
raised at high temperatures (29°) have a lower proportion of cells
in which the variegating gene is inactivated than do cultures
raised at lower temperatures (22°). Although the effect is
measurable and reproducible, it is not always striking. Being
poikilotherms, drosophilids develop more rapidly at 29° than at 22°.
The extent of variegation is correlated with length of
developmental time (see below), which could explain the temperature
effect. It has also been proposed that variegation involves the
assembly of macromolecular complexes and that in some instances
high temperatures do not favor the formation of such complexes
(Zuckerkandl, 1974). A third explanation would be that at 29°
flies are physiologically stressed and individuals showing strong
variegation would be selected against. At this time there is no
evidence to support or refute any of these three hypotheses.

It has been known for some time that flies developed in
crowded cultures show stronger variegation than those developed in
non-crowded cultures (Hinton, 1949). Because of competition for
nutrients, flies raised in crowded cultures have a longer
developmental time and this has been invoked as the explanation for
the enhancement of variegation. Proponents of the hypothesis that
the extent of variegation reflects the ability of a cell to
assemble heterochromatin argue that a longer cell cycle associated with prolonged development would allow more time for assembly to occur. Regardless of the underlying mechanism, the evidence linking strength of variegation to developmental time is somewhat equivocal. Flies reared at pH 2.6 which results in a prolonged developmental time show enhanced variegation (Michailidis et al., 1988). Some mutations which cause delayed development also show enhancement of PEV; however, there are some mutations which don’t (Michailidis et al., 1988).

There are chemicals which have been shown to modify PEV and also influence developmental time. A number of agents which delay development by interfering with DNA synthesis have been correlated with enhancement of PEV (Schultz, 1956). However, propionate and butyrate, both of which prolong developmental time, suppress variegation of the white gene in Drosophila melanogaster (Mottus et al., 1980; Rushlow et al., 1984). Butyrate is a potent inhibitor of histone deacetylase (Candido et al., 1978) and is known to affect chromatin compaction (Annunziato et al., 1988). Although it is tempting to conclude that butyrate modifies variegation through an alteration in chromatin structure, this chemical has been shown to have a wide spectrum of effects on other cellular processes which may be responsible for the modification (Boffa, 1981; Christman et al., 1980).

In addition to being sensitive to environmental and chemical influences, the PEV phenomenon can be modified by a number of well described genetic factors. Strength of variegation is very
sensitive to the ploidy of the Y chromosome. Males with extra Y chromosomes (XYY males) exhibit a suppressed phenotype (Gowen and Gay, 1934) while the absence of a Y chromosome (XO males) enhances variegation (Spofford, 1976). The effect appears to be attributable to the amount of Y chromosome present rather than any specific sites (Dimitri and Pisano, 1989). Zuckerkandl (1974) and others have proposed that PEV is sensitive to intracellular availability of structural components of heterochromatin and that the Y chromosome acts as a sink for these components. The entire Y chromosome in Drosophila melanogaster is packaged as heterochromatin. Zuckerkandl's proposal finds support in the observation that deficiencies and duplications of autosomal heterochromatin affect variegation in the same way as deficiencies and duplications of the Y chromosome (Spofford, 1976).

The proposal that PEV is sensitive to intracellular levels of the structural components of heterochromatin was examined by testing the sensitivity of PEV to the dosage of the histone gene cluster (Khesin and Leibovitch, 1978; Moore et al., 1983). Strains which are haploid for the histone gene cluster exhibit strong suppression of PEV in rearrangements which variegate for the white gene (Khesin and Leibovitch, 1979; Moore et al., 1983). However, in similar studies, no effect was observed at the rosy locus (Rushlow and Chovnick, 1984). It has been proposed that chromatin formation is sensitive to the availability of a number of chromatin components and that heterochromatin, because it replicates late, is more sensitive (Moore et al., 1983).
Since PEV is sensitive to the dosage of histone proteins, this sensitivity may extend to the dosage of genes encoding other chromosomal proteins involved in chromatin condensation. A large number of single-site modifiers of the PEV phenotype have been isolated, which may identify proteins of this class. These are described in detail in the section titled Dominant modifiers of PEV.

The structural basis for position-effect variegation

Variegation is not associated with somatic loss of the variegating locus, but rather, a reduction in the accumulation of nascent transcripts. This has been observed for variegating alleles of the Hsp70 gene (Henikoff, 1981), the rosy gene (Rushlow et al., 1984), the Sgs4 gene (Kornher and Kauffman, 1986) and the brown gene (Henikoff and Dreesen, 1989). It is not yet clear whether the reduction in nascent transcription at the variegating locus is due to under-replication, reduced accessibility of the locus to transcriptional regulators or transcriptional interference from a promoter located within the adjacent heterochromatin.

If unique probes for variegating genes are hybridized to salivary gland DNA there is no evidence of underreplication for the white gene (Hayashi et al., 1990), the Hsp70 gene (Henikoff, 1981) or the rosy gene (Rushlow et al., 1984). However, underreplication has been detected at the Sgs4 locus in T(1;4)wm258-21 (Koernher and Kauffman, 1986) and at the yellow locus in Dp(1;f)1187 (Karpen and Spradling, 1990). Although these latter studies implicate
underreplication as one mechanism for variegation it cannot be invoked to explain variegation in diploid tissue. The white gene variegates in pigment cells of the eye while the nod gene variegates in germline cells, two tissues which are neither polytenized nor polyploid (Karpen and Spradling, 1990, Zhang and Hawley, 1990).

Another plausible explanation for the reduction in nascent transcription associated with a variegating gene would be an inaccessibility of the locus to transcriptional regulators. A number of groups have described a positive correlation between the strength of a variegating rearrangement and the number of salivary gland cells in which the chromosomal segment containing the affected gene adopts an altered cytological morphology (Hartmann-Goldstein, 1967; Henikoff, 1981; Reuter et al., 1982; Zhimulev et al., 1986; Hayashi et al., 1990). The affected segment becomes dark-staining, compact and unbanded, features reminiscent of β-heterochromatin. In a series of in situ hybridizations to salivary gland chromosomes, the white gene has been shown to be less accessible to DNA probes in variegating rearrangements than in non-variegating strains (Hayashi et al., 1990).

Since variegating genes are invariably associated with broken segments of heterochromatin, a simple interpretation of the observations described above would be that variegation is the result of a euchromatic segment of DNA taking on characteristics of heterochromatin. In diploid tissue, variegation would be caused by overcompaction of the DNA while in polytenized tissue it would be
caused by underreplication. In this regard, it is interesting that euchromatic sequences juxtaposed to heterochromatin become late replicating (Prokopyeva-Belgovskaya, 1947; Ananiev and Gvozdev, 1974), a feature normally associated with heterochromatin.

An intriguing, alternative explanation for variegating phenotypes is that transcription from a promoter within the heterochromatin (ie. in a transposable element) is interfering with transcription at the affected euchromatic locus (Spradling and Karpen, 1990). The heterochromatic breakpoints of several variegating rearrangements are closely associated with satellite and middle-repetitive DNA sequences (Tartof et al., 1984) and it has been proposed that transcription may initiate within these sequences. In several rearrangements which completely restore wild-type function to the variegating loci, these elements are still associated with the gene implying that variegation originates from deeper within the heterochromatin. However, Reuter et al. (1985) show that in 48 of 51 wild-type revertants of a variegating rearrangement, an affected gene can still be made to variegate in the presence of a strong Enhancer of PEV (see Dominant modifiers of PEV). This suggests that variegation can in fact originate from relatively small sequences, adjacent to the chromosomal breakpoint. The revertants characterized by Tartof (1984), do not respond to strong E(var) loci (T. Grigliatti, personal communication).

**Dominant Modifiers of position effect variegation**

Genetic modifiers of PEV either reduce (Su(var)s) or increase
(E(var)s) the number of cells in which the variegating gene is inactivated. A large number of single-site genetic modifiers of PEV have been described (Reuter and Wolff, 1981; Sinclair et al., 1983; Sinclair et al., 1989; Locke et al., 1988; Wustmann et al., 1989). The majority of these have been identified in segmental aneuploidy studies (Locke et al., 1988; Wustmann et al., 1989). Locke et al. (1988), use 12 chromosomal rearrangements to define four independent loci and from this extrapolate to predict the existence of 20-30 dosage-sensitive modifiers of PEV in the Drosophila genome. In a more extensive study, Wustmann et al. (1989) describe 38 independent loci in an area covering 30% of the autosomal complement. They predict the existence of 120-150 such loci, a number closely approximated by several other studies conducted on a smaller scale (Henikoff, 1979; Reuter et al., 1987; Szidonya and Reuter, 1988). Altogether, the aneuploidy studies identified 44 dosage dependent modifying loci which can be conveniently divided into four classes: haplo suppressor/triplo enhancer, haplo enhancer/triplo suppressor, haplo enhancer and haplo suppressor. The reciprocal haplo/triplo phenotypes of the loci which fall into the first two categories suggests that they may play an important role in the chromatin assembly process.

A model has been proposed to explain the sensitivity of PEV to the dosage of a number of single-site modifiers (Locke et al., 1988). Arguing that heterochromatin formation involves the self-assembly of multimeric complexes and that Su(var) loci identify components of these complexes, Locke et al. (1988) point out that
the law of mass action dictates that the dose of a single component
can effect the rate of assembly exponentially. If none of the
components are rate-limiting, the rate would be determined by the
total number of elements in the complex. Although an attractive
hypothesis, it may only apply to the limited number of modifiers
which show reciprocal haplo/triplo effects.

In addition to modifiers of PEV identified by segmental
aneuploidy, two large collections of ethylmethane sulfonate (EMS)
induced $Su(var)$ mutations have been described. One collection
consists of 12 mutations on the second chromosome and eleven
mutations on the third chromosome (Reuter and Wolff, 1981) while
the second collection identifies 16 on the second and 33 on the
third (Sinclair et al., 1983). Smaller collections of $E(var)$ loci
have also been described (Reuter and Wolff, 1981; Sinclair et al.,
1989). The location of these loci correspond well with the loci
identified in the aneuploidy studies and their phenotypes indicate
that the majority of mutations are amorphs or strong hypomorphs.
The elaboration of a complementation map comprised of both $Su(var)$
collections is the focus of a collaborative effort between the two
groups (R. Mottus, personal communication).

Typically, $Su(var)$ mutations exhibit a dominant phenotype,
often restoring nearly full activity to a variegating gene. They
exert their influence over a broad range of variegating genes
showing no preference for particular types of rearrangements
(Hayashi et al., 1990; Reuter et al., 1986; Sinclair et al., 1983;
1991). Their suppressing ability is often sex-specific (Hayashi et
al., 1990; Reuter et al., 1986; Sinclair et al., 1991) and allele-specific, some alleles showing stronger suppression than others. Not all of the suppressor genes are essential. Reuter et al. (1986) report that all of their third chromosome suppressors are either homozygous lethal or sterile while Sinclair et al. (1983), note that all of their third chromosome suppressors are homozygous viable. These two collections may not comprise the same loci, or alternatively, the results may reflect differences in the strength of particular alleles. The germ-line requirement for suppressor genes has been examined for alleles of two loci, Suvar(2)1 and Suvar(3)3 (Szabad et al., 1988). In both instances mitotic recombination in the germ-line tissue indicates that both genes are required for the development of the germ-line as well as the soma.

Suvar(2)1 is one of the first described Su(var)s and has been the subject of a great deal of study. The Suvar(2)1° allele increases the transcriptional capacity of chromosomes in vitro (Khesin and Bashkirov, 1979) indicating that the gene has an effect on euchromatin function. Larvae which are heterozygous or homozygous for the Suvar(2)1 gene exhibit hyperacetylation of histone H4 which is correlated with an increased accessibility of the DNA to endogenous endonucleases (Dorn et al., 1986). It has been proposed that the gene may encode a histone deacetylase or a product which can control the accessibility of histones to deacetylases (Dorn et al., 1986). In this regard, it is interesting that strains carrying mutant alleles of Suvar(2)1 exhibit reduced viability when grown on sodium butyrate (Reuter et
al., 1986; Sinclair et al., 1991), a known inhibitor of histone deacetylase (Candido et al., 1978), while strains carrying other strong Su(var) loci such as Suvar(3)1, Suvar(3)2 and Suvar(3)9 do not exhibit the same sensitivity.

There have been several studies describing small collections of enhancers of position effect variegation (E(var)s; Tartof et al., 1984, Sinclair et al., 1989). E(var) mutations are dominant and they enhance in a general manner, their effect being independent of the heterochromatic breakpoint and euchromatic gene involved. These genes are interesting in that they may identify proteins involved in the establishment of euchromatin domains.

**Cloned Su(var) loci**

Three dominant Su(var) loci have been cloned, Suvar(3)7, Suvar(3)6 and Suvar(2)5. Suvar(3)7 is a modifier of the haplo-suppressor/triplo-enhancer class located at 87E on the Drosophila polytene map. This locus exhibits a dosage dependent effect from one to five copies of the gene as determined by germ-line transformations (Reuter et al., 1990). The encoded protein is phosphorylated, chromatin associated (G. Reuter, personal communication) and contains 5 widely spaced zinc finger motifs which may allow it to interact with widely spaced segments of DNA (Reuter et al., 1990).

The Suvar(3)6 locus at 87B is a modifier of the haplo-suppressor class. The locus exhibits no triplo effects even when two copies are introduced by germ-line transformations (G. Reuter,
personal communication). *Suvar(3)6* encodes the protein phosphatase, PP1. Interestingly, mutations for *Suvar(3)6* are epistatic over the enhancer effect of an extra copy of *Suvar(3)7*, a protein which contains over 40 potential phosphorylation sites (G. Reuter, personal communication).

The *Su(var)205* locus located at 29A is another modifier of the haplo-suppressor/triplo-enhancer class. Cytological interval 29A is also the location of the gene which encodes the heterochromatin associated protein, HP1. Since two alleles of *Su(var)205* have lesions in the HP1 gene, it is likely that these two genes are allelic (Eissenberg et al., 1990; G. Reuter, personal communication). HP1 associates specifically with regions of β-heterochromatin although the protein sequence does not contain any known DNA binding motifs. A 37 amino acid region in the amino terminus is homologous to a similar domain in the *Polycomb* locus (Paro and Hogness, 1991). *Polycomb* is a member of a family of genes which act as repressors of many homeotic loci including those in the bithorax complex (Zink and Paro, 1989, Paro and Hogness, 1989). Like HP1, the *Pc* product is a known component of chromatin (Zink and Paro, 1989) and may be part of a heterochromatin like complex (Paro, 1990). Interestingly, a number of the members of the *Polycomb-like* group of genes are enhancers and suppressors of PEV (D. Sinclair, N. Clegg, T. Grigliatti and H. Brock personal communication).
Recessive Modifiers of position-effect variegation

Although virtually all modifiers of PEV which have been described exhibit dominant phenotypes, this is a reflection of the assays used, not a general characteristic of all modifying loci. There have been no screens described for recessive modifiers of PEV although many genes (particularly those defining enzymatic functions) involved in determining chromatin structure would not be expected to exhibit dominant phenotypes.

Two recessive su(var) loci have been reported. The genes mfs(2)31 and wdl were identified as essential genes in division 31 of the Drosophila polytene chromosome map (Sandler, 1977). Because of their proximity to a cluster of Su(var) genes and a second cluster of genes which are thought to interact with heterochromatin (da-abo group; see Sandler, 1977), these mutations have been tested for their ability to modify PEV (Sinclair et al., 1991). Although neither gene exhibits a dominant phenotype, both show strong recessive suppression of white variegation in the rearrangement In(1)w4.

In addition to being a modifier of PEV, the mfs(2)31 strain exhibits a variety of recessive phenotypes including male and female sterility, abnormal bristles and prolonged development. The failure of flies to survive when the mutation is uncovered by a deficiency indicates that the gene also has an essential function. A number of abnormalities have been observed in the testes of homozygote males including, absence of motile sperm, spermatids with two associated centrioles and spermatids with abnormally sized
nuclei (Lindsley et al., 1980). It has been proposed that \textit{mfs(2)31} encodes a function required for proper centriole migration since improper positioning of centrioles and spindle poles may result in macro- and micronuclei being formed (Lindsley et al., 1980).

D. SUMMARY

It is becoming increasingly apparent that an important principle governing the regulation of gene expression through development involves local alterations in chromatin structure (Gyurkovics et al., 1990). This realization has intensified the search for proteins which initiate and maintain such structural changes. Since many of these proteins will have close and strong associations with chromatin, they may be identified by assays which are specific for non-histone chromosomal proteins. This thesis describes the use of one such assay, PEV, to identify a gene which may encode a non-histone chromosomal protein in \textit{Drosophila melanogaster}. This protein may be involved in establishing two cytologically differentiable states of chromatin, heterochromatin and euchromatin. The \textit{mfs(2)31} gene differs from previously cloned modifiers of PEV in that mutations exhibit no dominant phenotypes. Chapter 2 of this thesis describes the cytogenetic localization of the \textit{mfs(2)31} gene and the isolation of new alleles. Chapter 3 contains a complete phenotypic description of the locus with a particular emphasis on the ability of mutant alleles to modify variegating backgrounds. Chapter 4 contains a molecular analysis of the locus in which the putative \textit{mfs(2)31} protein sequence is
compared to previously described NHPs.
CHAPTER 2: CYTOGENETIC ANALYSIS OF SUBDIVISIONS 31D-E

Introduction

The \textit{mfs(2)31} locus is a recessive suppressor of position-effect variegation (\textit{su(var)}) located in subdivision 31E of the \textit{Drosophila} polytene chromosome map. Sandler (1977) placed \textit{mfs(2)31} in 31B-31F, a 35 band region, based on its failure to complement \textit{Df(2L)J27} while Sinclair \textit{et al.} (1991) further localized it to the 20 chromomereres comprising 31D-E by its failure to complement \textit{Df(2L)J77} and \textit{Df(2L)J106}.

We have had an ongoing interest in the \textit{mfs(2)31} locus because of its \textit{su(var)} phenotype. Several \textit{Su(var)} genes are known to encode non-histone chromosomal proteins. Although \textit{mfs(2)31} is the first reported example of a recessive suppressor of PEV, the ability to expand the study of this locus was hampered by several considerations. First, the existence of only a single allele severely limits the phenotypic and developmental analysis. Second, the relative paucity of well characterized deficiencies and complementation groups in the 31D-E region makes it difficult to assess various strategies for a molecular analysis of the locus.

To deal with these substantial limitations, three initial objectives were identified: 1) Further cytological subdivision of 31D-E by the characterization of new deficiencies to map \textit{mfs(2)31} more precisely: 2) A more precise genetic, as well as cytogenetic, localization of the \textit{mfs(2)31} locus relative to previously cloned genes in the region: 3) Isolation of new alleles of \textit{mfs(2)31} which
should include point mutations for genetic and developmental analysis as well as insertional mutations to aid in the molecular analysis. It was anticipated that all these objectives could be achieved through a comprehensive cytogenetic and genetic analysis of region 31D-E; the region defined by the overlap of \( Df(2L)J27 \), \( Df(2L)J77 \) and \( Df(2L)J106 \), the smallest deficiency subinterval known to contain \( mfs(2)31 \).

The 31D-E region is contained within division 31, an anomalous segment of the polytene chromosome colloquially referred to as the "gooseneck" because of its thin underreplicated appearance (Lefevre, 1976). Although the distinct morphology of division 31 makes it a useful cytological landmark for chromosome arm 2L, the region is poorly banded making the precise localization of deficiency breakpoints extremely difficult. Diffuse banding coupled with apparent underreplication give the region a heterochromatin-like appearance. Interestingly, division 31 has been identified as a binding site for an antibody directed towards HPI, a non-histone chromosomal protein which is primarily associated with heterochromatin (James et al., 1989).

HPI was first identified in a nuclear protein fraction of \textit{Drosophila melanogaster} (James et al., 1986). A full description of the gene which encodes HPI (\textit{Su(var)205}) is provided in Chapter 1. Immunofluorescent labelling of an antibody specific for HPI revealed that on salivary gland polytene chromosomes, the protein is associated with \( \beta \)-heterochromatin and discrete segments of euchromatin including division 31 (James et al., 1989). Although
the HP1 antibody binds to division 31 with a characteristic banding pattern, the protein contains no previously described DNA binding motifs. This banding could reflect the presence of repetitive heterochromatic DNA sequences within division 31 or alternatively, HP1 could be involved in the coordinate regulation of several non-contiguous genes within this interval. These two hypotheses are not mutually exclusive, the latter being particularly intriguing since amongst the genes which have been localized to division 31 are several known modifiers of PEV, a phenotype shared by the gene encoding HP1 (Su(var)205; Sinclair et al., 1983).

There have been four genes mapped to 31D-E: daughterless (da), cdc2, 1(2)54 and mfs(2)31. The da gene was cloned and mapped by in situ hybridization to 31E (Cronmiller et al., 1988) during the course of the investigation reported here. It is a member of a network of genes which have been identified in the Drosophila sex determination pathway (Cline, 1989). This essential locus was named for the inability of hypomorphic alleles to produce female offspring. The gene has been shown genetically to be a regulator of Sex-lethal, the primary sex-determining switch in Drosophila, as well as an essential gene in the development of the peripheral nervous system (Cline, 1989). A molecular analysis of the locus has revealed that the da transcript encodes a transcription factor of the helix-loop-helix class (Cronmiller et al., 1988; Caudy et al., 1988). da alleles show neither dominant nor recessive suppression of PEV and have no known functional relationship to mfs(2)31 (Sinclair et al., 1991).
Subsequent to the initiation of these studies, the *Drosophila* homolog of the Yeast *cdc2* gene was localized to subdivision 31E (Lehner and O'Farrell, 1990), mapping within 5 kb of the *da* locus (Cronmiller et al., 1988). The *cdc2* family of genes encode kinases whose activity is required during the cell cycle both to initiate DNA synthesis (G₁-S transition) and to initiate mitosis (G₂-M transition), two functions which are genetically separable in Yeast (for review see Nurse, 1990).

Single alleles of *l(2)54* and *mfs(2)31* were isolated in a screen for essential genes uncovered by *Df(2L)J39* (Sandler, 1977). Sandler and his colleagues were hoping to add to a previously identified group of closely-linked genes which interact with X chromosome heterochromatin. The group extends from 31D to 32D and includes *da*, abnormal-oocyte (*abo*) and *hold-up* (*hup*). The precise number and distribution of genes located within this cluster cannot be determined since no systematic genetic characterization of this large cytological interval has been described. *l(2)54* is a recessive lethal mutation and has not been subject to any further genetic analysis.

As a homozygote, the *mfs(2)31* mutation is semi-lethal but sterile in both sexes. Because of the recessive sterility, homozygotes cannot be tested for abnormal sex ratios amongst their progeny. An abnormal sex ratio amongst progeny is a characteristic of the *da-abo* cluster mutations. A complete description of this pleiotropic locus can be found in Chapter 3.

In a subsequent analysis of a number of dominant suppressors
of PEV which map to division 31 (Sinclair et al., 1991), all members of the da-abo cluster were tested for an ability to modify PEV. The genes were tested because of their apparent sensitivity to cellular levels of heterochromatin, a property shared by Su(var) genes (see Chapter 1). Although no members of the da-abo group are dominant modifiers of PEV, mfs(2)31 was found to be a strong recessive suppressor of white variegation in In(1)wm. This pleiotropic effect is attributable to the mfs(2)31 locus since rare survivors over Df(2L)J106 show the same phenotype.

The mfs(2)31 locus is the first reported example of a recessive suppressor of PEV. The novelty of this gene coupled with an ability to localize it to a cytological interval (31D-E) for which molecular entry points (da and cdc2) are available make it an excellent candidate for further genetic and molecular analysis. Such an analysis would be greatly facilitated by a cytogenetic study on the chromosomal segment in which mfs(2)31 is located. The location of the gene in a region of the chromosome which, in itself, is of interest to the study of Su(var)s (region of HP1 binding) provides further impetus for such a study. In addition, it may be possible to determine whether the heterochromatin-like appearance of division 31 at the cytological level correlates with a lack of mutable loci.

This chapter contains a complete cytogenetic analysis of 31D-E. New deficiencies are described which divide the region into smaller subintervals. Saturation mutagenesis brings the total number of complementation groups within the region to 25, 9 of
which map to an interval containing $mfs(2)31$, $da$ and $cdc2$. The relative orientation of these three genes was determined with respect to the centromere. Three new alleles of $mfs(2)31$ were generated, one of which was recovered from a screen which utilized $P$ element mediated hybrid-dysgenesis. As anticipated, this analysis made possible the further genetic and molecular analyses of the $mfs(2)31$ locus described in chapters 3 and 4.
Materials and Methods

Stocks

Unless described below, information pertaining to stocks can be found in Lindsley and Zimm (1992). The study utilized three series of deficiency chromosomes generated by the reversion of the neomorphic, rumple-winged phenotype of Jammed (J). The first series, consisting of Df(2L)J2, Df(2L)J27, and Df(2L)J39, was kindly provided by L. Sandler (see Mange and Sandler, 1973). Series two, consisting of Df(2L)J77 and Df(2L)J106, was provided by J. Lengyel (see Salas and Lengyel, 1984). The third series which included Df(2L)J1, Df(2L)J3, Df(2L)J16 and Df(2L)J17, was generated by D. Sinclair (unpublished). A single deficiency, Df(2L)G2, was generated by the author (unpublished) in a screen for lethal alleles of Su(var)216. The list of deficiencies and their breakpoints, as estimated from cytological examination, are shown in Table 1.

A number of female sterile mutations map to division 31 based on their failure to complement Df(2L)J2 (Schupbach and Wieschaus, 1986; 1989; 1991). The following mutant strains were kindly provided by T. Schupbach: trunk (trk), ltoDG25, ltoRU26, ltoPI23, erratic (err), mat(2)earlyQM47 and mat(2)synPJ50. The following recessive lethal and female sterile mutations were provided by L. Sandler: da, dal, mfs(2)31, hup and 1(2)54 (see Mange and Sandler, 1973; Sandler, 1977). The da2 allele was provided by C. Cronmiller while female-sterile-2-rosy-4 (fs(2)ry4) was provided by A. Spradling. The Su(var)216 mutation is described in Sinclair et
<table>
<thead>
<tr>
<th>Rearrangement</th>
<th>Reference</th>
<th>Cytology</th>
</tr>
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<tbody>
<tr>
<td>$Df(2L)J2$</td>
<td>MANGE and Sandler 1973</td>
<td>31A3-32A</td>
</tr>
<tr>
<td>$Df(2L)J39$</td>
<td>MANGE and Sandler 1973</td>
<td>31D-32B</td>
</tr>
<tr>
<td>$Df(2L)J27$</td>
<td>Sandler 1984</td>
<td>31D-31E</td>
</tr>
<tr>
<td>$Df(2L)J77$</td>
<td>SALAS and LENGYEL 1984</td>
<td>31D-31E</td>
</tr>
<tr>
<td>$Df(2L)J106$</td>
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<td>31D-31E</td>
</tr>
<tr>
<td>$Df(2L)J1$</td>
<td>THIS STUDY</td>
<td>31B-31D</td>
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<tr>
<td>$Df(2L)J3$</td>
<td>THIS STUDY</td>
<td>31D-31F</td>
</tr>
<tr>
<td>$Df(2L)J16$</td>
<td>THIS STUDY</td>
<td>$In(2L)30C-D;31E$ associated with a deficiency in 31E</td>
</tr>
<tr>
<td>$Df(2L)J17$</td>
<td>THIS STUDY</td>
<td>not visible</td>
</tr>
<tr>
<td>$Df(2L)G2$</td>
<td>THIS STUDY</td>
<td>31D;31F</td>
</tr>
</tbody>
</table>
al. (1991). A summary of these mutations and their origins can be found in Table 2.

Several collections of recessive lethal mutations in the 31 region have also been included in this study. The first collection consists of sixteen gamma-induced lethal alleles, uncovered by Df(2L)J2 (cited in Brock, 1989). Brock (1989), used ethyl-methane sulfonate (EMS) to induce a second collection of 40 lethal mutations which are also uncovered by Df(2L)J2. In a screen of 5000 second chromosomes exposed to gamma-irradiation, Harrington (1990) identified 8 lethal mutations which fail to complement Df(2L)J27. One lethal allele, uncovered by both Df(2L)J2 and Df(2L)J77 was isolated by Clegg (1991) in a screen which used P element mediated hybrid dysgenesis as a mutagen source.

Culture conditions

Fly cultures were raised on a cornmeal-agarose-sucrose medium supplemented with tegosept as a mold inhibitor. Tetracycline and ampicillin or tetracycline and streptomycin were added as antibiotics. All experiments were performed at 25° unless otherwise indicated.

Cytology

Black cells (Bc) is a dominant larval marker on the second chromosome. Males carrying a Bc chromosome and the chromosome of interest, were crossed to wild-type virgin females at 17°. Larvae carrying the mutant chromosome (Bc') were selected for cytological

37
<table>
<thead>
<tr>
<th>Mutation</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>Su(var)216</td>
<td>SINCLAIR et al., 1983; 1991</td>
</tr>
<tr>
<td>fip</td>
<td>DE VALOIR et al., 1991</td>
</tr>
<tr>
<td>trk</td>
<td>SCHÜPBACH and WIESCHAUS, 1986</td>
</tr>
<tr>
<td>bsk</td>
<td>NÜSSLEIN-VOLHARD, WIESCHAUS and KLUDING, 1984</td>
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<td>SCHÜPBACH and WIESCHAUS, 1991</td>
</tr>
<tr>
<td>ltoDG25</td>
<td>SCHÜPBACH and WIESCHAUS, 1991</td>
</tr>
<tr>
<td>da</td>
<td>CLINE, 1989</td>
</tr>
<tr>
<td>mfs(2)31</td>
<td>LINDSLEY, GOLDSTEIN and SANDLER, 1980</td>
</tr>
<tr>
<td>pim</td>
<td>NÜSSLEIN-VOLHARD, WIESCHAUS and KLUDING, 1984</td>
</tr>
<tr>
<td>l(2)54/</td>
<td>SANDLER, 1977; SCHÜPBACH and WIESCHAUS, 1989</td>
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<tr>
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</tr>
<tr>
<td>earlyQM47</td>
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<tr>
<td>err</td>
<td>SCHÜPBACH and WIESCHAUS, 1989</td>
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<td>mat(2)</td>
<td>SCHÜPBACH and WIESCHAUS, 1989</td>
</tr>
<tr>
<td>synPJ50</td>
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</tr>
<tr>
<td>ltoPI23</td>
<td>SCHÜPBACH and WIESCHAUS, 1991</td>
</tr>
<tr>
<td>cdc2</td>
<td>SINCLAIR et al., 1991</td>
</tr>
<tr>
<td>wdl</td>
<td>SANDLER, 1977</td>
</tr>
<tr>
<td>hup</td>
<td>SANDLER, 1977</td>
</tr>
<tr>
<td>dal</td>
<td>SANDLER, 1977</td>
</tr>
</tbody>
</table>
examination. Salivary glands were dissected from 3rd instar larvae in *Drosophila* ringers (6.5 g NaCl, 0.14 g KCl, 0.12 g CaCl₂, 0.20 g NaHCO₃, 0.01 g NaH₂PO₄ in 1000 ml dH₂O; pH 7.2) and were then transferred immediately to a drop of aceto-alcohol (3 parts absolute alcohol: 1 part glacial acetic acid) on a clean acid-washed slide. Following a two minute fixation in the aceto-alcohol, a drop of aceto-orcein stain (3% in 45% acetic acid) was added directly to the glands. Glands were stained for 20 minutes and then excess stain was removed by rinsing carefully with 45% acetic acid. A single drop of lacto-aceto-orcein stain (3% in 1:1 glacial acetic acid and lactic acid) was added followed by a coverslip. The glands were squashed and then examined under phase contrast optics.

**Genetic screens**

Three genetic screens were conducted to isolate new lethal mutations in the 31D-E region. The protocols for these screens are summarized in Figures 1 and 2.

The first two screens were designed to generate EMS induced lethal mutations uncovered by *Df(2L)J27* and *Df(2L)J106* respectively (see Figure 1). Males which were homozygous for a genetically marked second chromosome (*b pr cn*), were fed EMS (0.025 M) by the method of Lewis and Bacher (1986), and mated, *en masse*, to *Tft/CyO* females. Male progeny with the genotype *b pr cn/CyO* were collected and individually mated (at 29°) to harems of 3-5 *Df(2L)J27/CyO* (screen #1) or *Df(2L)J106/CyO* (screen #2) virgin females. The
Figure 1: Screens for EMS induced lethal mutations

Males

EMS

$25^\circ$

$\frac{b \ pr \ cn}{b \ pr \ cn}$

Females

$\frac{Tft}{CyO}$

$\frac{b \ pr \ cn}{CyO}$

$\frac{Df(2L)J27}{CyO}$ Screen 1

$\frac{Df(2L)J106}{CyO}$ Screen 2

$29^\circ$

Establish Balanced Stocks

Score for Absence.
Figure 2: Screen for $P$ element induced lethal mutations

Males

\[
\frac{b \text{ pr cn}}{b \text{ pr cn}}; \quad + \quad \times \quad \frac{Sp}{CyO}; \quad \frac{\triangle 2-3 \text{ Sb}}{TM6 \ Ubx} \quad 25^\circ
\]

\[
\frac{b \text{ pr cn}}{CyO}; \quad \frac{\triangle 2-3 \text{ Sb}}{birm} \quad \times \quad \frac{birm}{birm} ; \quad + \quad \frac{ry}{ry} \quad 16^\circ
\]

\[
\frac{b \text{ pr cn}}{birm}; \quad \frac{\triangle 2-3 \text{ Sb}}{ry} \quad \times \quad \frac{Tft}{CyO} ; \quad + \quad + \quad 16^\circ
\]

\[
\frac{b \text{ pr cn}^*}{CyO}; \quad + \quad \times \quad \frac{Df(2L)J77}{CyO} ; \quad + \quad + \quad 25^\circ
\]

Establish Balanced Stocks

Score for Absence.
presence of a lethal mutation on the b pr cn chromosome, which is uncovered by either of the two deficiencies, was indicated by the absence of b pr cn/Df(2L)J27 or Df(2L)J106 progeny (Cy'). Stocks of putative lethal mutations were established from balanced siblings (b pr cn/CyO) and were re-tested at 22° to identify temperature-sensitive mutations.

A third screen was conducted using P element mediated hybrid dysgenesis to generate insertional lethal mutations uncovered by Df(2L)J77 (Figure 2). This screen utilized the Δ2-3 mutagenizing system as described by Robertson et al., (1988). Males which were homozygous for the b pr cn chromosome were mated en masse to females with the genotype Sp/CyO; Δ2-3 Sb/TM6, Ubx. The gene Δ2-3 is a P factor with the inverted repeats deleted; it synthesizes transposase but cannot move. The b pr cn/+; Δ2-3 Sb/+ males were collected and mated en masse to birm/birm; ry/ry females (the birm strain contains approximately 17 P elements which can respond to transposase when it is provided from another source). This cross and the next were done at 16° to minimize somatic mobilization of P elements. Dysgenic males of genotype b pr cn/birm; Δ2-3 Sb/ry were collected for mass matings to Tft/CyO females. The b pr cn/CyO; ry/+ males were then collected and individually mated to harems of Df(2L)J77/CyO virgin females (25°). As was the case in screens #1 and #2, a lethal mutation was indicated by the absence of b pr cn/Df(2L)J77 progeny. Stocks of putative lethal mutations were established from balanced siblings.
Complementation

New and previously identified mutations in division 31 were crossed to the collection of deficiencies available for the region. Alleles were assigned to a cytological interval based on either lethality, sterility or a visible phenotype. Failure to complement was based on a minimum of 50 flies scored although in the vast majority of cases 200-300 flies were examined. Once placed within a cytological interval, alleles were sorted into complementation groups by inter se crosses. If a cytological interval was determined by earlier rounds of crosses to have a large number of complementation groups, a strong representative allele from each group was designated as the tester strain for subsequent crosses. In the case of some sterile mutations, assignment to a group was based on a failure to complement a single allele.

Mapping by Recombination

In order to determine the order of complementation groups with respect to the centromere, lethal strains carrying different genetic markers on the mutant chromosome were crossed. Females heterozygous for both mutations were collected and crossed, en masse, to either $Df(2L)G2/Cy0$ or $Df(2L)J77/Cy0$ males. Putative $Cy'$ recombinants were identified by the presence of straight-winged flies. These were collected and retested for their ability to complement the deficiency chromosome. Gene order was inferred by the genetic markers on the recombinant chromosome. The genetic markers used were $black$ ($b$), $purple$ ($pr$) and $cinnabar$ ($cn$) located
at map positions 48.5, 54.5 and 57.5 respectively (Lindsley and Zimm, 1992), all of which are proximal to the 31 region. The closest marker, $b$, is located at map position 48.5. In some cases only two recombinant chromosomes were recovered.
Results

Cytology

Five new deficiencies within division 31 were identified and characterized: \textit{Df(2L)J1}, \textit{Df(2L)J3}, \textit{Df(2L)J16}, \textit{Df(2L)J17}, and \textit{Df(2L)G2}. The breakpoints for these deficiencies are listed in Table 1 along with the previously described deficiencies for the region: \textit{Df(2L)J2}, \textit{Df(2L)J39}, \textit{Df(2L)J27}, \textit{Df(2L)J77} and \textit{Df(2L)J106}. As previously mentioned, division 31 has a very diffuse banding pattern making the precise determination of rearrangement breakpoints very difficult. Nonetheless, careful cytological examinations coupled with recent molecular localizations of genes within this cytological division have facilitated the drawing of a new, reasonably accurate, cytological map (Figure 3). Non-visible deficiencies (i.e. \textit{Df(2L)J17}) have not been placed on this map although their breakpoints can be inferred from strictly genetic considerations. What follows is a rationalization of the breakpoints which have been determined for the new deficiencies.

\textit{Df(2L)J1} is a visible deficiency contained completely within division 31. Cytologically, it removes the distal two thirds of 31D which sets the proximal breakpoint in proximal 31D. This is confirmed genetically by its failure to uncover the da gene, which has been localized by \textit{in situ} hybridization to distal 31E (Cronmiller \textit{et al.}, 1988). Although the distal breakpoint falls within the extremely poorly banded 31B-C region, the failure of the deficiency to complement the \textit{fs(2)ry4} gene, which was localized by \textit{in situ} hybridization to 31B (personal observation), makes 31B the
Figure 3: Cytological map for division 31. Dashed lines represent regions of uncertainty.
most likely site of the distal breakpoint.

*Df(2L)J3* is a visible deficiency in the proximal end of division 31. Proximally, it deletes the sharply banded 31F1 but not the 32A1 doublet thus placing the proximal breakpoint within 31F. The distal breakpoint does not appear to remove the proximal most band of 31D although genetically, the deficiency uncovers the *da* gene. This suggests that the distal breakpoint is in 31E but very close to the 31D-E boundary.

*Df(2L)J16* is a small deficiency within subdivision 31E. It is associated with an inversion whose distal breakpoint is at the 30C-D boundary and whose proximal breakpoint falls within 31E. The three distinct bands which comprise all of 31D are clearly visible within the inversion loop thus setting the distal deficiency breakpoint near the 31D-E boundary. This was confirmed genetically by the failure of the deficiency to complement the *da* gene. The 31F1 band is not deleted thus localizing the deficiency to 31E.

*Df(2L)J17* is not visible. Both the proximal most band of 31D and band 31F1 are not removed by this deficiency. Genetically, it appears to be smaller than *Df(2L)J16*, uncovering the distal most complementation groups.

*Df(2L)G2* is the only known deficiency for the 31 region which was not isolated by reverting the dominant phenotype of the neomorphic mutation *Jammed (J).* It was isolated in a screen for gamma induced lethal alleles of *Su(var)216* (unpublished). It is a small visible deficiency in the proximal end of the 31 region. Its distal breakpoint falls within 31D, although a more precise
localization was not possible. Genetically, it appears to have a very similar distal breakpoint to \textit{Df(2L)J39}, uncovering all of the same complementation groups. Proximally, it deletes 31F1 but not the 32A doublet placing the proximal breakpoint within 31F.

**Screens**

Lethal mutations in the 31 region were generated in seven independent screens conducted in our lab. Four of the screens have been described previously while three were performed as part of this current study. The results of the new screens are summarized in Table 3.

Screen #1 used the potent mutagen ethyl methanesulfonate (EMS) to generate lethal mutations uncovered by \textit{Df(2L)J27}. A total of 10,000 chromosomes were examined from which 27 lethal alleles were recovered (frequency = 0.0027). Screen #2 used EMS to generate lethal mutations uncovered by \textit{Df(2L)J106}. This screen also produced 27 mutations but in only 5000 chromosomes examined (frequency = 0.0054). None of the mutations recovered from Screens #1 and #2 are temperature-sensitive.

Screen #3 was designed to generate insertional mutations in the 31D-E region. The \textit{A2-3} mutagenizing system (Robertson et al., 1988) was employed to jump \textit{P} elements into the \textit{b pr cn} chromosome. Twelve thousand five hundred chromosomes were screened for failure to complement \textit{Df(2L)J77}. Four lethal mutations were recovered for a mutation frequency of 0.0003.
### TABLE 3

**Summary of genetic screens**

<table>
<thead>
<tr>
<th>Screen</th>
<th>Tester strain</th>
<th>Mutagen</th>
<th>Chromosomes screened</th>
<th>Mutations recovered</th>
<th>Mutation Frequency</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Df(2L)J27</em></td>
<td>EMS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10000</td>
<td>27</td>
<td>0.0027</td>
</tr>
<tr>
<td>2</td>
<td><em>Df(2L)J106</em></td>
<td>EMS</td>
<td>5000</td>
<td>27</td>
<td>0.0054</td>
</tr>
<tr>
<td>3</td>
<td><em>Df(2L)J77</em></td>
<td>HD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12500</td>
<td>4</td>
<td>0.00032</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>27500</td>
<td>58</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> ethyl methanesulfonate

<sup>b</sup> *P* element hybrid dysgenesis
Complementation

The 58 new alleles were combined with 65 previously identified lethal mutations in division 31 (see Materials and Methods) and 19 mutations isolated on the basis of other phenotypes (see Materials and Methods and Table 2), to generate a pool of 142 testable strains. Mutations were initially screened for those which failed to complement $Df(2L)J106$, $Df(2L)J77$ and $Df(2L)J27$. The overlap between these three deficiencies defines the smallest cytological interval to which the $mfs(2)31$ gene had been previously assigned. For the purposes of this study, only mutations which failed to complement all three deficiencies were examined further. A total of 97 mutant strains fell into this category. These were then tested against the newly characterized deficiencies in the region: $Df(2L)J16$, $Df(2L)J1$, $Df(2L)J17$, $Df(2L)J3$ and $Df(2L)J2$. Three of these deficiencies ($Df(2L)J16$, $Df(2L)J17$ and $Df(2L)J11$) appear, genetically, to have breakpoints within the overlap of $Df(2L)J106$, $Df(2L)J77$ and $Df(2L)J27$ (see Figure 4). Thus, it was possible to assign all 97 mutant strains to one of six cytogenetic intervals (intervals 1-6 in Figure 4).

The mutations were broken down by inter se complementation into 25 complementation groups, 9 of which are represented by a single allele. The smallest intervals, #1 and #4, contain single complementation groups while the largest interval, #5, contains 9. Fourteen of the complementation groups have not been previously identified. New alleles were generated for ltoDG25, err, pim, da, cdc2, l(2)54 and mfs(2)31. ltoDG25 and err had been previously
Figure 4: Cytogenetic map of region 31D-E. Lethal complementation groups have been named by conventional nomenclature but are abbreviated in the figure i.e. \( l(2)31Db = Db \). Total number of alleles and commonly used synonyms are provided in parentheses.
identified as maternal effect female sterile mutations. The isolation of lethal alleles of these genes indicates an essential function not restricted to the female germline.

The mfs(2)31 gene was localized to the largest subinterval: #5. This interval contains nine complementation groups including the previously cloned genes, da and cdc2. All groups within this interval exhibited a simple complementation pattern. There are two mutations which were generated in P element screens which map to this interval. The single mutation generated by Clegg (1991) is in the cdc2 complementation group while a second mutation isolated in screen #3 (this study) was assigned to the mfs(2)31 complementation group. In total, three new alleles of mfs(2)31 were generated, one in a P element screen (screen #3) and two in an EMS screen (screen #1). These new alleles are described in detail in Chapter 3.

The interval to which mfs(2)31 was localized is defined by the proximal breakpoints of Df(2L)J16 and Df(2L)J17. The next most proximal interval is defined by the proximal breakpoints of Df(2L)J27 and Df(2L)J16. This interval, with its eight complementation groups, is contained completely within subdivision 31E (see Figure 3). If a random dispersal of genes is assumed within 31E, the data indicates that mfs(2)31 is located in the distal half of this subdivision. This is supported by the observation that the previously cloned genes cdc2 and da, both in the same interval as mfs(2)31, have been localized by in situ hybridization to 31E1-E3 (Cronmiller et al., 1988; Lehner and O'Farrell, 1990).
Interval 31D-E can be defined approximately by the overlap between $Df(2L)J27$, $Df(2L)J106$ and $Df(2L)J77$. All screens described within this study overlap within this region. However, because all screens were done independently using a variety of deficiencies and mutagens, an empirical determination of the degree of saturation of this region is not possible. Nonetheless, the existence of 9 complementation groups out of 25 with only one allele would suggest that the region is not saturated. This is supported by the recent identification of new complementation groups within the region (Randy Mottus, personal communication).

Mapping by Recombination

The smallest cytological interval into which $mfs(2)31$ can be placed (interval #5 on Figure 4) contains two previously cloned genes: $da$ and $cdc2$. The position of these two genes relative to one another has been determined molecularly (Clegg, 1992), but not genetically. Genetically, $mfs(2)31$ has been mapped proximally to $da$ but has not been mapped relative to $cdc2$ (Lindsley et al., 1980). A precise positioning of $mfs(2)31$ relative to the two cloned genes could be of great value for the molecular analysis of this gene. More specifically, it would allow for the orientation of a molecular walk.

Genetic mapping was used to determine the order of $da$, $cdc2$ and $mfs(2)31$ relative to the centromere. The results of the individual pair-wise mapping experiments are provided in Table 4 and these are summarized in the genetic map provided in Figure 5.
TABLE 4

Mapping by recombination

<table>
<thead>
<tr>
<th>Cross</th>
<th>Flies Scored</th>
<th>Recomb.</th>
<th>Mapa Distance</th>
<th>Order</th>
</tr>
</thead>
<tbody>
<tr>
<td>pim x cdc2</td>
<td>30562</td>
<td>7</td>
<td>0.046</td>
<td>pim - cdc2</td>
</tr>
<tr>
<td>Eb x cdc2</td>
<td>78755</td>
<td>2</td>
<td>0.005</td>
<td>Ec - cdc2</td>
</tr>
<tr>
<td>da x cdc2</td>
<td>39270</td>
<td>2</td>
<td>0.010</td>
<td>cdc2 - da</td>
</tr>
<tr>
<td>mfs(2)31 x da</td>
<td>66181</td>
<td>8</td>
<td>0.024</td>
<td>da - mfs(2)31</td>
</tr>
<tr>
<td>Ef x cdc2</td>
<td>31128</td>
<td>7</td>
<td>0.045</td>
<td>cdc2 - Ef</td>
</tr>
<tr>
<td>Ef x mfs(2)31</td>
<td>58775</td>
<td>5</td>
<td>0.017</td>
<td>mfs(2)31 - Ef</td>
</tr>
</tbody>
</table>

a map distance = ((Recomb. X 2)/Parentals) X 100. The number of parental types was estimated from the number of Cy siblings.
Figure 5: Recombination map for complementation groups located in cytogenetic interval #5. More commonly used synonyms are provided in parentheses. *pim* is located in cytogenetic interval #4.
An initial mapping experiment was done with \textit{pim} and \textit{cdc2}, two genes located within adjacent cytological subintervals. This was done to verify the orientation of the cytogenetic map relative to the centromere. Although this orientation can be inferred by the pattern of complementation with respect to \textit{Df(2L)J1}, the difficulties with cytology in the region made this verification advisable. As predicted by the cytological map, \textit{pim} maps distal to \textit{cdc2} (0.046 map units). The \textit{mfs(2)31} mutation has previously been mapped proximal to \textit{da}, although no map distance has been reported (Lindsley et al., 1980). We confirm this orientation placing \textit{mfs(2)31} 0.024 map units proximal to \textit{da}. A mapping experiment between \textit{da} and \textit{cdc2} yielded only two recombinants placing \textit{cdc2} 0.01 map units distal to \textit{da}. This low frequency of recombination reflects the physical distance between these two genes < 3 kb. This order was confirmed recently in an unrelated study in which three recombinants were recovered (D. Sinclair, personal communication).

In an unrelated study, I am conducting a complete ordering of the complementation groups in subdivision 31E. At the current time two additional complementation groups have been placed on the map: \textit{1(2)31Eb} and \textit{1(2)31Ef}. Their map positions relative to \textit{da}, \textit{cdc2} and \textit{mfs(2)31} are indicated in Figure 5.
Discussion

To aid in the genetic and molecular characterization of the mfs(2)31 locus, a cytogenetic analysis has been conducted in the smallest cytological interval to which it has been localized, 31D-E. This adds 31D-E to a growing number of regions in the Drosophila genome which have been intensively characterized in this manner. There are three objectives which can be met through such an analysis, a more precise cytological localization of the gene, an orientation of the locus with respect to molecular markers in the region and the isolation of new mutant alleles, particularly ones which are the result of insertional events. In addition, a cytogenetic analysis within division 31 may provide a clue as to why the region has specific associations with HP1, a heterochromatin associated protein.

The majority of 31D-E is encompassed by the overlap of Df(2L)J27, Df(2L)J106 and Df(2L)J77. The elaboration of a cytogenetic map for this region involved two steps, the cytological characterization of new deficiencies for the region and the placement of new and previously identified loci onto an updated cytological map. It was hoped that new deficiencies would subdivide the relatively large cytological interval into which mfs(2)31 had been mapped. From amongst a collection of revertants of the Jammed phenotype several new rearrangements were identified with breakpoints in the 31D-E region. These new rearrangements were combined with previously described ones to generate a new cytological map for division 31 (Figure 3 and Table 1).
A total of 142 mutations were examined for complementation with the battery of rearrangements described in Table 1. Nineteen of the mutations were provided by other laboratories (Table 2), sixty-five are from our collections and fifty-eight are new mutations induced in this study. The mutation frequencies for the new EMS screens are 0.0027 and 0.0054. A survey of recent literature indicates that these frequencies are typical for f₂ EMS lethal screens over similarly sized euchromatic deficiencies (0.0026, Wohlwill and Bonner, 1991; 0.009, Belote et al., 1990; 0.0036, Kimble et al., 1990). A screen of 12,500 second chromosomes exposed to P element mutagenesis identified four lethal mutations uncovered by Df(2L)J77. The Δ2-3 system has been shown to be a potent mutagenizing system producing 1-3 insertional events per chromosome arm per generation (Robertson et al., 1988). This predicts over 200 insertional events into 31D-E in a screen of this magnitude. The low rate of recovery of lethal mutations cannot be attributed to a lack of genetic activity within the region since there are at least 28 lethal complementation groups uncovered by Df(2L)J77. A previous screen of 14,000 chromosomes utilizing P element mediated hybrid dysgenesis generated only two mutational events uncovered by Df(2L)J77 (Clegg, 1992). Although the results of these two screens would suggest that region 31D-E may be somewhat refractory to insertional mutagenesis, there is no good empirical data available which would suggest that the observed mutation frequencies are atypical for the Drosophila genome.

A new cytogenetic map was generated for region 31D-E (Figure
4) providing valuable information for further studies on \textit{mfs(2)31}. With regard to a more precise cytogenetic localization of this \textit{su(var)} locus, the 31D-E interval is now divided into six smaller cytological subintervals containing as few as one and as many as nine lethal complementation groups. A total of twenty-five complementation groups were identified, nine of which are defined by a single allele. This large number of complementation groups represented by a single allele suggests that the region has not been saturated for essential genes. This was recently verified by the isolation of new lethal complementation groups within the region (R. Mottus, personal communication). The \textit{mfs(2)31} gene was localized to a genetically large subinterval containing eight other complementation groups. These include the previously cloned genes \textit{da} and \textit{cdc2}, both of which have been localized by \textit{in situ} hybridization to distal 31E (Cronmiller \textit{et al.}, 1988; Lehner and O'Farrell, 1990). Since at least 1/2 of the complementation groups which map to 31E are proximal to \textit{mfs(2)31}, the locus is probably located in the distal half of this subdivision.

Since \textit{mfs(2)31} is contained within the same cytogenetic interval as two previously cloned genes, \textit{da} and \textit{cdc2}, the orientation of these three genes was determined with respect to the centromere. The gene order, moving distally to proximally, is \textit{cdc2 - da - mfs(2)31}. The map distance between \textit{da} and \textit{mfs(2)31} is roughly three times greater than the distance between \textit{da} and \textit{cdc2}, although the \textit{cdc2 - da} distance was based on a low number of recombinants recovered. Since the distance between \textit{cdc2} and \textit{da} is
less than 3 Kb (Clegg, 1992), we infer that mfs(2)31 is quite close to these two genes on the molecular map. A genomic walk from da, away from cdc2, would cross the proximal breakpoint of Df(2L) J17, defining the segment of DNA within which the mfs(2)31 locus should be contained.

Although a number of the complementation groups contain 10 or more alleles only three additional alleles of mfs(2)31 were identified. Two alleles were recovered in the new EMS screens (Figure 1) while a third allele was isolated in the P element screen (Figure 2). The isolation of a P induced allele of mfs(2)31 was fortuitous since our analysis of the region indicates that although it is very active genetically, it may be refractory to P element mutagenesis. These new mutations, along with Sandler’s (1977) original allele, are described in detail in Chapter 3.

Prior to this study, very few genetic functions had been confirmed in division 31. This was of interest since not only does the region have a cytological morphology reminiscent of β-heterochromatin but it is also one of the few segments of euchromatin which is associated with HP1, a Drosophila protein which associates primarily with regions of β-heterochromatin (James et al., 1989). However, unlike heterochromatin, division 31 does not appear to have a lower level of genetic activity than other segments of euchromatin. Twenty-five lethal complementation groups have been identified in 31D-E alone. This number is in excess of the bands identified in the region although it is not atypical for Drosophila euchromatin (Lefevre and Watkins, 1985). In terms of
total gene numbers, patterns of complementation, accessibility to mutagens and frequency of recombinational events, division 31 is more typical of euchromatin than heterochromatin. Although, this cytogenetic analysis does not provide any indication why HP1 would associate specifically with this region, the level of genetic activity demonstrated for 31D-E may not be typical for all of division 31.

Based on this cytogenetic analysis, a comment can be made with regard to the relationship between mfs(2)31 and the remainder of the da-abo region cluster of genes (Sandler, 1977). The mfs(2)31 locus has been included in this cluster based on its physical proximity to the other genes and its phenotypic similarity to another member of the group, hup. The results of this study bring into question the first of these two criteria. Although, the da and mfs(2)31 genes, both members of the proposed cluster, map to the same cytogenetic interval they are genetically separable from the remainder of the cluster by a minimum of ten complementation groups. Although these could represent as yet undescribed members of a functionally related group of genes, the size of such a cluster would be unprecedented.
CHAPTER 3: GENETIC ANALYSIS OF THE mfs(2)31 LOCUS

Introduction

Position-effect variegation (PEV) occurs when a chromosomal rearrangement establishes a close association between a broken piece of heterochromatin and a euchromatic gene (for review see Chapter 1). As a consequence of this rearrangement, the gene is transcriptionally inactivated in some cells while remaining competent in others. The determination of transcriptional competence is made early during development and, for the most part, is propagated clonally. If the affected gene encodes a product which acts in a cell autonomous manner, a variegated phenotype may be observed. Although the molecular basis of the inactivating event is not known, the inactivation is not a consequence of somatic mutation since complete wild-type activity can be restored to a variegating gene by removing it from the heterochromatic breakpoint (Judd, 1955). The most durable hypothesis to date proposes that inactivation results from the aberrant packaging of the variegating gene as heterochromatin (Prokofyeva-Belgovskaya, 1941). The involvement of chromatin in the variegating phenotype is well illustrated by the sensitivity of the phenomenon to the ploidy of sex chromosome heterochromatin (Gowen and Gay, 1934) and the histone gene cluster (Khesin and Leibovitch, 1978; Moore et al., 1979).

Since its earliest description, it has been proposed that the PEV phenomenon may provide an effective genetic assay for genes
involved in the establishment and maintenance of different states of chromosomal compaction. As is the case with the histone genes, the variegating phenotype may be sensitive to the ploidy of other loci which encode structural components of chromatin. Several large collections of single-locus modifiers of PEV have been described (Reuter and Wolff, 1981; Sinclair et al., 1983; Reuter et al., 1986) of which the best characterized are the dominant suppressors of PEV, the Su(var) family. Mutant alleles of Su(var) genes, when placed in trans with a variegating rearrangement, exhibit strong, dominant suppression of PEV, restoring the phenotype to an almost wild-type appearance. It has been proposed that at least a subset of the Su(var) genes will encode non-histone chromosomal proteins (NHPs) which are responsible for the establishment and maintenance of heterochromatin (Spofford, 1976). A reduction in the availability of NHPs would reduce the probability that any given cell can produce sufficient heterochromatin to inactivate the variegating gene.

This chapter contains a phenotypic analysis of a novel type of recessive su(var) locus, mfs(2)31. mfs(2)31 is a male and female recessive sterile mutation which was isolated in a screen for alleles of the maternal-effect genes daughterless (da; Bell, 1954) and abnormal-oocyte (abo; Sandler et al., 1968; Sandler, 1977). Sandler and his colleagues (1968) had observed a phenotypic similarity between hypomorphic alleles of the da and abo genes. Mothers which are homozygous for hypomorphic da alleles produce no female progeny while mothers which are homozygous for hypomorphic
abo alleles, and which carry the normal complement of sex chromosomes, produce primarily female progeny if crossed to males carrying attached XY chromosomes (no free Y). Both maternal effects can be modified by varying the amount of sex chromosome heterochromatin in either the mutant mother or her offspring. Sandler proposed that both of these loci modify the function of genes located within heterochromatin either by regulating their expression or by interacting with their products directly (Sandler, 1968).

In order to isolate new alleles of da and abo, Sandler (1977) screened for EMS-induced lethal mutations uncovered by Df(2L)J39. Both da and abo mutations fail to complement this large deficiency for divisions 31 and 32 of chromosome arm 2L. In a screen of 455 second chromosomes, single alleles of five new loci were isolated and described: da-abo like (dal), wavoid-like (wdl), hold-up (hup), l(2)54 and mfs(2)31 (originally designated mfs48). In the hup, wdl, and dal strains the progeny of homozygote mothers exhibit an abnormal sex ratio, a phenotype which is sensitive to the maternal X heterochromatin constitution. Because of the similar phenotypes and the close-linkage of hup, wdl, dal, da and abo, Sandler (1977) proposed that they constituted a large cluster of functionally related genes within the so-called da-abo region on chromosome arm 2L.

Because of their respective homozygous lethal and homozygous sterile phenotypes, the l(2)54 and mfs(2)31 strains could not be tested for abnormal sex ratios or sensitivity to maternal X
heterochromatin constitution. However, in a subsequent study (Lindsley et al., 1980), mfs(2)31 was added to the da-abo cluster based on two criteria. First, recombination mapping places mfs(2)31 between the da and abo genes, thus physically localizing it to the cluster. Second, a more detailed analysis of the mutant strain reveals some phenotypic similarities with hup, a previously described member of the group. Both hup and mfs(2)31 are lethal as hemizygotes but semi-lethal as homozygotes. Viable homozygotes are completely sterile at 28.5° while showing varying degrees of fertility at 23°. The testes of male homozygotes have been examined in both mutant strains. At 23°, spermatogenesis appears normal, the testes are full of cells and the seminal vesicles contain motile sperm. In contrast, at 28.5° there are no motile sperm in the seminal vesicles of either mutant strain. In addition, a variety of defects are apparent in the later stages of spermiogenesis, beginning shortly after meiosis. Spermatids are observed with both macronuclei and micronuclei and there are occasional nuclei with two basal bodies attached. In wild-type strains, spermatids contain a single nucleus of uniform size and each spermatid receives only a single centriole which attaches to the nucleus and acts as a basal body. Lindsley and his colleagues (1980) propose that both mutations have defects in the systems responsible for segregation of the centrioles during meiosis. Abnormally sized nuclei would result from an incorrect alignment of spindle poles and centrioles prior to division. Since the mfs(2)31 locus has a similar phenotype to hup and is located between da and
abo, it was included in the da-abo cluster.

In an unrelated study (Sinclair et al., 1991), a group of dominant suppressors of PEV had been mapped to division 31 and were uncovered by Df(2L)J39. Since Su(var) genes are sensitive to intracellular levels of heterochromatin, a phenotype shared by the da-abo region cluster, it was of interest to know whether members of the cluster exhibit a Su(var) phenotype. The mutant strains have been tested, both as heterozygotes and as homozygotes, for an ability to suppress white variegation in In(1)w.

This inversion on the X chromosome moves the white gene (w) from its distal euchromatic location to a position adjacent to a breakpoint within centric heterochromatin. Since the result is a mottled eye, this rearrangement serves as an excellent reporter gene for the action of second-site modifiers of PEV. None of the da-abo region cluster of genes show any appreciable dominant suppression when placed in trans with the In(1)w rearrangement. However, two members of the group, mfs(2)31 and wdl, show strong suppression as homozygotes. The phenotype in the mfs(2)31 strain is attributable to the mfs(2)31 locus since rare mfs(2)31/Df(2L)J106 survivors also exhibit strong suppression.

mfs(2)31 and hup are the first reported examples of recessive modifiers of PEV. To date, there has been no attempt to screen for such mutations, primarily because of the ease with which dominant modifiers can be obtained. The standard assay for modifiers of PEV has been to screen for dominant enhancers and suppressors of the w variegating rearrangement. Large collections of modifiers have
been isolated in this manner, and several loci have now been examined molecularly (reviewed in Chapter 1).

In addition to the building blocks necessary to produce heterochromatin, a cell would require enzymatic functions to assemble, disassemble and maintain the integrity of the complex. Throughout the cell cycle, chromatin exhibits a variety of changes in degrees of compaction although the systems responsible for effecting these changes have not yet been identified. There is also mounting evidence that fundamental developmental processes such as differentiation, determination, and maintenance of transcriptional competence are intimately tied to alterations in chromatin environment (Pfeifer et al., 1987; Paro, 1990). It is unlikely that all the enzymatic functions required to give chromatin its dynamic character would be contained within the structural elements themselves. Hence, some of these transient factors in chromatin packaging may not necessarily be identifiable by dominant suppression of PEV. Nonetheless, PEV could be used as an assay for genes involved in these processes. The genes which encode proteins which modify chromatin through more transient enzymatic interactions are likely to differ from previously described Su(var) genes in two ways. First, unless such proteins are involved in rate-limiting steps in enzymatic pathways, mutations within these genes are more likely to exhibit recessive rather than dominant phenotypes with respect to PEV. Second, important modifiers of chromatin assembly could have such a profound impact on cellular activity that they are likely to be
essential loci, only exhibiting a \textit{su(var)} phenotype in viable hypomorphic alleles. Both criteria are met by the \textit{mfs}(2)31 mutation.

This chapter describes \textit{mfs}(2)31, a novel type of \textit{su(var)} locus located in cytological subdivision 31E. In addition to the original \textit{mfs}(2)31 mutation (\textit{mfs}(2)31^1), three new alleles (\textit{mfs}(2)31^2, \textit{mfs}(2)31^3 and \textit{mfs}(2)31^4) have been isolated (see Chapter 2). The four alleles will be referred to hereafter as \textit{mfs}^1, \textit{mfs}^2, \textit{mfs}^3 and \textit{mfs}^4. The \textit{mfs}^2 allele was induced in a screen for \textit{P} element insertions while \textit{mfs}^3 and \textit{mfs}^4 were induced in screens which utilized EMS as a mutagen. The isolation of new alleles of \textit{mfs}(2)31 makes possible a more complete genetic description of this pleiotropic locus.

Although none of the \textit{mfs}(2)31 alleles showed dominant phenotypes with respect to PEV, surviving combinations of \textit{mfs}(2)31 hypomorphic alleles exhibited recessive suppression of PEV in all the variegating backgrounds in which they were tested. An examination of ovaries from homozygous females revealed that the female sterility is associated with a delay in vitellogenesis. Although the gene is early acting (all alleles exhibiting larval lethality), an examination of the sterility phenotype under a variety of temperature regimes indicates that the gene product may have an adult function. A high frequency of reversion of \textit{mfs}^2 was observed when the allele was outcrossed under dysgenic conditions. This allele carries a \textit{P} element insertion in subdivision 31E which has been used for the molecular analysis of the \textit{mfs}(2)31 locus.

68
described in Chapter 4.
Materials and Methods

Stocks

Descriptions of all special chromosomes and mutations used in this study can be found below and/or in Lindsley and Zimm (1992). *mfs*¹ is an EMS-induced male and female sterile mutation which maps to division 31 because of its failure to complement *Df(2L)J39* and *Df(2L)J77*. This mutant strain was kindly provided by L. Sandler. The isolation of three new alleles of *mfs(2)31* is described in Chapter 2: *mfs*², *mfs*³ and *mfs*⁴. *mfs*² was recovered in a *P* element screen while *mfs*³ and *mfs*⁴ are EMS-induced. All three mutations were induced on an isogenic *b pr cn* chromosome. *Df(2L)J77* is a visible deficiency for region 31D-E which has been described elsewhere in this study (see Table 1).

Two collections of second chromosome *Su(var)* mutations have been tested for allelism with *mfs(2)31*. The first series consists of strongly suppressing alleles which were isolated in an EMS screen for dominant modifiers of PEV (Sinclair et al., 1983): *Su(var)204*, *Su(var)205*, *Su(var)212*, *Su(var)213* and *Su(var)214*. The second series are weak *Su(var)* mutations which have been mapped to division 31 by recombination (G. Reuter, personal communication): *Sup20*, *Sup34*, *Sup37*, *Sup39*, *Sup41*, *Sup46* and *Sup47*. The first series was selected from our lab collection while the second series was kindly provided by G. Reuter.

*In(1)w⁵¹b*, *In(1)w⁵³* and *In(1)w⁵⁴* (hereafter referred to as *w⁵¹b*, *w⁵³* and *w⁵⁴* respectively) are X chromosome inversions which variegate for the distally located *white (w)* gene. In the cases of *w⁵¹b* and
$w^{na}$, the variegation inducing regions remain near the centromere and
the affected euchromatic loci are brought near them while in $w^{n\prime}$ the
variegation inducing region is removed from the centromere and
brought near the distally affected loci. Additional information on
the breakpoints of these rearrangements can be found in Tartof et
al. (1984). The $w^{51b}$ and $w^{n\prime}$ strains rapidly accumulate modifiers
in laboratory stocks showing considerably weaker variegation than
is observed following several rounds of out-crossing. To
circumvent this problem, chromosomes were used which had been
maintained as heterozygotes with a $w$ chromosome for seventeen
generations ($w/y \times w^{bottled}/w$) and which had been selected at each
generation for strong variegation. These strains were kindly
provided by V. Lloyd.

**Culture conditions**

Fly cultures were raised on a cornmeal-agarose-sucrose medium
supplemented with p-aminobenzoic acid as a mold inhibitor.
Tetracycline and ampicillin or tetracycline and streptomycin were
added as antibiotics. All experiments were performed at 25° unless
otherwise indicated.

**Complementation**

Genetic complementation among strains was determined as
follows. Two mutant strains, each carrying the dominantly marked
second chromosome balancer $\text{In}(2LR)\text{CyO}$ ($\text{CyO}$), were crossed. Failure
to complement was indicated by the absence of $\text{Cy}^+$ progeny in a
minimum of one hundred and fifty flies scored. Viability is expressed as $Cy^+$ flies recovered / $Cy^*$ flies expected where $Cy^*$ flies expected = $Cy^+$ flies / 2. Where two mutant alleles complemented, $Cy^+$ flies of both sexes were examined for fertility and visible phenotypes.

**Fertility tests**

Fertility tests on viable $mfs(2)31$ hypomorphs were performed as follows. Parental crosses ($mfs/CyO \times mfs/CyO$) were at either $25^\circ$ or $29^\circ$. The $Cy^+$ flies were collected within 12 hours of eclosion and tested for fertility at either $22^\circ$, $25^\circ$, or $29^\circ$ by mating with $+/+$ ($Oregon-R$) control males. Typically the flies were tested in groups of 25 with a minimum of 100 flies tested per sex and strain. Cultures were cleared of adults after seven days and were then observed until all viable progeny had eclosed.

**Lethal phases**

The lethal phase was determined for each of the four $mfs(2)31$ alleles. $mfs(2)31 / +$ males were crossed to $Df(2L)J77 / +$ females at $25^\circ$. All progeny from this cross should survive except those with the genotype $mfs(2)31 / Df(2L)J77$. Following a twelve hour egg lay, eggs were collected, counted and transferred to fresh vials. A minimum of five vials were established for each strain with approximately 100 eggs per vial. Embryonic death was recorded at 72 hours; white eggs were recorded as unfertilized while darkened eggs were recorded as deaths. Larval death was determined
by failure to pupate while pupal death was determined by failure to eclose. Total deaths for a particular developmental stage were compared to the percentage of deaths recorded in the appropriate control crosses.

**Pigment Assays**

All flies used for pigment assays were raised at 22°, in 1/2 pint milk bottles with a maximum 50 flies per bottle. Flies of the correct genotype were collected within 24 hours of eclosion, kept at 22° for 48 hours, and then frozen at -70° for at least 30 minutes. A minimum of 25 flies per sex per genotype were collected. Immediately after removing the flies from the freezer, they were decapitated by banging the vials on a pounding pad. For each genotype, five heads were put into the well of a microtiter plate (Nunc microtiter plates), 5 times for a total of 25 flies per genotype. The heads were sonicated vigorously for five seconds in 30 ul of sonicating solution (1% NH<sub>4</sub>OH, 0.25 M β-mercaptoethanol) with care taken to ensure that all heads remained immersed.

Slides were prepared by attaching Whatman 3mm filter paper to one side with glue sticks providing adequate adhesion. Five microliters of solution from each well was spotted onto a prepared slide (Whatman side) using a Gilson P20 pipetman. Slides were protected from bright lights and were not read until dry. The amount of pigment in each spot was determined fluorometrically using an MPS-1 Zeiss microscope. The values obtained were averaged and expressed as a percentage of Oregon-R control flies.
Ovary dissections

Virgin mfs1/mfs1 females were collected from cultures which had been developed at 25°. The flies were held at 25° for the appropriate period of time prior to dissection. Ovaries were dissected and photographed in Drosophila ringers (6.5 g NaCl, 0.14 g KCl, 0.12 g CaCl2, 0.20 g NaHCO3, 0.01 g NaH2PO4 in 1000 ml dH2O; pH 7.2). Photographs were interpreted according to the drawings of King (1970).

Thick sections of ovaries

Freshly dissected ovaries from seven day old virgin females (25°) were fixed in glutaraldehyde (2.5% in 0.1 M cacodylate buffer) for eight hours and then washed in cacodylate buffer overnight. Postfix was in 1% osmium tetroxide for 1 hour followed by a rinse in distilled water. Next, the tissue was serially dehydrated on the following schedule: 10 min in 50% ethanol: 10 min in 70% ethanol: 10 min in 85% ethanol: 10 min in 95% ethanol: 2 X 10 min in 100% ethanol. The ovaries were transferred to 50% propylene oxide in absolute ethanol for 30 minutes followed by 2 X 30 minutes in 100% propylene oxide. Embedding was done serially on the following schedule: 30 min in 50% spurr resin / 50% propylene oxide: 30 min in 75% spurr resin / 25% propylene oxide: 30 min in 100% spurr resin. Polymerization was in an oven at 60° for 16 hours. Ovaries were sectioned and stained with toluidine blue (1% toluidine blue : 1% aqueous borax).
Reversion screen

The mfs\textsuperscript{2} allele was recovered in a P element screen (see Chapter 2). To determine whether or not the mutation was the result of an insertional event, the allele was outcrossed under dysgenic conditions and then selected for viability in trans with a deficiency which uncovers the locus. The protocol for this screen for revertants can be found in Figure 6. The mfs\textsuperscript{2} b pr cn males were crossed en masse to females carrying the Δ2-3 Sb chromosome, a potent source of transposase activity. Dysgenic males carrying both the mfs\textsuperscript{2} b pr cn chromosome and the Δ2-3 Sb chromosome were collected and crossed to Df(2L)J77 females. A germ-line reversion of the mutation is indicated by the presence of exceptional Cy\textsuperscript{+} progeny. Such progeny have the genotype mfs\textsuperscript{2} b pr cn/Df(2L)J77, a normally lethal combination of second chromosomes.

In situ hybridization to polytene chromosomes

Black cells (Bc) is a dominant larval marker on the second chromosome. Males carrying a Bc chromosome and an mfs\textsuperscript{2} b pr cn chromosome were crossed to wild-type virgin females at 17°. Late third instar larvae carrying the mfs\textsuperscript{2} b pr cn chromosome (Bc\textsuperscript{+}) were selected for in situ hybridizations. Salivary glands were dissected in Drosophila ringer, fixed for 45 seconds in 45% acetic acid and then squashed in 10 ul of a solution of 1 part lactic acid: 2 parts distilled water: 3 parts glacial acetic acid. The chromosomes were allowed to flatten overnight at 4° and were then flash frozen in liquid nitrogen. The coverslips were removed using
Figure 6: Screen for revertants of $mfs(2)31^2$.

$$
\begin{align*}
\text{mfs}^2 \, b \, pr \, cn \, CyO ; & \quad + \quad \times \quad Sp \, CyO ; \quad \triangledown \quad 2-3 \, Sb \, Ubx \\
\downarrow & \\
\text{mfs}^2 \, b \, pr \, cn \, CyO ; & \quad \triangledown \quad 2-3 \, Sb \, CyO ; \quad + \quad \times \quad Df(2L)J77 \, CyO ; \quad + \\
\downarrow & \\
\text{mfs}^2 \, b \, pr \, cn \, Df(2L)J77 ; & \quad + \, + \\
\end{align*}
$$

REVERTANT
a sharp razorblade and the slides were immediately immersed in pre-chilled (-20°) 95% EtOH which was then allowed to come to room temperature. The slides were removed from the ethanol, air-dried and stored at 4° until needed.

Immediately prior to incubation with a biotinylated probe, the slides were immersed for 10 minutes in 250 ml of 0.1 M triethanolamine-HCl solution (pH 8.0) to which 310 ul of acetic anhydride had been added. The slides were then washed (2 X 5 minutes) in 2 X SSC and dehydrated sequentially in 70% EtOH (2 X 5 minutes) and 95% EtOH (2 X 5 minutes). The chromosomes were air-dried and then denatured in freshly prepared 0.07 M NaOH. They were again washed and sequentially dehydrated as described above. The slides were air-dried and the probe was added immediately.

Nick-translated probes (1 ug of DNA) were prepared using biotin-11 dUTP (BRL) and the BRL Bionick labelling system according to the manufacturers instructions. Probes were purified by a single ethanol precipitation and re-suspended in 117 ul water. They were denatured by boiling for 5 minutes and were then plunged into ice water. The following was added to the denatured probe: 40 ul 20 X SSC, 40 ul 10% dextran sulfate and 4 ul 50 X Denhardt’s (50 X Denhardt’s reagent contains 5 g of Ficoll, 5 g of polyvinylpyrroloidone (PVP), 5 g of bovine serum albumin (BSA), and H2O to 500 ml). Approximately 20 ul of probe was added to each slide which was then covered with a small coverslip and sealed with rubber cement. Chromosomes were incubated for 12 hours in a moisture chamber at 58°. The coverslips were then removed and the
slides were washed sequentially as follows: 3 X 20 min in 2 X SSC at 53°, 2 X 10 min in 2 X SSC at room temperature.

Hybridization was detected using the BRL Blugene kit and phase contrast microscopy. Slides were incubated for 10 min in Buffer 1 (0.1 M Tris (pH 7.5), 0.1 M NaCl, 2 mM MgCl₂, 0.05% Triton X-100 (Sigma)) at room temperature and 20 min in Buffer 2 (Buffer 1 plus 2% BSA) at 42°. The solution was moved to room temperature and allowed to sit for an additional 10 minutes. One hundred ul of a solution of 2 ul strepavidin-alkaline phosphatase conjugate in 1 ml Buffer 1 was added to each slide which were then incubated at room temperature for 2 hours. Slides were washed for 2 X 3 min in Buffer 1 and 1 X 3 min in Buffer 3 (0.1 M Tris (pH 9.5), 0.1 M NaCl, 50 mM MgCl₂). One hundred ul of a solution of 4.4 ul NBT and 3.3 ul BCIP in 1 ml Buffer 3 was added to each slide. Slides were covered with a large coverslip and incubated overnight in a darkened moisture chamber at room temperature. The coverslips were removed, the slides were washed briefly in distilled water and the chromosomes were examined under water with phase contrast optics.
Results

Complementation of mfs(2)31 with Su(var) loci

A number of dominant suppressors of PEV have been localized to division 31, either by recombination or by deficiency mapping. Although mfs\textsuperscript{i} exhibits no dominant suppression, it may be a weak allele of one of these previously described modifiers. Complementation tests were conducted between mfs\textsuperscript{i} and representative alleles of closely linked Su(var) loci, the results of which are summarized in Table 5. Transheterozygotes were examined for fertility and visible phenotypes.

Su(var)212 is a strong dominant suppressor which maps to the right arm of chromosome 2 (Sinclair et al., 1983). Although its map position would indicate that it is not allelic to mfs(2)31, it was included in the complementation tests as a control for general interactive phenotypes between mfs(2)31 and Su(var) loci. Su(var)212 and mfs\textsuperscript{i} exhibited no phenotypes when placed in \textit{trans}.

Suvar(2)1 alleles show sex specific lethality and sterility in \textit{trans} with Df(2L)J2 but fully complement Df(2L)J106, Df(2L)J27 and Df(2L)J77 (Sinclair et al., 1991). This pattern of complementation is consistent with the recent isolation of Suvar(2)1 alleles which are associated with P element insertions in 31B-C (G. Reuter, personal communication). Heteroallelic combinations of Suvar(2)1 exhibit a variety of phenotypes including eye discoloration and defective wings. To test for allelism between mfs(2)31 and Suvar(2)1, mfs\textsuperscript{i} was crossed to two of the stronger Suvar(2)1 alleles: Su(var)213 and Su(var)214. Transheterozygotes were
<table>
<thead>
<tr>
<th>Cross</th>
<th>Viability</th>
<th>Fertility</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>(mfs^1/CyO \times b\l t\ r1/CyO)</td>
<td>0.98 (381)</td>
<td>Fertile</td>
<td>Fertile</td>
<td></td>
</tr>
<tr>
<td>(mfs^1/CyO \times Su(var)212/CyO)</td>
<td>1.17 (766)</td>
<td>Fertile</td>
<td>Fertile</td>
<td></td>
</tr>
<tr>
<td>(mfs^1/CyO \times Su(var)213/CyO)</td>
<td>0.80 (642)</td>
<td>Fertile</td>
<td>Fertile</td>
<td></td>
</tr>
<tr>
<td>(mfs^1/CyO \times Su(var)214/CyO)</td>
<td>0.92 (1132)</td>
<td>Fertile</td>
<td>Fertile</td>
<td></td>
</tr>
<tr>
<td>(mfs^1/CyO \times Su(var)204/CyO)</td>
<td>0.76 (999)</td>
<td>Fertile</td>
<td>Fertile</td>
<td></td>
</tr>
<tr>
<td>(mfs^1/CyO \times Su(var)205/CyO)</td>
<td>0.97 (880)</td>
<td>Fertile</td>
<td>Fertile</td>
<td></td>
</tr>
<tr>
<td>(mfs^1/CyO \times Sup20/CyO)</td>
<td>0.81 (246)</td>
<td>ND</td>
<td>Fertile</td>
<td></td>
</tr>
<tr>
<td>(mfs^1/CyO \times Sup34/CyO)</td>
<td>0.87 (205)</td>
<td>ND</td>
<td>Fertile</td>
<td></td>
</tr>
<tr>
<td>(mfs^1/CyO \times Sup37/CyO)</td>
<td>0.76 (185)</td>
<td>ND</td>
<td>Fertile</td>
<td></td>
</tr>
<tr>
<td>(mfs^1/CyO \times Sup39/CyO)</td>
<td>0.86 (297)</td>
<td>ND</td>
<td>Fertile</td>
<td></td>
</tr>
<tr>
<td>(mfs^1/CyO \times Sup41/CyO)</td>
<td>0.86 (408)</td>
<td>ND</td>
<td>Fertile</td>
<td></td>
</tr>
<tr>
<td>(mfs^1/CyO \times Sup46/CyO)</td>
<td>0.85 (292)</td>
<td>ND</td>
<td>Fertile</td>
<td></td>
</tr>
<tr>
<td>(mfs^1/CyO \times Sup47/CyO)</td>
<td>1.08 (277)</td>
<td>ND</td>
<td>Fertile</td>
<td></td>
</tr>
</tbody>
</table>

---

\(^a\) Viability = \((Cy^+ \text{ progeny recovered}) / (Cy^+ \text{ progeny expected})\) from the cross \(mfs^1/CyO \times Su(var)/CyO\). Total progeny scored are provided in parentheses.

\(^b\) ND = Not done
counted, examined for visible phenotypes and tested for fertility. Based on all these criteria, \textit{mfs(2)31} fully complemented \textit{Suvar(2)1}.

\textit{Su(var)204} has been mapped by recombination to division 31 (Sinclair et al., 1991). Since this mutation is homozygous viable and fertile and shows no phenotype over deficiencies for the region, its precise cytological location is not known. As is the case with \textit{Suvar(2)1}, transheterozygotes of \textit{mfs(2)31} and \textit{Su(var)204} were completely viable and fertile, showing no visible phenotypes.

\textit{Su(var)205} has been cloned and localized to cytological subdivision 29A. This gene encodes the non-histone chromosomal protein HP1, a protein which specifically associates with division 31 on salivary gland polytene chromosomes (James et al., 1989). Although \textit{Su(var)205} is not allelic to \textit{mfs(2)31}, the possibility exists that it regulates the expression of genes within division 31 and thus may exhibit a phenotype in \textit{trans} with \textit{mfs(2)31} alleles. A complementation test between these two mutations did not provide any evidence of such an interaction.

\textit{Sup20, Sup34, Sup37, Sup39, Sup41, Sup46,} and \textit{Sup47} are weak dominant suppressors of PEV which map by recombination to division 31 (G. Reuter, personal communication). These are homozygous viable strains which have not been tested for allelism nor have they been tested against division 31 deficiencies. Strains which were heterozygous for these mutations and the \textit{mfs\textsuperscript{i}} chromosome were viable and exhibited no visible phenotypes. The female transheterozygotes were all fertile but the males were not tested.

To summarize, \textit{mfs(2)31} was tested for allelism with all
available dominant suppressors of PEV which have been localized to division 31. Using viability, male and/or female fertility, and visible phenotypes as a criteria, \textit{mfs(2)31} does not appear to be allelic to any previously described \textit{Su(var)} loci.

\textit{mfs(2)31 inter se complementation}

Hypomorphic alleles can be useful for determining a gene's function, particularly if they exhibit phenotypes at well defined stages in development or in easily recognizable tissues. Similarly, alleles which represent complete loss-of-function at a locus can sometimes be used to determine the earliest stage at which a gene product acts. In order to determine the relative strengths of the four \textit{mfs(2)31} alleles and identify viable allelic combinations, a complementation matrix was elaborated for the \textit{mfs(2)31} locus (Table 6). In no case did the parental source of the mutation influence viability. Similarly, no differences in viability were observed if the crosses were done both at 25° and at 29°.

All \textit{mfs(2)31} alleles were recovered on the basis of their failure to complement a deficiency for division 31 and all showed complete lethality when tested on a larger scale over \textit{Df(2L)J77} (see Table 6). Although rare survivors of \textit{mfs} in \textit{trans} with \textit{Df(2L)J106} have been previously described (Sinclair et al., 1991; D. Sinclair, personal communication), no survivors over \textit{Df(2L)J77} were recovered in 10,000 flies scored.

The only \textit{mfs(2)31} allele which survived as a homozygote was
## TABLE 6
Complementation matrix for *mfs*(2)31a

<table>
<thead>
<tr>
<th></th>
<th>mfs1</th>
<th>mfs2</th>
<th>mfs3</th>
<th>mfs4</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>DfJ77</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29°</td>
<td>0.00 (4721)</td>
<td>0.00 (2763)</td>
<td>0.00 (3714)</td>
<td>0.00 (1527)</td>
</tr>
<tr>
<td>25°</td>
<td>0.00 (5716)</td>
<td>0.00 (1825)</td>
<td>0.00 (1165)</td>
<td>0.00 (1450)</td>
</tr>
<tr>
<td><em>mfs1</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29°</td>
<td>0.50 (2658)</td>
<td>0.73 (4896)</td>
<td>0.00 (1651)</td>
<td>0.00 (2110)</td>
</tr>
<tr>
<td>25°</td>
<td>0.48 (1684)</td>
<td>0.68 (3331)</td>
<td>0.00 (1944)</td>
<td>0.00 (1794)</td>
</tr>
<tr>
<td><em>mfs2</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29°</td>
<td>0.00 (1700)</td>
<td>0.00 (2768)</td>
<td>0.00 (1231)</td>
<td></td>
</tr>
<tr>
<td>25°</td>
<td>0.00 (1296)</td>
<td>0.00 (2368)</td>
<td>0.00 (1138)</td>
<td></td>
</tr>
<tr>
<td><em>mfs3</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29°</td>
<td>0.00 (878)</td>
<td>0.00 (878)</td>
<td>0.00 (1106)</td>
<td></td>
</tr>
<tr>
<td>25°</td>
<td>0.00 (910)</td>
<td>0.00 (910)</td>
<td>0.00 (1230)</td>
<td></td>
</tr>
<tr>
<td><em>mfs4</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29°</td>
<td>0.00 (182)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25°</td>
<td>0.00 (423)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Data is recorded as (Cy' progeny recovered)/(Cy' progeny expected) from the cross *mfs*/CyO X *mfs*/CyO. Total number of flies scored is provided in parentheses.
the original \( mfs^1 \) mutation. Homozygotes of both sexes exhibited
the small, slender bristles and sterility previously described by
Sandler (1977). These flies were recovered at a frequency of 50% of expected, which is substantially higher than the previously reported value of 25% (Lindsley et al., 1980). This discrepancy may be attributable to culture conditions, however it is possible that a portion of the homozygous progeny were missed in previous determinations of viability, since \( mfs^1 \) homozygotes have a very prolonged developmental period, eclosing several days later than their heterozygote siblings. The other alleles of \( mfs(2)31 \) were completely lethal both as homozygotes and as hemizygotes. The \( mfs^4 \) strain has a mild reduction in fertility in both sexes although it can be maintained as a balanced stock. The reduced fertility is reflected in the relatively low number of heterozygotes recovered in the test for homozygous viability.

The only other viable combination of \( mfs(2)31 \) alleles was
\( mfs^1/mfs^2 \). Like the \( mfs^1 \) homozygotes, these flies have shortened thoracic bristles in both sexes and are male sterile. They differ from \( mfs^1/mfs^1 \) flies in that the females are fertile and the frequency of recovery is somewhat higher: 0.68 vs 0.48 at 25°.

A reasonable interpretation of this complementation matrix is that \( mfs^1 \) and \( mfs^2 \) are hypomorphic alleles while \( mfs^3 \) and \( mfs^4 \) approximate the complete loss-of-function phenotype at the \( mfs(2)31 \) locus. Since all the alleles are lethal as hemizygotes, and three are lethal as homozygotes, the gene would appear to have an essential function. Although \( mfs^1 \) is the only allele to survive as
a homozygote, it is difficult to judge the relative strengths of \(mfs^1\) and \(mfs^2\). Compared to the \(mfs^1\) homozygotes, the \(mfs^1/mfs^2\) allelic combination survives better and has less severe fertility problems. It is plausible that \(mfs^2\) is the weaker allele and only fails to survive as a homozygote because of secondary lesions.

**Fertility of \(mfs(2)31\) alleles**

The failure of \(mfs(2)31\) hypomorphs to elaborate wild-type bristles coupled with their severe fertility problems suggests that the gene product may function late in development or in adulthood. It has been reported that the sterility associated with \(mfs^1\) homozygotes is temperature-sensitive (Lindsley et al., 1980) although the nature of the sensitivity has not been well defined. If there is an adult temperature-sensitive period for sterility, it would indicate a germ-line associated adult function for the gene product.

Temperature shift experiments were conducted on \(mfs(2)31\) hypomorphs and fertility was examined under a variety of temperature regimes. Flies were reared at either 25° or 29°, collected within 24 hours of eclosion and tested for fertility at either 22°, 25° or 29°. This protocol made it possible to distinguish between sensitivity due to developmental temperature and sensitivity due to adult temperature. The results of this study are summarized in Table 7.

Regardless of the temperature regime, all males that were tested were sterile, thus providing no evidence for, or against, an
<table>
<thead>
<tr>
<th>Genotype(^a)</th>
<th>Temp. Reared</th>
<th>Temp. Tested</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>(mfs^1/mfs^1)</td>
<td>29(^\circ)</td>
<td>29(^\circ)</td>
<td>Sterile; Lay no eggs</td>
<td>Sterile; Lay no eggs</td>
</tr>
<tr>
<td></td>
<td>25(^\circ)</td>
<td>Sterile</td>
<td>Sterile; Lay no eggs</td>
<td></td>
</tr>
<tr>
<td></td>
<td>22(^\circ)</td>
<td>Sterile</td>
<td>Sterile; 1 or 2 eggs</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25(^\circ)</td>
<td>29(^\circ)</td>
<td>Sterile; 1 or 2 eggs</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25(^\circ)</td>
<td>Sterile</td>
<td>Sterile; 1 or 2 eggs</td>
<td></td>
</tr>
<tr>
<td></td>
<td>22(^\circ)</td>
<td>Sterile</td>
<td>sterile; small, egg lay</td>
<td></td>
</tr>
<tr>
<td>(mfs^1/mfs^2)</td>
<td>29(^\circ)</td>
<td>29(^\circ)</td>
<td>Sterile; large, egg lay</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25(^\circ)</td>
<td>Sterile</td>
<td>Semi-sterile; 1 or 2 eggs hatch and develop into adults</td>
<td></td>
</tr>
<tr>
<td></td>
<td>22(^\circ)</td>
<td>Sterile</td>
<td>Semi-sterile; 1 or 2 eggs hatch and develop into adults</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25(^\circ)</td>
<td>29(^\circ)</td>
<td>Sterile; Large, egg lay; Eggs hatch but die as 1st instar larvae; A few pupate but no eclosion</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25(^\circ)</td>
<td>Sterile</td>
<td>Fertile</td>
<td></td>
</tr>
<tr>
<td></td>
<td>22(^\circ)</td>
<td>Sterile</td>
<td>Fertile</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) The \(mfs^1\) allele was induced on an \textit{Oregon-R} chromosome while \(mfs^2\) is on a \(b pr cn\) chromosome.
adult function for the gene product. The results of the female fertility tests were somewhat more informative. In the case of mfs\(^1\) homozygotes, females were sterile under all conditions tested although they differed in their ability to deposit eggs. The ability to lay eggs depended both on the temperature at which the flies were reared and the temperature at which they were tested. Thus, if flies were reared at 29° and tested at 22° they only produced one or two eggs per vial (from 25 females), whereas if they were reared at 25° and tested at 22° they produced 50-100 eggs per vial. A similar effect was observed if the adult temperature was varied. Thus, flies reared at 25° and tested at 29° produced only one or two eggs whereas flies reared at 25° and tested at 22° produced 50-100 eggs per vial. The control strain (Oregon-R) was completely fertile under all temperature regimes. It would appear that the ability of the sterile females to deposit eggs is sensitive both to developmental temperature and the temperature of adult cultures. Interestingly, egg deposition in mfs\(^1\) homozygotes and mfs\(^1\)/mfs\(^2\) heteroallelic females resembles the lethality phenotype.

The pattern of temperature-sensitivity was also observed in the fertility tests of mfs\(^1\)/mfs\(^2\) heteroallelic females. The mfs\(^1\)/mfs\(^2\) females were highly fecund, that is they produced substantial amounts of eggs regardless of the temperature regime examined. However, the ability of the eggs to hatch and develop into adults depended both on developmental temperature and the temperature at which the adults were maintained. Thus, flies
reared at 29° and tested at 22° produced only one or two eggs which were able to hatch and develop into adults while flies reared at 25° and tested at 22° were completely fertile. The fertility phenotype also varied as a consequence of temperature at which adults were maintained. Flies which were reared at 25° and tested at 29° were completely infertile while siblings which were tested at 22° were completely fertile. The control strain (b pr cn/+) was fertile under all temperature regimes.

To summarize, the degree of sterility of mfs(2)31 hypomorphs (females only) is sensitive both to the temperature at which the flies are developed and the temperature at which they are maintained as adults. This latter observation indicates that the germ-line associated function of the mfs(2)31 gene product continues into adulthood. The more severe sterility observed in the mfs1 homozygotes is correlated with a lack of oviposition in females.

Studies on mfs(2)31 oogenesis

The sterility phenotype of the mfs1 homozygotes is associated with a failure to deposit eggs. Whether the lack of fecundity reflects a failure in one of the systems controlling oviposition or an inability of the flies to produce mature eggs is not clear. However, the lengthy temperature-sensitive period favors the latter hypothesis. Indeed, all elements of the reproductive system, with the exception of the ovaries, are morphologically normal in mfs1/mfs1 females raised at 29°. Thus, a defect in one of these
structures is an unlikely source of the sterility phenotype. In contrast, oogenesis is an ongoing process in the adult involving a balance and interaction between a number of regulatory factors (for review see Mahowald and Kambysellis, 1980). Since the fidelity of these adult processes is of utmost importance in controlling the timing and rate of egg maturation and deposition, defects in a number of these processes may exhibit a sensitivity to temperature.

As a preliminary step in the investigation of the reproductive defect in mfs\(^1\) homozygote females, the temporal pattern of ovarian development was examined in adults. Ovaries were dissected from mfs\(^1\)/mfs\(^1\) virgins at 3 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 10 days and 21 days post-eclosion and compared to ovaries from similarly staged Oregon-R females. Representative ovaries from the various stages are shown in Figure 7.

At 3 hours post-eclosion (plate A), ovaries from mfs\((2)31\) and Oregon-R females are virtually indistinguishable. The vitellariums appear normal in number and morphology as do the lateral and common oviducts (see arrow). As is expected for this stage in development, there is no evidence of vitellogenesis in either the mutant or wild-type ovaries.

Over the next 24 hours (plates b and c), the morphology of mfs\(^1\)/mfs\(^1\) and Oregon-R ovaries becomes strikingly different. In the wild-type ovaries, vitellogenesis commences and many stage 9-11 oocytes are identifiable. In contrast, there is no indication that vitellogenesis is occurring at this time in mfs\(^1\) homozygotes. The ovaries of mfs\(^1\) homozygotes become moderately enlarged, but this
Figure 7: Ovarian development in mfs¹/mfs¹ adults raised at 25°. Ovary(s) on left of plates are from mfs¹/mfs¹ while ovary(s) on right are from Oregon-R. Plates E and F show only a single wild-type ovary for comparison while plates G and H contain only single ovaries from both strains. Plate A: 3 hrs post-eclosion (arrow indicates intact common and lateral oviducts), Plate B: 12 hrs post-eclosion, Plate C: 24 hrs post-eclosion, Plate D: 48 hrs post-eclosion, Plate E: 72 hrs post-eclosion, Plate F: 96 hrs post-eclosion (arrow indicates stage 10-12 ovarian cyst), Plate G: 10 days post-eclosion, Plate H: 3 weeks post-eclosion. Plate I: Thick section through 96 hr mfs¹/mfs¹ ovary showing a stage 9 ovarian cyst.
appears to be due to the accumulation of oocytes at the pre-vitellogenic stages.

By 72 hours of development (plates D and E) the difference in morphology between wild-type and mutant ovaries has become even more pronounced. The ovaries of wild-type flies are completely mature; they contain many fully-developed eggs. In contrast, the mfs$^1$ ovaries appear arrested in development; the eggs appear to be essentially pre-vitellogenic. However, some yolk deposition maybe occurring in the mutant ovaries by 72 hours; there are a few opaque stage 8 oocytes (plate E).

At 96 hours (plate F), vitellogenesis in mfs$^1$ ovaries is more apparent. At this time, it is not uncommon to see individual ovarioles within which vitellogenesis has proceeded as far as stages 10-12 (see arrow in plate F) in the mutants. However, for the most part, vitellogenesis is proceeding very slowly and is still not evident in the majority of the ovarioles.

By day ten (plate G), the process of vitellogenesis has advanced in the mutant ovaries and eggs in the latter stages of development are present in virtually all the ovarioles. Although the wild-type virgins have commenced egg deposition, the mfs$^1$/mfs$^1$ females do not lay eggs at this time.

By three weeks (plate H), the mfs$^1$ homozygotes have still only deposited 1 or 2 eggs and the morphology of the ovaries has changed once again with respect to wild-type ovaries. Although fully formed eggs are clearly visible, there is no evidence of pre-stage 12 oocytes. This is presumably due to the reabsorption of these
oocytes, a phenomenon which has been described in Drosophila females which fail to undergo oviposition (Wilson, 1985; Giorgi and Deri, 1976).

The yolk proteins in developing Drosophila oocytes originate from two sources, the follicle cells and the cells of the fat bodies (Mahowald and Kambyseallis, 1980; Brennan et al., 1982). Vitellogins from the fat bodies are released into the hemolymph where they are transported to the oocytes and taken up by pinocytosis. Thus, failure to undergo vitellogenesis may reflect a defect in the fat bodies or follicle cells. Ovaries from 96 hour mfs₁ homozygotes were sectioned and examined. A representative section through a stage 9 oocyte (the first stage in which yolk deposition is detectable) is shown in plate I (Figure 7).

In all ovarian sections examined, no defects were detected at any stage in oogenesis. In particular, the follicle cell, nurse cell and oocyte morphology appear to be normal at the onset of vitellogenesis (stage 9). By stage 9 of oogenesis, the majority of the follicle cells have moved over the rapidly growing oocyte and assumed a columnar morphology, while the remainder have become thin and squamous covering the 15 nurse cells. As can be seen in Figure 7, the follicle cells of mfs₁ homozygotes have proliferated, migrated properly and assumed a wild-type morphology. The nuclei are visible and positioned normally in the columnar follicle cells. The border cells, a group of anterior follicle cells which migrates as a cluster to the anterior surface of the oocyte, are morphologically normal and correctly positioned in the mfs₁
homozygotes.

The correct number of nurse cells are present in the mfs
ovarian cysts and their orientation and size with respect to the
oocyte appears normal. As is the case with stage 9 wild-type nurse
cells, polyploid chromosomes are easily distinguished, dispersed
throughout the nurse cell nuclei.

Within the oocyte cytoplasm, protein yolk spheres are observed
at a density equivalent to similarly staged wild-type oocytes. As
expected, the oocyte nucleus has migrated to a position adjacent to
the nurse cells at the future antero-dorsal position in the egg.

To summarize, the sterility in mfs homozygotes is associated
with a delay in the onset of vitellogenesis. Yolk deposition is
first detected 72 hours following eclosion and the ovaries do not
achieve full maturity for an additional six days. Although fully
mature eggs are detected in the ovaries after 10 days, and the
morphology of the common and lateral oviducts appears normal,
oinposition is rare. It is not clear whether the delay in
vitellogenesis and the failure to oviposit are the consequence of
the same or independent events. Thick sections through ovaries of
mfs homozygotes revealed no obvious morphological defects at any
stage in ovarian development.

Lethal phases for mfs(2)31

In addition to an adult germ-line associated function, the
mfs(2)31 product acts early in development as evidenced by the
failure of strong alleles to survive to adulthood. Strong alleles
of essential loci can be useful for determining the earliest stage at which the essential function is occurring. Weaker alleles can also be useful for defining the function of a gene product, particularly if they die at well-defined stages in development i.e. the time of elaboration of a particular tissue. As part on an ongoing analysis of the various functions of the mfs(2)31 locus, the lethal phase was determined for all four mutant alleles. The alleles were tested in trans with Df(2L)J77 and the crosses were designed such that only the mfs(2)31/Df(2L)J77 progeny should die.

The results of the lethal phase analysis are summarized in Table 8. The Oregon-R chromosome is the control for mfs1 while b pr cn is the control for the remainder of the alleles. These are the chromosomes upon which these mutations were induced. The crosses were done in one direction only using Df(2L)J77 females, and thus the results should not reflect any differences in allele specific maternal effects. All four alleles exhibit one principle phase of lethality during larval development. Although mfs1 and mfs2 appear to be weaker alleles based on their pattern of complementation, this is not reflected in the lethal phase analysis.

mfs(2)31 alleles suppress position -effect variegation

It has been previously reported that mfs1 homozygotes suppress the white (w) variegation associated with the w' rearrangement, a phenotype which has also been observed in rare mfs1/Df(2L)J106 survivors of both sexes (Sinclair et al., 1991). Although this
<table>
<thead>
<tr>
<th>Strain</th>
<th>Total Scored</th>
<th>% Lethality</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Embryonic</td>
<td>Larval</td>
<td>Pupal</td>
</tr>
<tr>
<td>Oregon-R</td>
<td>505</td>
<td>0.0</td>
<td>4.0</td>
<td>2.7</td>
</tr>
<tr>
<td>b pr cn</td>
<td>594</td>
<td>2.7</td>
<td>8.0</td>
<td>7.8</td>
</tr>
<tr>
<td>mfs$^1$</td>
<td>585</td>
<td>2.7</td>
<td>35.5</td>
<td>4.6</td>
</tr>
<tr>
<td>mfs$^2$ b pr cn</td>
<td>457</td>
<td>3.9</td>
<td>39.6</td>
<td>4.8</td>
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<tr>
<td>mfs$^3$ b pr cn</td>
<td>691</td>
<td>2.3</td>
<td>25.3</td>
<td>8.5</td>
</tr>
<tr>
<td>mfs$^4$ b pr cn</td>
<td>849</td>
<td>4.5</td>
<td>25.0</td>
<td>7.0</td>
</tr>
</tbody>
</table>

$^a$ All chromosomes were tested over Df(2L)J77.
mutation exhibits no dominant phenotype with respect to PEV, interallelic complementation indicates that it is a weak allele. This raises the possibility that stronger alleles such as \( mfs^3 \) and \( mfs^4 \) may exhibit dominant phenotypes. In addition, since only one \( mfs(2)31 \) allele has been tested for suppression, it is formally possible that the \( su(var) \) phenotype is a consequence of a tightly linked second site mutation which is also uncovered by \( Df(2L)J106 \). Alternatively, the phenotype could be a peculiarity associated with the one \( mfs(2)31 \) allele, if for example it is acting as an antimorph or neomorph with respect to suppression. Gain-of-function alleles have been previously described for other \( Su(var) \) loci (Sinclair et al., 1983; 1991). In order to address all of these issues, the four \( mfs(2)31 \) alleles were tested for dominant and recessive (where testable) suppression of PEV in a variety of variegating backgrounds. The results of this study are summarized in Table 9.

The four \( mfs(2)31 \) alleles were tested for their ability to suppress the \( white \) variegation associated with \( w^{ma}, w^{51b} \) and \( w^{ma} \). These are X-chromosome inversions with proximal breakpoints in the centric heterochromatin. Since the distal breakpoints in \( w^{51b} \) and \( w^{ma} \) are distal to the \( white \) gene, these rearrangements move the variegating locus to the variegation inducing region. In contrast, the distal breakpoint of \( w^{ma} \) is proximal to the \( white \) gene thus moving the variegation inducing region to the affected locus.

The \( w^{ma} \) and \( w^{51b} \) strains rapidly accumulate modifiers in balanced stocks becoming such weak variegators that they are of
## TABLE 9

**Pigment assays**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sex</th>
<th>$W^m^4$</th>
<th>$W^m5^1^b$</th>
<th>$W^m^3^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ / +</td>
<td>♂</td>
<td>5.7 ± 2</td>
<td>31.5 ± 4</td>
<td>14.7 ± 4</td>
</tr>
<tr>
<td></td>
<td>♀</td>
<td>25.3 ± 1</td>
<td>18.9 ± 2</td>
<td>64.7 ± 6</td>
</tr>
<tr>
<td>b pr cn / +</td>
<td>♂</td>
<td>6.3 ± 2</td>
<td>34.2 ± 4</td>
<td>18.5 ± 3</td>
</tr>
<tr>
<td></td>
<td>♀</td>
<td>21.2 ± 2</td>
<td>14.2 ± 1</td>
<td>67.0 ± 5</td>
</tr>
<tr>
<td>mfs$^1$ / +</td>
<td>♂</td>
<td>7.1 ± 1</td>
<td>39.3 ± 3</td>
<td>22.4 ± 2</td>
</tr>
<tr>
<td></td>
<td>♀</td>
<td>25.4 ± 3</td>
<td>22.8 ± 2</td>
<td>66.7 ± 5</td>
</tr>
<tr>
<td>mfs$^2$ b pr cn / +</td>
<td>♂</td>
<td>6.1 ± 1</td>
<td>34.7 ± 4</td>
<td>21.5 ± 2</td>
</tr>
<tr>
<td></td>
<td>♀</td>
<td>30.5 ± 7</td>
<td>11.3 ± 1</td>
<td>69.1 ± 4</td>
</tr>
<tr>
<td>mfs$^3$ b pr cn / +</td>
<td>♂</td>
<td>8.3 ± 1</td>
<td>37.7 ± 2</td>
<td>15.9 ± 1</td>
</tr>
<tr>
<td></td>
<td>♀</td>
<td>13.1 ± 5</td>
<td>22.9 ± 4</td>
<td>65.4 ± 6</td>
</tr>
<tr>
<td>mfs$^4$ b pr cn / +</td>
<td>♂</td>
<td>11.1 ± 2</td>
<td>31.2 ± 5</td>
<td>17.3 ± 1</td>
</tr>
<tr>
<td></td>
<td>♀</td>
<td>29.7 ± 5</td>
<td>20.9 ± 2</td>
<td>74.4 ± 6</td>
</tr>
<tr>
<td>mfs$^1$ / mfs$^1$</td>
<td>♂</td>
<td>66.5 ± 9</td>
<td>nr$^b$</td>
<td>97.1 ± 2</td>
</tr>
<tr>
<td></td>
<td>♀</td>
<td>89.7 ± 5</td>
<td>45.9 ± 9</td>
<td>90.8 ± 6</td>
</tr>
<tr>
<td>mfs$^2$ / mfs$^2$ b pr cn</td>
<td>♂</td>
<td>50.0 ± 3</td>
<td>64.8 ± 8</td>
<td>44.5 ± 7</td>
</tr>
<tr>
<td></td>
<td>♀</td>
<td>28.7 ± 4</td>
<td>47.6 ± 3</td>
<td>68.2 ± 4</td>
</tr>
</tbody>
</table>

$^a$ Pigment levels are expressed as a percentage of Oregon-R. The measurements of $w^{m5^1b}$ and $w^{m3^b}$ females are for $w^{m5^1b}/w$ and $w^{m3^b}/w$ flies respectively. All measurements were done at 22°.

$^b$ No flies recovered.
little use for assaying suppression. Because of this, chromosomes were used which had been outcrossed for seventeen generations to \( w \) males, and which had been selected at every generation for strong variegation. To further reduce the pigment levels and thus make a more sensitive assay, pigment measurements were made on \( w^{51b}/w \) and \( w^{51}/w \) females rather than \( w^3/w^3 \) and \( w^{51b}/w^{51b} \).

Control pigment levels for the \( mfs^1/+ \) and \( mfs^1/mfs^1 \) strains are from a wild-type strain while levels for \( mfs^2/+ \), \( mfs^3/+ \), \( mfs^4/+ \) and \( mfs^1/mfs^2 \) are from a \( b \) \( pr \) \( cn/+ \) strain. When compared to their respective controls, none of the \( mfs(2)31 \) alleles exhibit dominant suppression in any of the rearrangements examined. In contrast, recessive suppression is associated with the \( mfs^1/mfs^1 \) and \( mfs^1/mfs^2 \) strains in all three variegating backgrounds. The phenotype is particularly strong in the \( mfs^1 \) homozygotes where near wild-type pigment levels are observed in \( w^4 \) and \( w^3 \) flies of both sexes and a moderate increase in pigment (twofold) is observed in \( w^{51b} \) females. No \( w^{51b}/y; mfs^1/mfs^1 \) males were recovered in this study.

Although substantial and consistent, the suppression associated with the \( mfs^1/mfs^2 \) strain is more moderate than in the \( mfs^1 \) homozygotes. Increased pigment levels on the order of magnitude of 25%-45% of \( Oregon-R \) (2-10 fold increases in pigment level) are observed for males in all three variegating backgrounds as well as in \( w^{51b} \) females. However, no suppression is observed in \( w^3 \) and \( w^4 \) females. This is not surprising since this allelic combination has a weak phenotype for female fertility.

The isolation of a second \( mfs(2)31 \) hypomorphic allele with a
su(var) phenotype confirms the initial observation of Sinclair et al. (1991) that lesions at the \textit{mfs}(2)31 locus are associated with suppression of PEV. Since these mutations have been isolated on the basis of lethality rather than suppression, it is unlikely that they are both gain-of-function alleles. It is more reasonable to conclude that loss-of-function at the \textit{mfs}(2)31 locus produces a su(var) phenotype. The absence of a dominant phenotype in even the strongest alleles would indicate that the gene product is not as dosage-sensitive as are the majority of previously described dominant modifiers of PEV. Finally, the su(var) phenotype does not appear to be due to a specific interaction between \textit{mfs}(2)31 and the \textit{w}^d inversion since suppression has been observed in a variety of variegating backgrounds.

\textbf{\textit{mfs}^2 has a \textit{P} element in 31E}

The \textit{mfs}^2 mutation is a weak allele of \textit{mfs}(2)31 which was isolated in a \textit{P} element screen. A \textit{P} element probe was hybridized to salivary gland polytene chromosomes from the \textit{mfs}^2 strain. The \textit{in situ} hybridization revealed an element in subdivision 31E on chromosome arm 2L (Figure 8), the known cytogenetic location of \textit{mfs}(2)31 (see Chapter 2).

\textbf{Reversion of \textit{mfs}^2}

\textit{P} element insertions often produce weak mutant phenotypes. However, it has been previously reported that a substantial proportion of mutations induced in \textit{P} element screens are
Figure 8: In situ hybridization to salivary gland polytene chromosomes of a $mfs^2 b pr cn/+ \text{ strain}$. A $P$ element specific probe reveals homology in cytological subdivision 31E (arrow).
attributable to chromosomal rearrangements. It is possible to distinguish between these two classes of mutation by out-crossing the mutant chromosome in a dysgenic background and measuring the frequency of reversion of the mutant phenotype. Under dysgenic conditions, reversion frequencies are considerably higher for insertional mutations than for mutations caused by rearrangements.

The A2-3 Sb chromosome is a potent source of transposase activity which was used to revert the lethal phenotype associated with mfs$^2$/Df(2L)J77 transheterozygotes (see Figure 6). A total of 2500 chromosomes were examined from which nine exceptional mfs$^2$/Df(2L)J77 flies were identified. This represents a reversion frequency of over 1% which is at least 5 orders of magnitude higher than spontaneous reversion frequencies (Ashburner, 1989). All revertants were recovered from different bottles and are likely to have arisen from independent events. The putative revertant chromosomes were established in balanced stocks and all nine complemented Df(2L)J77 and mfs$^4$ on subsequent retests. These revertants are described in Table 10.

Of the nine revertants recovered, five were male and four were female. Three of the females and one of the males were Sb indicating that they still carried the A2-3 Sb chromosome. The revertants were tested as homozygotes and all were viable and fertile in both sexes. This strongly argues against the mfs$^2$ chromosome carrying any second site lethal and/or sterile mutations. Stocks established from female and/or Sb revertants have not been used for any subsequent analyses because of the
TABLE 10
Revertant description

<table>
<thead>
<tr>
<th>Revertant Strain</th>
<th>Sex</th>
<th>Stubble</th>
<th>Homozygotes</th>
</tr>
</thead>
<tbody>
<tr>
<td>$mfs^{R1} b pr cn$</td>
<td>♂</td>
<td>+</td>
<td>viable/fertile</td>
</tr>
<tr>
<td>$mfs^{R2} b pr cn$</td>
<td>♂</td>
<td>+</td>
<td>viable/fertile</td>
</tr>
<tr>
<td>$mfs^{R3} b pr cn$</td>
<td>♂</td>
<td>+</td>
<td>viable/fertile</td>
</tr>
<tr>
<td>$mfs^{R4} b pr cn$</td>
<td>♂</td>
<td>+</td>
<td>viable/fertile</td>
</tr>
<tr>
<td>$mfs^{R5} b pr cn$</td>
<td>♂</td>
<td>−</td>
<td>viable/fertile</td>
</tr>
<tr>
<td>$mfs^{R6} b pr cn$</td>
<td>♀</td>
<td>+</td>
<td>viable/fertile</td>
</tr>
<tr>
<td>$mfs^{R7} b pr cn$</td>
<td>♀</td>
<td>−</td>
<td>viable/fertile</td>
</tr>
<tr>
<td>$mfs^{R8} b pr cn$</td>
<td>♀</td>
<td>−</td>
<td>viable/fertile</td>
</tr>
<tr>
<td>$mfs^{R9} b pr cn$</td>
<td>♀</td>
<td>−</td>
<td>viable/fertile</td>
</tr>
</tbody>
</table>
possibility of unwanted recombinational and transpositional events.

Three of the stocks established from male \textit{Sb}' revertants were selected for further analysis: \textit{mfs}^{S1}, \textit{mfs}^{S2} and \textit{mfs}^{S3}. All three revertant chromosomes were placed in a \textit{w^{d}} background and examined for bristle, sterility and \textit{su(var)} phenotypes. The revertant chromosomes were tested as homozygotes, as heterozygotes with a wild-type chromosome and in \textit{trans} with the \textit{mfs}^{I} and \textit{mfs}^{2} alleles. The results of these crosses are summarized in Table 11.

When tested in \textit{trans} with a wild-type chromosome the three revertants have normal thoracic bristles and are fertile in both sexes. Although all three have eye pigment levels at least as low as control levels (\textit{b pr cn/+}), the levels in \textit{mfs}^{S1} females are particularly low showing an enhanced effect (4.2\% vs 21.2\% in the controls).

None of the revertant chromosomes exhibit any aberrant phenotypes when examined as homozygotes. Although the pigment levels for these strains are very low, this is also the case for the control strain (\textit{b pr cn/b pr cn}) and probably reflects the presence of two other mutations on the chromosome which influence pigment deposition, \textit{pr} and \textit{cn}.

When crossed to \textit{mfs}^{I} and \textit{mfs}^{2}, the revertant chromosomes act as if the \textit{mfs}^{2} mutation is no longer present. The revertants survive over \textit{mfs}^{I} and exhibit no abnormal phenotypes. Over \textit{mfs}^{I}, they show none of the phenotypes associated with \textit{mfs}^{I}/\textit{mfs}^{2} transheterozygotes: male suppression, slender bristles and male sterility. The complete reversion of the \textit{mfs}(2)31 phenotype is
<table>
<thead>
<tr>
<th>Strain</th>
<th>Sex</th>
<th>Pigment (\text{wm}^3)^a</th>
<th>Bristle</th>
<th>Fertility</th>
</tr>
</thead>
<tbody>
<tr>
<td>b pr cn/+</td>
<td>♂</td>
<td>6.3 ± 2</td>
<td>+</td>
<td>Fertile</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>21.2 ± 2</td>
<td>+</td>
<td>Fertile</td>
</tr>
<tr>
<td>b pr cn/b pr cn</td>
<td>♂</td>
<td>6.3 ± 2</td>
<td>+</td>
<td>Fertile</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>8.2 ± 1</td>
<td>+</td>
<td>Fertile</td>
</tr>
<tr>
<td>mfs^{R1}b pr cn/+</td>
<td>♂</td>
<td>2.4 ± 1</td>
<td>+</td>
<td>Fertile</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>4.2 ± 1</td>
<td>+</td>
<td>Fertile</td>
</tr>
<tr>
<td>mfs^{R2}b pr cn/+</td>
<td>♂</td>
<td>3.1 ± 1</td>
<td>+</td>
<td>Fertile</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>12.1 ± 4</td>
<td>+</td>
<td>Fertile</td>
</tr>
<tr>
<td>mfs^{R3}b pr cn/+</td>
<td>♂</td>
<td>6.8 ± 1</td>
<td>+</td>
<td>Fertile</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>22.1 ± 5</td>
<td>+</td>
<td>Fertile</td>
</tr>
<tr>
<td>mfs^{R1}b pr cn/mfs^{R1}b pr cn</td>
<td>♂</td>
<td>3.8 ± 1</td>
<td>+</td>
<td>Fertile</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>2.6 ± 1</td>
<td>+</td>
<td>Fertile</td>
</tr>
<tr>
<td>mfs^{R2}b pr cn/mfs^{R2}b pr cn</td>
<td>♂</td>
<td>3.7 ± 1</td>
<td>+</td>
<td>Fertile</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>3.9 ± 1</td>
<td>+</td>
<td>Fertile</td>
</tr>
<tr>
<td>mfs^{R3}b pr cn/mfs^{R3}b pr cn</td>
<td>♂</td>
<td>2.8 ± 1</td>
<td>+</td>
<td>Fertile</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>3.7 ± 1</td>
<td>+</td>
<td>Fertile</td>
</tr>
<tr>
<td>mfs^{R1}b pr cn/mfs^{2}b pr cn</td>
<td>♂</td>
<td>3.6 ± 2</td>
<td>+</td>
<td>Fertile</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>2.6 ± 1</td>
<td>+</td>
<td>Fertile</td>
</tr>
<tr>
<td>mfs^{R2}b pr cn/mfs^{2}b pr cn</td>
<td>♂</td>
<td>1.5 ± 1</td>
<td>+</td>
<td>Fertile</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>5.1 ± 1</td>
<td>+</td>
<td>Fertile</td>
</tr>
<tr>
<td>mfs^{R2}b pr cn/mfs^{2}b pr cn</td>
<td>♂</td>
<td>3.6 ± 1</td>
<td>+</td>
<td>Fertile</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>4.0 ± 2</td>
<td>+</td>
<td>Fertile</td>
</tr>
<tr>
<td>mfs^{R1}b pr cn/mfs^1</td>
<td>♂</td>
<td>6.4 ± 1</td>
<td>+</td>
<td>Fertile</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>15.5 ± 1</td>
<td>+</td>
<td>Fertile</td>
</tr>
<tr>
<td>mfs^{R2}b pr cn/mfs^1</td>
<td>♂</td>
<td>8.4 ± 1</td>
<td>+</td>
<td>Fertile</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>11.5 ± 1</td>
<td>+</td>
<td>Fertile</td>
</tr>
<tr>
<td>mfs^{R3}b pr cn/mfs^1</td>
<td>♂</td>
<td>6.9 ± 1</td>
<td>+</td>
<td>Fertile</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>21.1 ± 3</td>
<td>+</td>
<td>Fertile</td>
</tr>
</tbody>
</table>

* Pigment levels are expressed as a percentage of Oregon-R.
illustrated in Figure 9.

In no case examined are the various \textit{mfs}(2)31 mutant phenotypes genetically separable; this provides compelling evidence that all the phenotypes are a consequence of a single lesion at the \textit{mfs}(2)31 locus. Removing the \textit{P} element insert from the \textit{mfs}^2 allele reverts all the phenotypes associated with the \textit{mfs}(2)31 locus. The high frequency of reversion of \textit{mfs}^2 in a dysgenic background indicates that this allele results from a \textit{P} element insertion.
Figure 9: Reversion of \( su(var) \) and bristle phenotypes in a hybrid-dysgenesis induced revertant of \( mfs^2 \).

Photographs are of adult males at 48 hrs post-eclosion:

A. \( w^{m4}/Y; +/+ \)
B. \( w^{m4}/Y; mfs^1/mfs^1 \)
C. \( w^{m4}/Y; mfs^1/mfs^2 \ b \ pr \ cn \)
D. \( w^{m4}/Y; mfs^1/mfs^{R1} \ b \ pr \ cn \).
Discussion

This chapter contains a phenotypic analysis of mfs(2)31, a suppressor of PEV which has been localized to cytological subdivision 31E on the left arm of chromosome 2. Since division 31 is known to contain one or more additional Su(var) loci that are both haplo- and triplo-abnormal (Sinclair et al., 1991), it is difficult to assess the nature of the mfs(2)31 mutation using duplications and deficiencies alone. For example, although the few duplications which are available for division 31 all show strong E(var) phenotypes, this cannot be directly attributed to the mfs(2)31 locus since these duplications also encompass several known dosage-dependant modifiers of PEV (Sinclair et al., 1991).

The deficiencies for 31E are somewhat more informative. Large deficiencies which extend distally into 31A exhibit strong suppression, presumably because they uncover Suvar(2)1, a haplo-dependant modifier which has been localized to 31B-C (Reuter et al., 1982; Sinclair et al., 1991). Smaller deficiencies which uncover 31D-E show moderate suppression while deficiencies which extend from 31D through 31F do not suppress at all (Sinclair et al., 1991). This pattern indicates that a moderately strong haplo-insufficient Su(var) locus is located in 31E, while acting epistatically to it, is an E(var) locus in 31F. In this regard, a strong E(var) strain which contains a P element inserted into 31F was recently isolated (G. Reuter, personal communication).

This current study does not indicate that the moderate haplo-insufficient Su(var) locus in 31D-E is mfs(2)31. Although complete
loss-of-function has not been formally demonstrated for any of the mfs(2)31 alleles, none of them exhibit any dominant suppression of PEV, regardless of the variegating background in which they are examined. Typically, modification of PEV is a dominant phenotype, a feature which reflects a bias in the method of isolating mutations rather than a functional characteristic of all modifying loci. The mfs(2)31 locus differs from all previously described modifiers in that suppression is only observed in viable combinations of hypomorphic alleles. There is some variability in the phenotype which may be attributable to intrinsic properties of the variegators and/or the individual alleles.

Although the data does not indicate that the mfs(2)31 gene is haplo-insufficient with respect to modifying PEV, suppression does appear to be associated with loss-of-function at this locus. In order to explain the sensitivity of the variegating phenotype to the dosage of many Su(var) loci, it has been proposed that these genes encode components of multimeric complexes whose assembly is required for the formation of heterochromatin (Locke et al., 1988). The absence of dominant phenotypes for the mfs(2)31 alleles suggests that, unlike the previously described modifiers of PEV, the dosage of the gene product is not rate-limiting with respect to chromatin assembly. However, the mfs(2)31 gene product has an essential function, and weak alleles of mfs(2)31 have a strong su(var) phenotype, suggesting that mfs(2)31 may have an important role in the chromatin assembly process.

The effective lethal phase for mfs(2)31 alleles occurs during
larval development. The larval period is a time of rapid and substantial cell growth in *Drosophila*, processes which are facilitated by polyploidization of most larval tissues. Although there is no direct evidence that the *mfs(2)31* gene product is required for protein synthesis and/or cell growth, there are phenotypic similarities between *mfs(2)31* and mutant strains which are known to be defective in these processes. *Minutes* are a class of haplo-insufficient mutation which are characterized by a prolonged developmental period and short, thin bristles (for review see Kay and Jacobs-Lorena, 1987). These phenotypes are also observed in viable *mfs(2)31* allelic combinations. Molecular evidence suggests that the *Minute* loci are genes which encode ribosomal proteins and that the phenotypes reflect failures during processes which require maximum rates of protein synthesis (Kay and Jacobs-Lorena, 1987).

Another process during *Drosophila* development which requires high rates of protein synthesis, and which is defective in *mfs(2)31* mutations, is the deposition of yolk proteins during vitellogenesis. Females which carry viable *mfs(2)31* allelic combinations exhibit temperature-sensitive sterility with a sensitive period extending into adulthood. The defect in oogenesis, as revealed by a temporal study of ovarian development, is associated with a delay in the onset of yolk deposition. Vitellogenesis does not begin in adult *mfs1* homozygotes until 72 hours post-eclosion and mature eggs are not observed in the ovaries for an additional seven days. In contrast, the wild-type ovary
reaches full maturity within 48 hrs of eclosion. The mutant strain does not undergo any oviposition and the developing oocytes are eventually reabsorbed. The relationship between oviposition and vitellogenesis in Drosophila is unclear and it is difficult to say whether the defects in these two processes is a consequence of the same or independent events.

Yolk protein synthesis in Drosophila occurs in both the follicle cells and the fat bodies, subsequent to the polyploidization of these respective cell populations (Mahowald and Kambysellis, 1980). The follicle cells produce a substantial fraction of the yolk proteins, transporting it directly into the rapidly growing oocyte (Brennan et al., 1981). I have examined thick sections through stage 9 ovarian cysts and found that the follicle cells of mfs1 homozygotes have a wild-type morphology at the onset of vitellogenesis indicating that there has not been a general failure on the part of these cells to proliferate and migrate properly.

Most of the yolk proteins are synthesized in the fat bodies, released into the hemolymph and transported into the oocytes by the pinocytotic activity of the oocyte itself (Mahowald and Kambysellis, 1980). These processes are under hormonal control with the Drosophila juvenile hormone (JH) being the primary controlling agent (Postlethwait et al., 1976; Kambysellis and Heed, 1974). Although the role that the mfs(2)31 gene product plays in vitellogenesis is not known, mutations in genes such as apterous (ap), whose products are required for the final stages of
histolysis of the fat bodies as well as the uptake of yolk proteins by the oocyte have similar phenotypes to *mfs(2)31* (Postlethwait and Weiser, 1973). Flies which are homozygous for *ap* mutations are male and female sterile and are defective in bristle elaboration. The female sterility is associated with a failure to deposit yolk in the developing oocyte.

Although it is tempting to conclude from the bristle and ovarian phenotypes that *mfs(2)31* mutant strains are unable to achieve maximum rates of protein synthesis, it is difficult to reconcile such a developmental defect with the observed abnormalities in centriole migration (Lindsley et al., 1980) and the suppression of PEV (Sinclair et al., 1991; this study). There is no evidence linking centriole behaviour with rates of protein synthesis while a low rate of protein synthesis would enhance rather than suppress PEV. The centriole phenotype could be explained if centriole migration is a prerequisite for polyploidization in *Drosophila*. A failure of either the follicle cells to achieve full polyploidy could slow down or delay yolk protein synthesis and its subsequent deposition. Polyploidy is also employed as a mechanism during larval development for sustaining rapid and substantial cell growth and as a mechanism for the elaboration of adult bristles (Beckingham and Rubacha, 1984).

The proposal that centriole migration and degradation is one of the triggers for the switch from a mitotic to an endomitotic cycle is not a new one (Mahowald and Kambysellis, 1980), but the evidence is still somewhat scant. During stages 1-7 of oogenesis,
centrioles migrate from the nurse cells to the oocyte where they are subsequently degraded. Although the reason for this migration is not known, it does signal the onset of polyploidization of the nurse cells. Similarly, the migration and subsequent degradation of centrioles prior to polyploidization has also been described in the follicle cells of developing ovarian cysts (Mahowald et al., 1979). Although centrioles have never been found in the terminally polyploid cells of insects, there has been no direct demonstration of a causal link between centriole behavior and entry into the endomitotic cycle.

Although underreplication of the variegating locus has been invoked as one cause of PEV (Karpen and Spradling, 1990), there are several reasons why the su(var) phenotype associated with mfs(2)31 mutations is probably not a consequence of ploidy of the affected gene. First, a mutation which reduces the copy number of a variegating locus is more likely to exhibit an e(var) phenotype than a su(var) phenotype. Second, and probably more important, mfs(2)31 alleles suppress PEV in the pigment cells of the eye, a tissue which is generally considered to be diploid.

Although a precise function of the mfs(2)31 gene product has yet to be defined, it is hoped that additional functional information can be obtained from a molecular analysis of the locus. Such an analysis is made possible by the isolation of mfs2, a P element induced allele of mfs(2)31. This allele reverts at a frequency of greater than 1% when passed through a dysgenic background and contains a P element insertion in subdivision 31E.
All of the phenotypes associated with mfs(2)31 mutations are co-reverted through mfs2 indicating that they are all the consequence of lesions in a single locus. Chapter 4 describes the use of this allele to identify a putative transcript for the mfs(2)31 gene.
CHAPTER 4: MOLECULAR ANALYSIS OF THE mfs(2)31 LOCUS

Introduction

The mfs(2)31 locus is located in cytological subdivision 31E on chromosome arm 2L of Drosophila melanogaster. Originally isolated as a recessive sterile mutation with a tightly linked bristle phenotype (Sandler, 1977), mfs(2)31 is a rare example of a recessive suppressor of position-effect variegation (su(var); Sinclair et al., 1991). We have had an ongoing interest in the mfs(2)31 locus because of its su(var) phenotype.

It has long been thought that the genes which can be mutated to modify PEV will encode non-histone chromosomal proteins (NHPs; Spofford, 1976); a proposal which has been recently borne out by the molecular analyses of several Su(var) loci. The protein which is encoded by Su(var)205 (HP1) was purified from a nuclear protein fraction and is known to associate with salivary gland polytene chromosomes (James and Elgin, 1986; James et al., 1989) while the predicted Suvar(3)7 protein sequence contains widely spaced zinc-finger motifs (Reuter et al., 1990). It is becoming increasingly apparent that PEV may be an effective tool for the genetic dissection of chromatin and/or the systems regulating its assembly. In this chapter we add mfs(2)31 to the relatively short list of PEV modifiers which have been analyzed at the molecular level.

Chapter 2 of this study describes the isolation of three additional alleles of mfs(2)31 including mfs2, a mutation which was recovered in a P element screen. The associated cytogenetic analysis of the 31D-E region indicates that this locus is most
probably located in the distal half of 31E. Chapter 3 contains an extended phenotypic analysis of the \textit{mfs}(2)31 locus including a description of the new alleles. The \textit{mfs}^2 allele is thought to be hypomorphic, in that it is the only allele to survive in \textit{trans} with the original \textit{mfs}^1 mutation. The \textit{mfs}^1/\textit{mfs}^2 heteroallelic survivors exhibit the same sterility, \textit{su}(\textit{var}), and bristle phenotypes observed in \textit{mfs}^1 homozygotes which strongly implies that all of these phenotypes are attributable to the \textit{mfs}(2)31 locus.

There are two reasons why we suspect that the \textit{mfs}^2 mutation is the result of an insertional event. First, the \textit{mfs}^2 strain contains a \textit{P} element inserted into subdivision 31E which is the most probable cytological location of the \textit{mfs}(2)31 locus (see chapter 3). Second, when passed through a dysgenic background, the allele reverts at a frequency of higher than 1\% (see chapter 3) which is at least five orders of magnitude higher than spontaneous reversion frequencies for \textit{Drosophila melanogaster} (Ashburner, 1989). All the phenotypes associated with \textit{mfs}(2)31 mutations, including the \textit{su}(\textit{var}) phenotype, are co-reverted through \textit{mfs}^2.

This chapter describes the use of the \textit{P} element containing \textit{mfs}^2 strain to identify a transcript which most likely corresponds to the \textit{mfs}(2)31 locus. The locus has been molecularly mapped with respect to the previously cloned and tightly linked \textit{da} and \textit{cdc}2 genes and a transcriptional profile of the region is described. A full length cDNA corresponding to the \textit{mfs}(2)31 transcript has been isolated and sequenced. The similarity of the deduced gene product to previously described proteins, and the implications of these
similarities with respect to the usefulness of PEV as a genetic assay, are discussed.
Materials and Methods

STOCKS

Complete descriptions of all strains which have been used for RNA and DNA isolations can be found in chapter 3 and/or in Lindsley and Zimm (1991).

RECOMBINANT CLONES

The pπ25.1 construct is a full length P element, plus flanking genomic DNA, cloned into pBR322 (described in O’Hare and Rubin, 1983). The pDmA2 construct contains a full length Drosophila actin gene plus flanking genomic DNA cloned into pBR322 (described in Fryberg et al., 1980). JT20, JT31 and JT35 are overlapping clones from subdivision 31E inserted into a modified CoSpeR vector. These cosmids, all of which encompass the da locus, were kindly provided by C. Cronmiller. The host bacterium for each of these recombinant clones is Escherichia coli (E.Coli) DH5α.

IN SITU HYBRIDIZATIONS

In situ hybridizations to polytene chromosomes were performed as previously described (see Chapter 3: Materials and Methods).

DNA ANALYSIS

Isolation of high-molecular weight genomic DNA

Genomic DNA was isolated using a modified procedure of Jowett (1988). Approximately 1-3 g of tissue were flash frozen in liquid
nitrogen and ground to a fine powder in a pre-chilled mortar and pestle. The powdered tissue was immediately transferred to 10 ml of lysis buffer (10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 50 mM EDTA, 1% SDS, 0.15 mM spermine, 0.5 mM spermidine) and Proteinase K (stock solution is 10 mg/ml in 50% glycerol) was added to a final concentration of 100 ug/ml. Following a two hour incubation at 37°, the mix was extracted twice with phenol (BRL: equilibrated with TE (pH 8.0)), twice with phenol/chloroform (1:1 v/v), and once with chloroform. The aqueous solution was overlayed with 5 ml of ice-cold 95% EtOH and the DNA was spooled out using a narrow bore glass Pasteur pipet. The DNA was suspended in 500 ul of TE (pH 8.0) and RNase (Sigma) was added to a final concentration of 100 ug/ml. Following a 60 minute incubation at 37°, the reaction was extracted once with phenol (as above), once with phenol/chloroform (1:1 v/v) and once with chloroform. The aqueous phase was overlayed with 500 ul of cold 95% EtOH and the DNA was spooled as above. Depending on yield, the DNA was suspended in 200-1000 ul of TE (pH 8.0) and stored at 4°.

**Isolation of plasmid and cosmid DNA**

Procedures for purifying either plasmid or cosmid DNA were identical in all respects. All protocols utilized modifications of the alkaline-lysis method of DNA isolation (Sambrook *et al.*, 1989).

**Small scale isolations:** Small scale DNA isolations were obtained using the Magic Miniprep DNA purification system.
Overnight cultures (1-3 ml of LM; 0.005% ampicillin) were pelleted, suspended in 200 ul of Resuspension Solution (50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 100 ug/ml RNase A) and then lysed with 200 ul of Cell Lysis Solution (0.2 M NaOH, 1% SDS). Bacterial debris was precipitated with 200 ul of Neutralization Solution (2.55 M KOAc (pH 4.8)) and removed by centrifugation at 15,000 rpm for 5 minutes. One ml of resuspended Purification Resin was mixed into the supernatant and the slurry was forced (by syringe) through a mini-column attached to a 3 ml disposable syringe barrel. Columns were washed with 3 mls of Column Wash Solution (200 mM NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM EDTA; diluted 50% with absolute ethanol) and dried by centrifugation. DNA was eluted in 50 ul of pre-heated (65°) TE buffer (pH 7.5).

**Medium scale isolations:** Overnight cultures (100 ml LM; 0.005% ampicillin) were pelleted and then suspended in 2.5 ml of Solution 1 (50 mM Glucose, 25 mM Tris (pH 8.0), 10 mM EDTA). Following a ten minute incubation on ice, the cells were lysed with 5 ml of freshly prepared Solution 2 (0.2 M NaOH, 1% SDS) and precipitated with 3.75 ml of 3 M KOAc. The bacterial debris was pelleted by centrifugation at 8000 rpm for 20 min in a Sorvall ss34 rotor. The clear supernatant was extracted once with phenol/chloroform (1:1) and once with chloroform prior to precipitation of the DNA with two volumes of 95% EtOH. The DNA was pelleted, washed with 70% ethanol and vacuum dried. The pellet was suspended in 300 ul of TE and RNase (Sigma) was added to a final concentration of 100 ug/ml. Following a 30 minute reaction time,
the RNase was precipitated with 1/2 volume 5M NH₄Ac (-70° for 20 minutes) and removed by centrifugation at 15,000 rpm for 5 min. The DNA was precipitated with two volumes of 95% EtOH, pelleted, vacuum dried and suspended in 900 ul of TE (pH 7.5). Plasmid DNA was precipitated (one hour on ice) with 600 ul of PEG solution (20% PEG, 2.5 M NaCl). The DNA was pelleted (15,000 rpm for 10 min), washed in 70% EtOH, vacuum-dried and suspended in 100 ul of TE.

Large scale isolations: Overnight cultures (500 ml of LM; 0.005% ampicillin) were pelleted and then suspended in 4 ml of Solution 1 (50 mM glucose, 25 mM Tris (pH 8.0), 10 mM EDTA). Following a ten minute incubation on ice, the cells were lysed with 16 ml of freshly prepared Solution 2 (0.2 N NaOH, 1% SDS). The bacterial debris was precipitated with 12 ml of 3 M KOAc and removed by centrifugation (20 min at 15,000 rpm in a Sorvall ss34 rotor). The DNA was precipitated in isopropanol (60% v/v), pelleted, washed in 70% EtOH, vacuum dried and suspended in 8 ml of TE (pH 8.0). The suspended DNA was prepared for centrifugation by the addition of eight grams of solid cesium chloride and 650 ul of ethidium bromide (10 mg/ml in H₂O). The solution was transferred to a Beckmann polyallomer 16 X 76 Quick-Seal centrifuge tube and the tube was heat-sealed. Centrifugation was at 45,000 rpm for 36 hours (20°) in a Sorvall Tft65.13 rotor in a Beckmann L8-80 ultracentrifuge. Supercoiled DNA was removed from the cesium chloride gradient using a 21 gauge needle attached to a 1 ml syringe barrel. The ethidium bromide was removed by repeated extractions with water saturated 1-butanol. The solution was
diluted three times with water and the DNA was precipitated with 2.5 volumes of 95% EtOH.

Isolation of bacteriophage DNA

Confluent plates of bacteriophage λ were prepared as follows. Approximately 3 X 10⁶ bacteriophage were mixed with 1.6 x 10⁸ bacterial cells and then incubated at 37° for 30 minutes. The mix was then diluted in 8 ml of melted top agar (cooled to 43°) and immediately spread on the surface of LM plates. The plates were incubated at 37° for 8 hours or until complete plate lysis occurred. The top agar was scraped from the surface of the plates into a solution of 25 ml λ-dil: 1 ml CHCl₃ and then left overnight at 4°. The debris was removed by centrifugation for 30 min at 2500 rpm in a desktop clinical centrifuge. The supernatant was transferred to fresh tubes and made 1 M NaCl (2.92 g/100 ml supernatant) and 10% polyethylene glycol (10 g/100 ml supernatant). The mixture was incubated for one hour in ice water to allow precipitation of the bacteriophage particles which were then pelleted by centrifugation at 8000 rpm for ten minutes (4°) and suspended in 0.5 ml of λ-dil.

Cesium chloride step gradients were prepared in the following manner. One ml of CsCl Solution 1 (5.0 M CsCl, 10 mM MgSO₄, 10 mM Tris (pH 8.0), 0.1 mM EDTA) was placed in the bottom of a Beckman 13 X 51 mm Ultra-Clear centrifuge tube. This was overlayed with 3 ml of CsCl Solution 2 (3.0 M CsCl, 10 mM MgSO₄, 10 mM Tris (pH 8.0), 0.1 mM EDTA) which in turn was overlayed with the 0.5 ml
bacteriophage solution. Centrifugation was in a Beckman SW50.1 rotor at 30,000 rpm for 1 hour (20°). Bacteriophage were removed from the gradient with a 21 gauge needle attached to a 1 ml syringe barrel. One volume of 1 M Tris (pH 8.0), 0.25 volumes of EDTA and 2.25 volumes of formamide were added directly to the solution. Following a 15 minute incubation at room temperature, the DNA was precipitated with 2.5 volumes of 95% EtOH, pelleted, washed in 70% EtOH and resuspended in 100 ul of TE.

Restriction endonuclease digests

Approximately 1-3 ug of DNA (plasmid, cosmid or genomic) was diluted in 16 ul of water. Two ul of the appropriate 10 X core buffer (BRL) and two ul of restriction enzyme (3-10 units) were added and the reaction was incubated at 37° for a minimum of one hour. Reactions were stopped by the addition of 5 ul of loading buffer (6 M Urea, 25% Sucrose, 50 mM EDTA, 0.4% xylene cyanol, 0.4% bromophenol blue).

Gel Electrophoresis

DNA was size fractionated on 0.6-1.2% agarose (BRL electrophoresis grade) gels. The gel buffer contained 40 mM Tris-HCl, 5 mM NaOAc, 1mM EDTA; solution is pH adjusted to 7.8, and 0.1 ug/ml ethidium bromide. Electrophoresis was at 1.5-4 v/cm for 1-18 hours. The gels were photographed using transmitted ultraviolet light for illumination.
DNA transfer from agarose gels to nylon membrane

DNA was transferred to nylon membranes using a modified procedure of Southern (1975). Gels were gently agitated in denaturing solution (0.4 N NaOH, 0.6 M NaCl) for 30 min at room temperature, rinsed briefly in H$_2$O and then neutralized in 1.5 M NaCl, 0.5 M Tris-HCl (pH 5.5), for an additional 30 minutes. The gel was then placed on a pre-soaked (20 X SSC) Whatman 3mm paper wick, which was suspended on a platform above a reservoir filled with 20 X SSC. The gel was overlayed first with a pre-wetted nylon membrane (as per manufacturers instructions), then two thicknesses of pre-soaked (20 X SSC) Whatman 3 mm paper and finally, a thick stack of paper towels. A weighted plexiglass plate was placed on top to ensure even transfer. Transfer was for 3-12 hours for plasmid and cosmid DNA and 36 hours for genomic DNA. The paper towels were replaced periodically as required. Following transfer, filters were air-dried for two hours and then the DNA was fixed to the membrane by exposure to ultraviolet light (254 nm) for five minutes.

RNA ANALYSIS

Isolation of developmentally staged Drosophila cultures

In order to observe the transcriptional profile of mRNA’s at different times in development, the following staged cultures were purified from the Oregon-R wild-type strain; 0-2.5 hour embryos, 0-6 hour embryos, 2.5-6 hour embryos, 6-9 hour embryos, 9-12 hour embryos, 12-24 hour embryos, 1st instar larvae, 2nd instar larvae,
3rd instar larvae, early pupae, late pupae and adult male and females. In addition, 0-6 hour embryo’s were isolated for the *mfs*¹, *mfs*², *mfs*³, *mfs*⁴, b pr cn, *mfs*⁵, *mfs*⁶, *mfs*⁷, *mfs*⁸, and *mfs*⁹ strains. Population cages were established for each strain at 25°. Egg lays were on plates made up of 1% agar, 5% malt vinegar and 5% EtOH. A live yeast paste (Fleischmann’s) was spread on the surface of the plates to encourage oviposition. Synchronized cultures were obtained in the following manner.

**Embryonic collections:** The duration of an egg-lay corresponded to the length of the embryonic period for which tissue was required i.e. 3 hours for 3-6 hour embryos, 12 hours for 12-24 hour embryos. Embryos were aged at 25° for the appropriate period of time and were then collected and concentrated by gentle manipulation on a fine nylon mesh. Embryos were transferred to microcentrifuge tubes, weighed and flash frozen by immersion in liquid nitrogen. Embryos were stored at -70° until needed.

**Larval and pupal collections:** Egg-lays for larval and pupal collections were for 24 hours on standard cornmeal-agar fly media in the bottom of 6 X 12" Tupperware containers. Subsequent to egg-lays, the containers were removed from the population cages, sealed and stored at 25°. First instar larvae were gently brushed from the surface of the food while second and third instars were separated from the food by floatation in 10% sucrose. Appropriately staged pupae were selected visually.
Isolation of Poly(A)+ RNA

To ensure inactivation of contaminating nucleases and prevent subsequent contamination, all solutions were treated with 0.1% Diethylpyrocarbonate (DEP) where possible. In addition, all glassware was washed using DEP treated water and baked overnight at 250°.

Whole RNA was extracted using a modified procedure of Jowett (1988). Approximately 200-1000 mg of pre-frozen tissue were immersed in liquid nitrogen and ground to a fine powder using a mortar and pestle. The powder was immediately transferred to 4 ml of pre-heated phenol (65°) saturated with 0.2 M NaOAc (pH 5.0). The solution was vortexed and then 3 ml of NaOAc and 0.8 ml of 10% SDS was added. Following a 5 minute incubation at 65°, the solution was again vortexed and allowed to cool to room temperature. Four mls of chloroform were added, the solution was vortexed again, and the aqueous phase was separated by centrifugation. The aqueous phase was extracted once with phenol/chloroform (1:1 v/v) and once with chloroform alone. The RNA was precipitated with 2.5 volumes of 95% EtOH and stored at -20° under ethanol.

Poly(A)+ RNA was isolated from the whole RNA by chromatography on Oligo(dT)cellulose columns. Oligo(dT)cellulose (Pharmacia), which had been equilibrated in 1 X Binding Buffer (10 mM Tris (pH 7.5), 0.4 M NaCl, 5 mM EDTA, 0.5% SDS), was used to prepare 0.5 ml columns in 12 ml Bio-rad econo-columns. The prepared columns were washed first with 5 volumes of 0.1 M NaOH, then 10 volumes of
sterile water and finally 10 volumes of 1 X Binding Buffer.

RNA (stored under ethanol) was pelleted, resuspended in 10 ml 
H₂O (DEP) and then diluted in an equal volume of 2 X Binding 
Buffer. The RNA solution was heated to 65° for five minutes and 
then loaded on the column. The column was then washed, first with 
10 volumes of Binding Buffer and then with an additional 10 volumes 
of Binding Buffer containing no SDS. The RNA was eluted in 3 X 1 
ml fractions with Elution Buffer (10mM Tris (pH 7.5), 1 mM EDTA). 
The eluted RNA solution was made 0.3 M NaOAc and the RNA was 
precipitated with 2.5 volumes of 95% EtOH. The poly(A)+ RNA was 
stored at -20° as a precipitate in ethanol.

Gel electrophoresis of RNA

Poly(A)+ RNA was size fractionated on formaldehyde (0.66 M) 
denaturing gels. Two grams of agarose were dissolved in a solution 
of 175 ml DEP treated water and 20 ml 10 X MOPS Buffer (0.2 M MOPS, 
50 mM NaOAc, 10mM EDTA; pH to 7.0). The solution was cooled to 50° 
prior to adding 10.2 ml of 37% de-ionized formaldehyde. The gel 
was poured and allowed to set for 1 hour before use. The wells 
were flushed prior to the loading of samples.

Approximately 5 ug of RNA (stored as a precipitate in ethanol) 
was pelleted and resuspended in 11 ul water, 2 ul 10 X MOPS Buffer, 
7 ul formaldehyde, 20 ul formamide and 2 ul 1 mg/ml Ethidium bromide. The sample was heated to 55° for five minutes, 2 ul of 
sterile loading buffer (6 M urea, 25% sucrose, 50 mM EDTA, 0.4% 
xylene cyanol, 0.4% bromophenol blue) were added and then the
sample was loaded onto the gel. Electrophoresis was at 60 V for 8 hours. The gels were photographed using transmitted ultraviolet light.

RNA transfer from formaldehyde gels to nylon membranes

The formaldehyde gels were prepared for transfer by soaking for 2 X 20 minutes in 10 X SSC. In all other respects transfers were performed as described above for agarose gels (see DNA transfer from agarose gels to nylon membrane).

HYBRIDIZATION TO NYLON MEMBRANES

In all respects, hybridization to DNA and RNA filters was conducted in the same manner.

Gel purification of probes

To prepare probes for radiolabelling, plasmid or bacteriophage DNA was cut with the appropriate restriction endonuclease and size-fractionated on agarose gels. A small gel slice containing the fragment of interest was excised from the gel and transferred to a microcentrifuge tube. The DNA was extracted from the agarose by adhesion to glass beads (GENECLEAN; Bio 101) as per manufacturers instructions.

Radiolabelling of probes

DNA was radiolabelled to a specific activity of approximately $1.8 \times 10^9$ dpm/ug by random hexamer-primed DNA labelling (Feinberg
and Vogelstein, 1983) using the Boehringer Mannheim Random Primed DNA Labelling Kit. Radiolabelled fragments were purified on Sephadex columns (G50 DNA grade; Pharmacia) which were prepared as follows. A suspension of Sephadex in sterilized TE (pH 7.5) was used to build six inch columns in modified Polyethylene Transfer Pipets (Fisher Scientific). Columns were allowed to pack by gravity for 30 minutes. Radiolabelling reactions were stopped by addition of an equal volume of Column Loading Buffer (40 mM EDTA, 2% Blue Dextran). The reaction was loaded on the column and the blue fraction was collected. The radiolabelled fragment was boiled for five minutes and plunged into iced water prior to adding to hybridization bags (see below).

Filter Hybridization

Filters were sealed in plastic bags with 10 ml pre-hybridization buffer (5 X SSC, 5 X Denhardt’s, 0.5% SDS, 100 ug/ml herring sperm DNA) and incubated at 65° for one hour with gentle agitation in a shaking water bath. A prepared radiolabelled fragment (see above) was added directly to the bag. Hybridization was at 65° for 12-18 hours. High stringency washes were performed as follows: 2 X 1 hour in 2 X SSC, 0.1% SDS (65°) followed by 2 X 1 hour in 0.1 X SSC, 0.1% SDS (65°). Hybridization was detected by autoradiography.
LIBRARY CONSTRUCTION AND SCREENING

Construction of a bacteriophage library

To facilitate cloning of the mfs(2)31 gene, a bacteriophage λ library was prepared from mfs\(^2\) DNA. Two micrograms of EMBL3 vector DNA (Promega) were digested to completion with Eco R1 (10 units). The digested DNA was diluted to 100 ul with H\(_2\)O, made 0.3 M NaOAc, and precipitated with 2.5 volumes 95% EtOH.

Ten micrograms (ug) of high-molecular weight genomic DNA from the mfs\(^2\) strain was digested at 37° with 0.1 units of Eco R1. The time course of the reaction was monitored by removing 1 ug aliquots of DNA. The aliquots were inactivated immediately by making them 20 mM with respect to EDTA. One hundred ng of DNA from each aliquot was examined by electrophoresis on an agarose gel to determine which sample had the greatest quantity of DNA falling in the 15-20 kb range. This sample was retained while the others were discarded.

The partially digested genomic DNA was precipitated in 2.5 volumes 95% EtOH, washed with 70% EtOH and suspended in phosphatase buffer (50 mM Tris (pH 9.0), 1 mM MgCl\(_2\), 0.1 mM ZnCl\(_2\), 1 mM spermidine). One unit of calf alkaline phosphatase was added and the reaction was incubated at 37° for one hour. The reaction mix was extracted once with phenol/chloroform (1:1 v/v) and once with chloroform. The sample was made 0.3 M NaOAc and precipitated with 2.5 volumes of 95% EtOH.

Two ug of vector DNA and 0.5 ug of the dephosphorylated genomic DNA were co-precipitated and resuspended in 10 ul ligase
buffer (10 mM Tris (pH 7.5), 10 mM MgCl₂, 5 mM β-mercaptoethanol, 1 mM ATP). One unit of T4 DNA Ligase (BRL) was added and the reaction was incubated overnight at 16°. The ligated DNA was packaged using the Gigapack Plus (Stratagene) packaging kit according to the manufacturers instructions.

**Screening Bacteriophage libraries**

Two bacteriophage λ libraries were screened in this study. The first was an EMBL3 genomic library constructed from partially digested (Eco R1) mfs² DNA (see above). The host strain bacterium for this library is NM539 (E. coli). The second library screened was an imaginal disc cDNA library (Dr. G. Rubin) constructed in λgt10. This library was amplified on C600hf1A (E. coli). Both libraries were screened using a modified procedure of Benton and Davis (1977).

**Preparation of confluent plates:** A single colony of the appropriate host strain was grown overnight at 37° in 50 ml of LM. Five ml were used to inoculate a fresh 50 ml culture and was grown at 37° for 3-5 hours. The cells were pelleted at low speed in a desk-top clinical centrifuge, suspended in 25 ml 10 mM MgSO₄ and stored at 4° for up to 4 weeks.

Approximately 5 x 10⁴ bacteriophage were added to 100 ul of the host cells, and were then incubated at 37° for 30 minutes. The mix was then added to 8 ml of molten top agar which had been cooled to 53°. The top agar was immediately poured onto the surface of LM plates which were then incubated at 37° until lysed bacterial lawns
covered the plates.

**Plaque lifts:** Lysed plates were carefully overlayed with dry, pre-cut nitrocellulose membranes. The membranes were peeled off the plates, soaked in 0.4 N NaOH for 30 seconds, washed twice for 60 seconds in 1.5 M NaCl, 0.5 M Tris (pH 5.5) and then washed for 30 seconds in 2 X SSC. The filters were air-dried and baked for two hours at 80°. Duplicate filters were made for each plate. Hybridization of the filters to radiolabelled probes was conducted as previously described (see **Filter hybridization**). Following the detection of homology, the appropriate agarose plug was recovered from the plate and successive rounds of screening were conducted at higher bacteriophage dilutions until plaque-purity was achieved.

**Screening cosmid libraries**

A cosPneo library prepared from *Drosophila* DNA was generously provided by J. Leung. The DNA was prepared by partial *Mbo* I digestion (35-50 kb size range) and the library was amplified in the recA *E. coli* strain DH1 (Leung, 1988). The library was plated overnight on LM agar plates (0.005% ampicillin) and the DNA was blotted directly onto the surface of nitrocellulose filters using the following modified protocol of Grunstein and Hogness (1975).

Pre-cut nitrocellulose filters were overlayed on the surface of the plates, peeled-off and placed immediately (bacterial side up) on Whatman 3MM paper soaked with denaturing solution (0.5 M NaOH, 1.5M NaCl). Following 5 minutes on the denaturing solution,
the filters were transferred to 3MM paper soaked in neutralizing solution (1.5 M NaCl, 0.5 M Tris (pH 8.0)) for an additional 5 minutes. Filters were air-dried and baked at 80° for 2 hours. Hybridization of the filters to radiolabelled probes was as previously described (see **Filter hybridization**).

**Subcloning cosmid, plasmid and bacteriovirge DNA**

Fragments of interest were cut from the vector and separated on an agarose gel. The appropriate band was excised from the gel and purified using the Geneclean DNA purification kit (Bio 101) according to the manufacturers instructions. Approximately 50-200 ng of DNA was co-precipitated with 50 ng of pre-cut pUC 19 vector with compatible ends. The DNA was resuspended in 20 ul of 10 mM Tris (pH 8.0), 10 mM MgCl₂, 1 mM DTT and 1 mM ATP. One unit of T4 DNA ligase was added and the reaction was incubated overnight at 16°. Following incubation, 5 ul of the ligation mix was added to 100 ul of sub-cloning efficiency *E. coli* DH5α competent cells (BRL), which were then incubated for a further 30 minutes on ice. The cells were then heat shocked for 45 seconds at 42°, and returned to the ice for another 2 minutes. The mix was diluted with 1 ml of LM and incubated in a rotary shaker for 1 hour at 37°. Approximately 50-200 ul of cells were spread evenly on LM agarose plates (0.005% ampicillin) whose surfaces had been pre-treated with 40 ul of 3% X-gal solution. Recombinants were identified as white colonies and screened by mini-plasmid preparations.
DNA SEQUENCING

Sequencing reactions

All sequencing reactions were double-stranded using Puc 19 as the template vector. Four ug of plasmid DNA was denatured by suspending it in 20 ul water and 2 ul of a 2 M NaOH, 2 mM EDTA solution, and then heating the solution to 65° for 5 minutes. Following denaturation, the DNA was precipitated with 2 ul 2 M NH₄Ac and 55 ul 95% EtOH for 30 minutes at -20°. The denatured template was pelleted, washed in 70% EtOH, vacuum-dried and suspended in 7 ul water. One ul of each primer (3 pmoles) and 2 ul Annealing Buffer (50 mM NaCl, 10 mM MgCl₂, 40 mM Tris (pH 7.5)) were added to the denatured template DNA. Annealing was at 37° for 20 minutes followed by incubation at room temperature for an additional 10 minutes. The labelling reaction was prepared sequentially by combining in the following order: 10 ul annealed template, 1 ul 0.1 M DTT, 2 ul nucleotide mix (1.5 uM each of dCTP, dGTP and dTTp), 1ul (α-³⁵S)dATP and 2 ul (1.8 Units) T7 DNA polymerase. Following the addition of the T7 polymerase, the reaction was incubated at room temperature for seven minutes. Termination reactions were performed by adding 3.5 ul of the labelling reaction to four tubes, each of which contained 2.5 ul of a different termination mix. A termination mix consists of 80 uM each of 3 of the 4 dNTPs found in DNA and 8 ul of the fourth. The termination reactions were incubated at 37° for 10 minutes and then all enzymatic activity was stopped by the addition of 4 ul of Stop Solution (95% formamide, 20 mM EDTA, 0.5% bromophenol blue, 0.5%
Polyacrylamide gel electrophoresis

Fifty ml of gel solution was prepared by combining 23 g urea (final molarity is 7 M), 13.2 ml 5 X TBE (Sambrook, 1989), 5.5 ml Long Ranger Gel Solution (J. T. Baker) and bringing to volume with water. Polymerization was induced by the addition of 250 ul 10% ammonium persulphate and 25 ul TEMED.

Plates were cleaned of any debris with 95% EtOH. One plate was treated with Dimethylchlorosilane solution (BDH) and re-cleaned with ethanol. Plates (33 X 40 X 0.04 mm) were assembled according to manufacturers instructions. Sequencing reactions were loaded into sharktooth sample wells and separated by electrophoresis at 45 watts for 2-6 hours. Gels were dried for two hours prior to autoradiography.

Sequencing strategy

All sequencing reactions were double-stranded using pUC19 as the sequencing vector. Following initial sequencing reactions, using the universal forward and reverse primers, primers were synthesized for each successive reaction based on the sequence obtained from the previous reaction. Primers were spaced approximately 150-200 bp apart and covered both strands. Both the cDNAs and the corresponding genomic fragments were sequenced on both strands using the same set of primers.

Oligodeoxynucleotide primers used for sequencing were
synthesized by the UBC Oligodeoxynucleotide Synthesizing Facility. Deprotected primers were purified as described by Sawadogo and Van Dyke (1991): 100 ul of primer solution in 30% NH₄OH was vortexed vigorously in a 1.5 ml eppendorf tube with 1000 ul n-butanol and then centrifuged for 1 min at 12,000 rpm. The single n-butanol phase was discarded and the oligonucleotide pellet was dissolved in 100 ul water.
Results

Cloning of a \( P \) element from \( mfs^2 \)

To determine the number of \( P \) elements in the \( mfs^2 \) \( b \) \( pr \) \( cn/CyO \) strain, high molecular weight genomic DNA was digested with \( Bam \) \( HI \) and hybridized with a 900 bp \( Hind \) III fragment cut from the \( P \) element in \( pn25.1 \) (Figure 10). \( Bam \) \( HI \) does not cut within the complete \( P \) element and thus, is a useful restriction enzyme for determining the total number of \( P \) elements present in a genome. Two hybridization signals are detected in the \( mfs^2 \) DNA which are not present in the control lane: a strong signal at 1.2 kb and a weak signal at 9.0 kb (Figure 10). Although, several repeats of this experiment have failed to detect any additional elements, the weak hybridization at 9.0 kb is reproducible.

Since the \( mfs^2 \) \( b \) \( pr \) \( cn/CyO \) strain contains only two detectable \( P \) element homologies, it was not necessary to remove elements from this stock by recombination. An EMBL 3 genomic library, was constructed from partially digested \( (Eco \) RI) \( mfs^2 \) \( b \) \( pr \) \( cn/CyO \) DNA and was screened with the 900 bp \( Hind \) III \( P \) element probe described above. Out of 250,000 plaques screened, four positives were and plaque-purified through successive rounds of hybridization. DNA from the four \( \lambda \) clones was digested with \( Eco \) RI (Figure 11a), and hybridized to the 900 bp \( P \) element fragment used to screen the library (Figure 11b). Three of the four clones (\( \lambda2, \lambda3 \) and \( \lambda4 \)) have similar \( Eco \) RI restriction digest patterns and contain a 1.5 kb fragment which hybridizes strongly to the \( P \) element probe. The fourth clone (\( \lambda1 \)), has a different \( Eco \) RI restriction digestion
Figure 10: mfs\(^2\) b pr cn/CyO genomic DNA hybridized with the 900 bp Hind III fragment from \(\pi\)25.1.

<table>
<thead>
<tr>
<th>Lane</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\lambda)</td>
<td>mfs(^2) b pr cn/CyO</td>
</tr>
<tr>
<td></td>
<td>Hind III</td>
<td>Hind III</td>
</tr>
</tbody>
</table>

1  2

8.5 kb

1.5 kb
Figure 11: Bacteriophage λ clones hybridized with the 900 bp Hin dIII fragment from π25.1.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Clone</th>
<th>Restriction Enzyme</th>
</tr>
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<td>Hin dIII</td>
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<tr>
<td>2</td>
<td>λ1</td>
<td>Eco RI</td>
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<tr>
<td>3</td>
<td>λ2</td>
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</tr>
<tr>
<td>4</td>
<td>λ3</td>
<td>Eco RI</td>
</tr>
<tr>
<td>5</td>
<td>λ4</td>
<td>Eco RI</td>
</tr>
</tbody>
</table>

A. Photograph of ethidium bromide stained gel.
B. Photograph of autoradiogram.
pattern and contains a weak P element homology in an 8.5 kb fragment.

In situ hybridizations were done to Oregon-R salivary gland polytene chromosomes using probes made from the full length λ1 and λ2 clones. The λ2 probe hybridizes to the base of chromosome arm 2R (data not shown), while the λ1 probe hybridizes to subdivision 31E on chromosome arm 2L (Figure 12), the known cytological location of the mfs(2)31 locus. Since the λ1 probe does not hybridize to any other site in the Drosophila genome, I presume that the λ1 clone contains the P element which is inserted into 31E in the mfs2 strain (see Chapter 3).

**Mapping λ1.**

To facilitate the mapping of the P element in 31E relative to transcripts in the region, a restriction map was generated of λ1. Overlapping fragments from λ1 were subcloned into pUC19 and mapped using a battery of seven restriction enzymes: Pst I, Xba I, Sal I, Eco RI, Sst I, Hind III and Bam HI. Figure 13 describes the subcloned fragments from λ1 while Figure 14 contains the composite restriction map generated from these constructs. Regions of overlap between subclones and any mapping ambiguities have been resolved and/or verified by hybridization.

To determine the orientation of the P element with respect to the previously cloned da and cdc2 genes, subcloned fragments from λ1 were hybridized with probes made from the full length JT31 and JT35 constructs (Figure 15). JT31 and JT35 are overlapping
Figure 12: Localization of $\lambda_1$ to subdivision 31E by *in situ* hybridization.
Figure 13: Restriction maps for various fragments from lambda 1. All fragments have been cloned into Puc 19: \( R = Eco R1 \), \( S = Sal I \), \( B = Bam HI \), \( T = Sst I \), \( P = Pst I \), \( X = Xba I \), \( H = Hind III \).
Figure 14: Composite restriction map for Lambda 1. The lines at the top of the figure indicate the alignment of the various subcloned fragments. The thick bar indicates the region of $P$ element homology: $R = Eco RI$, $S = Sal I$, $B = Bam HI$, $T = Sst I$, $P = Pst I$, $X = Xba I$, $H = Hind III$. 

---

$1 kb$
Figure 15: Overlapping subcloned fragments from λ hybridized with B. JT31 and C. JT35.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Fragment</th>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>λ</td>
<td>Hind III</td>
</tr>
<tr>
<td>2</td>
<td>pB1.5</td>
<td>Bam HI</td>
</tr>
<tr>
<td>3</td>
<td>pR8.5</td>
<td>Eco RI</td>
</tr>
<tr>
<td>4</td>
<td>pP6.0</td>
<td>Pst I</td>
</tr>
<tr>
<td>5</td>
<td>pS5.0</td>
<td>Sal I</td>
</tr>
<tr>
<td>6</td>
<td>pB2.8</td>
<td>Bam HI</td>
</tr>
</tbody>
</table>

B (JT31)

C (JT35)
cosmids, which both encompass the da and cdc2 genes: JT31 extends 10 kb more proximally while JT35 extends 15 kb more distally (Clegg, 1992). The orientation of the two cosmids with respect to the centromere is inferred from the mapping experiments described in Chapter 2 which place the da gene proximal to the cdc2 gene. The plasmids pB2.9, pS5.0, pP6.0, pR8.5 and pB1.5 contain overlapping fragments which completely span λ1 (restriction maps are provided in Figure 13). While there is no homology between JT35 and these λ1 fragments (Figure 15b), JT31 does overlap, extending into the 6.0 kb Pst 1 fragment (pP6.0) which contains the P element homology (Figure 15c). This would indicate that the molecular order of the three genes is, moving distal to proximal, cdc2-da-mfs(2)31 (Figure 16), thus confirming the recombination mapping in chapter 2.

The P element homology in λ1 is completely contained within an 8.5 kb Eco RI (pR8.5) fragment. To further localize the P element sequences, this fragment was digested with Hind III and hybridized to the full length pn25.1 plasmid (Figure 17). The P element homology in pR8.5 is contained within a 1.0 kb Hind III fragment. The position of this fragment is indicated in Figure 14. Since a full length P element is 2.9 kb in length, the element in 31E must be degenerate.

To determine whether the P element homology in λ1 corresponds to a mobilizable element, high molecular weight genomic DNA was extracted from the following strains; b pr cn/CyO, mfs2 b pr cn/CyO, mfs mfs2 b pr cn/CyO, mfs mfs2 b pr cn/CyO, mfs mfs
Figure 16: Overlapping clones in subdivision 31E. The precise extent of overlap between JT31 and Lambda 1 has not been determined and thus is indicated with a dashed line. The location of cloned genes within the constructs is indicated. The orientation of the clones with respect to the centromere is inferred from the recombination mapping described in chapter 2.

\[ \text{cdc2} \quad \text{da} \quad \text{mfs(2)31} \]

JT35

JT31

Lambda 1

Centromere
b pr cn/CyO and mfs\textsuperscript{R5} b pr cn/CyO. The mfs\textsuperscript{R1}, mfs\textsuperscript{R2}, mfs\textsuperscript{R3}, mfs\textsuperscript{R4} and mfs\textsuperscript{R5} strains are hybrid dysgenesis induced revertants of the mfs\textsuperscript{2} mutation (see Chapter 3). Genomic DNA from each of these strains was doubly digested with Eco RI and Xba I and hybridized with the 4.0 kb Eco RI/Xba I fragment cut from pR8.5 (Figure 18). This fragment spans the region of \textit{P} element homology in \lambda l (see Figure 14). While this probe hybridizes to a single 4.0 kb band in the b pr cn/CyO control DNA (Lane 1), it hybridizes with equal intensity to bands of 4.0 kb and 4.5 kb in the mfs\textsuperscript{2} b pr cn/CyO DNA (Lane 2). This polymorphism could indicate the loss of either the Eco RI or Xba I sites on the mfs\textsuperscript{2} chromosome or alternatively, it could indicate the presence of a 500 bp duplication or insertion. Since the polymorphism is lost in all five revertant strains (Lanes 3-7), I feel that this result indicates the presence of a 500 bp mobilizable \textit{P} element contained within \lambda l.

**Transcriptional analysis of \lambda l and the mfs(2)31 locus.**

To identify transcripts which the inserted \textit{P} element may be interfering with, all the embryonic messages encompassed by \lambda l were mapped. Poly(A)\textsuperscript{+} RNA was isolated from 0-6 hr wild-type embryos and probed with contiguous fragments from \lambda l. The results of results of the transcriptional analysis are summarized in Figure 19. In cases where hybridization failed to detect any message, the filters were re-probed with pDmA2 (actin) to ensure that the transfer of the RNA was successful.

As indicated, a large number of embryonic transcripts were
Figure 17: Digested 8.5 kb Eco RI fragment from λ1 hybridized with pπ25.1.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Sample</th>
<th>Restriction Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>λ</td>
<td>Hind III</td>
</tr>
<tr>
<td>2</td>
<td>pR8.5</td>
<td>Eco RI</td>
</tr>
<tr>
<td>3</td>
<td>pR8.5</td>
<td>Eco RI/Hind III</td>
</tr>
<tr>
<td>4</td>
<td>pR8.5</td>
<td>Hind III</td>
</tr>
</tbody>
</table>

A. Photograph of ethidium bromide stained gel.
B. Photograph of autoradiogram.
Figure 18: *mfs(2)31 b pr cn/CyO* genomic DNA hybridized with a 4.0 kb *Eco RI/Xba I* fragment from λ1.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Lineage</th>
<th><em>Eco RI/Xba I</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>b pr cn/CyO</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><em>mfs² b pr cn/CyO</em></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td><em>mfs³ b pr cn/CyO</em></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td><em>mfs⁴ b pr cn/CyO</em></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td><em>mfs⁵ b pr cn/CyO</em></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td><em>mfs⁶ b pr cn/CyO</em></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td><em>mfs⁷ b pr cn/CyO</em></td>
<td></td>
</tr>
</tbody>
</table>

Lane 1: 4.5 kb 4.0 kb

Lane 2: 4.5 kb 4.0 kb
Figure 19: Transcriptional analysis of Lambda 1. The solid lines at the top of the figure represent the probes which were used to detect the messages indicated at the bottom of the figure. The sizes of the messages are indicated. The thick bar indicates the region of $P$ element homology. Abbreviations are the same as in Figure 14.
identified in this analysis. Of particular interest is the region in the immediate vicinity of the $P$ element insertion where no messages are detected within 7 kb of the distal end of the $P$ element containing fragment. The 1.5 kb Hind III/Eco RI fragment immediately proximal to the element detects 0.8 and 1.2 kb transcripts while the next most proximal fragment detects a 0.8 kb transcript only. It is possible that the 0.8 and 1.2 kb messages detected by the 1.5 kb Hind III/Eco RI fragment are differentially processed products of the same transcription unit while the 0.8 kb transcript detected by the 300 bp Eco R1 fragment is a separate message. Alternatively, there could be just one 0.8 kb message which lies proximal to the 1.2 but still encroaches into the 1.5 kb Hind III/Eco RI fragment. Although this analysis does not allow us to distinguish between these two possibilities, the 1.2 kb transcript lying immediately adjacent to the $P$ element insert is of particular interest.

The 1.5 kb Eco RI/ Hind III fragment, which is proximal to the $P$ insert, was used as a probe to screen a Drosophila disc cDNA library. Out of 250,000 bacteriophage screened, eight positive plaques were chosen at random and purified by successive rounds of amplification and screening. These cDNAs range in size from 0.8 kb to 1.2 kb (Figure 20a) and cross-hybridize with the 1.5 kb Eco RI/Hind III fragment which was used to screen the library (Figure 20b) as well as the largest cDNA: cDNA 6 (Figure 20c).

cDNA 6 was used as a probe to hybridize to Poly(A)$^+$ wild-type RNA from 0-6 hour embryos (Figure 21). Since this cDNA only
Figure 20: Putative mfs(2)31 cDNAs hybridized with B. pH1.5 and C. cDNA6.

<table>
<thead>
<tr>
<th>Lane</th>
<th>λ</th>
<th>Hind III</th>
<th>Eco RI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>λ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>cDNA1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>cDNA2</td>
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<tr>
<td>4</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>cDNA4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>cDNA5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>cDNA6</td>
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<td>8</td>
<td>cDNA7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>cDNA8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 21: Poly(A)+ RNA from 0-6 hour Oregon-R embryos hybridized with cDNA 6.
hybridizes to a 1.2 kb transcript, it is probable that the 0.8 and the 1.2 kb messages, detected by the 1.5 kb Eco RI/Hind III fragment, are adjacent transcripts rather than derivatives of the same transcription unit.

To determine if the 1.2 kb transcript which corresponds to cDNA 6 is disrupted in mfs(2)31 mutant strains, Poly(A)' RNA was isolated from all four alleles as well as the revertant strains mfsR1 and mfsR2. The RNA was hybridized to a probe made from the full length cDNA 6 (Figure 22a) and then the filter was stripped and reprobed with pDmA2 to verify equal loading (Figure 22b). In all cases but one, there is no significant alteration in levels of transcription of the 1.2 kb message nor are any additional messages detected. The single exception was in the mfs2 P element containing strain in which the level of transcription was significantly reduced. Since this reduction is not observed in either of the two revertant strains, I conclude that the P element in subdivision 31E is interfering with the expression of the adjacent 1.2 kb transcript.

To observe the developmental profile of the 1.2 kb transcript, Poly(A)' RNA was isolated from a number of developmentally staged Drosophila tissues: 0-2.5 hr embryos, 2.5-6 hour embryos, 6-9 hour embryos, 9-12 hour embryos, 12-24 hour embryos, 1st instar larvae, 2nd instar larvae, third instar larvae, early pupae, late pupae and adult males and females. Following hybridization with a cDNA 6 probe (Figure 23a), the filter was stripped and re-probed with pDmA2 (Figure 23b). The hybridization with pDmA2 reveals that
Figure 22: Poly(A)* RNA from the mfs(2)31 alleles hybridized with A. cDNA 6 B. pDmA2. RNA is from 0-6 hr embryos.

Lane 1  Oregon-R  
2  mfs^2 b pr cn/CyO  
3  mfs^4 b pr cn/CyO  
4  mfs^2 b pr cn/CyO  
5  mfs^1 b pr cn/CyO  
6  mfs^4 b pr cn/CyO  
7  mfs^2 b pr cn/CyO
Figure 23: Developmental profile of a putative mfs(2)31 transcript. Staged poly(A)+ RNA hybridized with A. cDNA 6 B. pDmA2. 1. 0-3 hr 2. 3-6 hr 3. 6-9 hr 4. 9-12 hr 5. 12-24 hr 6. 1st Instar 7. 2nd Instar 8. 3rd Instar 9. Early pupae 10. Late pupae 11. Adult male 12. Adult female.
there is a great deal of variability in the loading of this gel and thus, little can be said about the relative levels of expression of the 1.2 kb transcript. Nonetheless, it is clear that a single 1.2 kb message is expressed in all stages of development including the adults of both sexes. The expression in the larvae and the adults is consistent with the larval lethal period and adult function which have been demonstrated for mfs(2)31 (see Chapter 3).

**Sequencing mfs(2)31**

Since cDNA 6 hybridizes to sequences adjacent to the site of the $P$ element insertion and is the most likely candidate for the transcription unit which encodes mfs', it was sequenced in its entirety. Initially, the ends of all 8 cDNAs were sequenced to determine their alignment. All have identical 3' ends but differ at their 5' ends (data not shown) which indicates that the shorter cDNAs are probably not full length. cDNA 6 is 1230 bp long (Figure 24) and contains a single long open reading frame which encodes a protein 259 aa in length (30 kDa). There is a 400 bp untranslated 5' leader which contains numerous stop and start codons in all three reading frames, and a short 3' untranslated sequence (70 bp) which contains both a polyadenylation signal and a poly(A) tail.

The 30 Kd protein predicted by the cDNA 6 sequence is quite basic with 66 of the 259 residues being either lysines or arginines. Beginning at the amino terminus, the basic residues are dispersed somewhat randomly throughout the first 180 aa. The protein sequence also contains nine potential phosphorylation sites...
Figure 24: The complete sequence of cDNA 6. The amino acid sequence of the mfs(2)31 gene is presented above the nucleotide sequence beginning at +384 and ending at +1160. The polyadenylation signal is underlined and potential sites of phosphorylation are indicated by asterisks.

AAGGTAAGTTTTTGGGGTTGTTACATTTTAAGCATCGGTTAAGTGAAAATAAACATACAAATACATACTTAAAGTAGTCCTTAACACTTG^90
TAAACTCTTGAGATGAAATTACATGTACATTGTTGATTGAACTGTAATGTTATTTAAAAATTTCACTAAACTTATGAAAAATTTACTTTTTTGC
AAACTCTTGAGATGAAATTACATGTACATTGTTGATTGAACTGTAATGTTATTTAAAAATTTCACTAAACTTATGAAAAATTTACTTTTTTGC
^180
TCTTAAAAAGTGCCGCATGAACTGGAATACTTTTAAGCATCGGTTAAGTGAAAATAAACATACAAATACATACTTAAAGTAGTCCTTAACACTTG
^270
GATCGCAGGTGCTCGGGTTCGAAGGAAAACAAAAGTGTGCGATTTAAAAATTTCACTAAACTTATGAAAAATTTACTTTTTTGC
^360
M F Q H Y N E R H R K R K L Y S R R L D Y F E
^450
GCTTAAGATAATTAAAATTTAACCGCAACATCGGTTAAGTGAAAATAAACATACAAATACATACTTAAAGTAGTCCTTAACACTTG
^540
R K R K E A R L R G R R L D Y E E
^630
GCTTAAGATAATTAAAATTTAACCGCAACATCGGTTAAGTGAAAATAAACATACAAATACATACTTAAAGTAGTCCTTAACACTTG
^720
G A L P H Y L L D R G I O S S A K V L S N M I K Q K R K E K
^810
GCTTAAGATAATTAAAATTTAACCGCAACATCGGTTAAGTGAAAATAAACATACAAATACATACTTAAAGTAGTCCTTAACACTTG
^900
^990
GCTTAAGATAATTAAAATTTAACCGCAACATCGGTTAAGTGAAAATAAACATACAAATACATACTTAAAGTAGTCCTTAACACTTG
1170
G K V V W G K Y A Q V T N N P E N D G V I N A V L L V
^1230
GCTTAAGATAATTAAAATTTAACCGCAACATCGGTTAAGTGAAAATAAACATACAAATACATACTTAAAGTAGTCCTTAACACTTG

158
for either protein kinase C or casein kinase II, which are randomly dispersed throughout its length. Although this protein has no similarities with any previously described *Drosophila* genes, a comparison of the predicted protein sequence with sequences in the EMBL/Genbank data base suggests that cDNA 6 may encode a microtubule associated protein (Figure 25). There is 26% similarity between cDNA 6 and mouse microtubule associated protein 1B (MAP1B) over a 130 amino acid stretch. The similarity with MAP1B is in a very basic domain which contains many copies of the sequence KKEE and KKEI/V at randomly dispersed intervals. Since this segment is responsible for the binding of MAP1B to microtubules (Noble et al., 1989), it may indicate an equivalent function for the cDNA 6 encoded protein. Alternatively, since the two sequences are both rich in basic residues, this similarity may simply be fortuitous.

Although there is no similarity between the cDNA 6 encoded protein and any non-histone chromosomal proteins, it has mild similarity (22% over 60 aa) in its carboxy terminus with the mammalian histone H1 protein. Even though histone H1 is also a very basic protein, this similarity is not attributable to random matches of basic residues. Although this similarity is somewhat limited, it is notable because of the *su(var)* phenotype of *mfs(2)31* mutations.

To determine the location of the *P* element insert with respect to the transcription unit identified by cDNA 6, the ends of the 1.0 kb *Hind* III (pH1.0) and the 1.5 kb *Hind* III/Eco RI (pH1.5) genomic
Figure 25: Similarities with the deduced protein sequence of \textit{mfs(2)31}. In each case, the upper sequence corresponds to \textit{mfs(2)31}. Perfect matches are indicated by double dots while conserved changes are indicated by single dots.

**MOUSE MICROTUBULE-ASSOCIATED PROTEIN: MAP1B**

26.2\% identity in 130 aa overlap

<p>| | | | | | | | | | |</p>
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**MAMMALIAN HISTONE H1**

21.7\% identity in 60 aa overlap

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| 40 | 50 | 60 | 70 |
| PPVSELITKAVAAASKERSGVSLAAAKLAAAGYVEKNSRIKLGLAALVSGLVSIQT |

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160
fragments were sequenced. The Hind III site, which is shared by these adjacent fragments, corresponds to the internal Hind III site which is found 75 bp from the end of intact P elements. Thus, the Hind III end of the 1.5 kb fragment begins with the terminal 75 bp of a P element sequence. Beginning 37 bp downstream of this element are sequences which are identical to the 5' end of cDNA 6 (Figure 26). Partial sequencing of the 1.0 kb Hind III fragment revealed that the P element is approximately 600 bp in length and contains all of the sequences which are considered necessary for transposition (Figure 27). Although the element has not been sequenced in its entirety, it contains an internal tract of A's and T's, at least 85 bp in length, which is not P element sequence.

To determine the structure of the mfs(2)31 transcription unit, a wild-type copy of the gene is required. The 1.5 kb Hind III/Eco RI fragment from pH1.5 was used as a probe to screen a cosmid library. Out of 25,000 colonies screened, a single cosmid was isolated and purified: cos50. If cos50 DNA is digested with Bam HI and Sal I and hybridized with the 1.5 kb Hind III/Eco RI fragment used to screen the library, the pattern of hybridization is the same as what would be predicted from a digestion of λ1 (compare Figure 14 with Figure 28). In addition, the hybridization of a series of overlapping fragments which span λ1 with a probe made from the complete cos50 construct indicates that these two clones are collinear (Figure 29). A 1.9 kb Eco RI/Hind III fragment, which hybridizes to cDNA 6, was subcloned out of cos50 into pUC19. This fragment, which was sequenced in its entirety (Figure 30),
Figure 26: The relationship between the P element insert and the 5' end of cDNA 6 in mfs². Compressed lettering indicates sequences which are identical to the 5' end of cDNA 6. P element sequences are underlined while double underlining indicates a reasonably good (5/7) insect cap site.

AGCTTCGGCT_TTCGACGGGA_CCACCTTATG_TTATTTCATC_ATGGTGTTT_CTTCCTTTCC
TTTGATTCTG_TTGCCGAAGC_AAGCTAAAGTTTTGCTGTTTACATTTTAAACATCGTTT
Figure 27: Partial sequence of the 1.0 kb Hin dIII from the plasmid pH1.0. Single underlining indicates P element sequences while double underlining indicates the non P element sequences which are within the insert.

CAGGCATCAA GCTTGTCAGG AGTGCTACCT GGATTTGGTA TTTTAATTTG TAAGAGTATT
AACAAATTAT CCTAGAACTT AAATTTGTAT ACATGTTTTT ATTTAAATAGG CGGATTTGGA
GCATCGCTTC TACTTTGTG CTTGGAAAAA GTATATGCAC ATTTTGGACC ACGAAAAATT
TACGGTTTTT AAATTTTTAT TTTTTGTTC CTTATGATG ATTTTTTTTT GATTTGTTAT
ATTTTATAAT TTTTGAATAA AGGAAAAAGT TATTTGATAT GGCTTCTGGT TCATCCAGT
TTATGTCCA TTCCACGCGT TCCAAAAATA AACGGTAGT TCAAAATATA AACAAAAAGTA
AACTCCGAAT GCAGCCCTGC GCTCAATTTT TCACTTCTCT GTCACGCTGT GTGTGTGTTC
CATGATGAAA TAACTAAAGG TGGTCCCAGTC GGCAAGAGAC ATCCACTTAA CGTATGCTTG
CAATAAGTGTC GAGTGAAGGG AATAGTATTC TGAGTGTCTGT AGTGAGTCTG AGTGAACAG
C.................. .................. .................. .................. .................. .................. ATATAA AATTATAAAT TTTATAAAT
TTAAAAATTA TATATTAAA TATTATTAA AATATATTAT ATTATATATA AAATATATTT
ATATTTTTAT TTTTTTTATT CCAGTAAGG GTTAATGGTTT CAAAAAAA ATTCTTCGCG
ACACAACCTT CCTCTCAACA AGCAAACGTG CACTGAATTTA AGTGATAC TTCGG

163
Figure 28: cos50 DNA hybridized with pH1.5.

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<tr>
<td>2</td>
<td>cos50</td>
<td>Bam HI</td>
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<tr>
<td>3</td>
<td>cos50</td>
<td>Sal I</td>
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A. Photograph of ethidium bromide stained gel.
B. Photograph of autoradiogram.
Figure 29: Overlapping fragments from λ1 hybridized with cos50.

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<td>5</td>
<td>pR8.5</td>
<td>Eco RI</td>
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<tr>
<td>6</td>
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<td>Bam H1</td>
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A. Photograph of ethidium bromide stained gel.
B. Photograph of autoradiogram.
Figure 30: The complete genomic sequence of the region encompassing the gene represented by cDNA 6. The sequence corresponding to cDNA 6 is underlined.

TAGAGTGACTGAGCGCTCAAGCTGCAAGGTTCGATGATTTGGATTTTATGTTACGCTGATTTTATGGGATATTTATGGAGCTGGAGATTTTGCTGTTATTTTATTTTATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
corresponds to the 1.5 kb Eco RI/Hind III and 1.0 kb Hind III fragments from λ1 (minus the P element insert). A comparison of the sequences of these three fragments indicates that the P element insertion is not associated with any rearrangement of genomic DNA. Comparing the sequence of cDNA 6 with this 1.9 kb fragment indicates that the transcription unit does not contain any introns.

The 5' end of the gene was examined for potential regulatory sequences (Figure 31). Although there is a good consensus sequence for an insect cap site at -16 from the 5' end of cDNA 6 (ATTCTGTT), the closest TATA box is at -103 (TATAAA). The TATAAA sequence falls within an imperfect (13/15) 15 bp direct repeat whose function is not known. If these are the promoter sequences of mfs(2)31, then cDNA 6 may not be full length and the P element may have inserted into the 5' untranslated leader sequence of the transcription unit. Although mfs2 is a hypomorphic allele and thus should be producing some functional message, a larger transcript has not been detected in the transcriptional analysis. It is formally possible that there is a small intron, 5' to cDNA 6, into which the element has inserted. There are several splice donor sites immediately 5' to the element and two splice acceptor sites between the site of the insert and the 5' end of cDNA 6. If this transcription unit contains an additional 5' exon it would have to be small since the size of the message is virtually identical to the size of cDNA 6. Since the 5' untranslated leader contains numerous stop and start codons in all three frames, it is unlikely that such an exon would be coding. It is more plausible that cDNA
Figure 31: Potential upstream regulatory sequences of \textit{mfs(2)31}. The sequence corresponding to cDNA 6 is indicated by compressed lettering. Single underlining indicates an imperfect (13/15) 15 bp direct repeat which contains the closest TATA box (TATAA). Double underlining indicates a reasonably good (5/7) insect cap site consensus sequence. An asterisk indicates the site of the \textit{P} element insertion.
6 is full length and that the P element has inserted 5' to the site of transcriptional initiation. In doing so, it may be interfering with the regulatory sequences of this transcription unit.
Discussion

This chapter describes the use of a $P$ element induced mutation to clone and sequence the $mfs(2)31$ locus of *Drosophila melanogaster*. There are several reasons why we are confident that we have identified the correct transcription unit. First, we have shown that the cloned $P$ element is responsible for the $mfs(2)31$ phenotype associated with the dysgenic allele $mfs^2$. Hybrid dysgenesis induced revertants of this allele are invariably associated with the excision of the cloned element. Second, the element has inserted immediately 5' to the transcriptional start site of a 1.2 kb message and reduces the level of this transcript. A transcriptional analysis of the region has failed to detect a message within 7 kb of the proximal end of the insert. Finally, although it is formally possible that the $P$ element is interfering with a second, more distant message, the cytogenetic analysis described in chapter 2 provides no evidence that the $mfs^2$ allele may be a double mutation. Although the 1.2 kb message is a strong candidate for the $mfs(2)31$ transcription unit, a formal proof would require either the sequencing of other $mfs(2)31$ alleles or a rescue of the mutant phenotype by $P$ element mediated germline transformations. Additional $mfs(2)31$ mutant alleles are being sequenced to verify the identity of this transcription unit.

A comparison of cDNA sequences with the corresponding genomic sequences from a wild-type and the $mfs^2$ strain indicate that the $P$ element has inserted 37 bp upstream of the transcriptional start site. Although this localization is based on the assumption that
the longest cDNA isolated (cDNA 6) is full length, there are several reasons why we feel that this assumption is correct. First, cDNA 6 is identical in size to the only transcript detected at this locus. Second, a transcriptional analysis in the region has failed to detect any messages within seven kb of the distal side of the insert. Finally, the apparent insertion of the element into 5' regulatory sequences is consistent with the transcriptional profile of \textit{mfs}^2\textit{b pr cn/CyO} heterozygotes. Although the amount of the 1.2 kb message is substantially reduced in this strain, no aberrant messages have been detected. This is particularly significant since the junctions of the \textit{mfs}^2 insertion do not conform to the eukaryotic splice consensus sequence making it unlikely that the element would be precisely excised during RNA processing.

The insertion of a \textit{P} element into the regulatory region of \textit{mfs}^2 is consistent with the genetic analysis of this mutation which shows that it has reduced, but not a zero level of function. However, one would not necessarily predict that a regulatory mutation would have a temperature-sensitive phenotype. An examination of the sterility associated with \textit{mfs}^1/\textit{mfs}^2 transheterozygotes revealed that the phenotype is temperature sensitive with a sensitive period which extends into adulthood. Based on the transcriptional analysis of these respective alleles, it now seems more likely that this temperature-sensitivity is attributable to the \textit{mfs}^1 mutation. The pattern of expression of the 1.2 kb transcript is unaltered in the \textit{mfs}^1 mutation, which
makes it more likely that it contains a lesion in the coding region of the gene.

The \textit{mfs(2)31} transcript is present at all stages of development as well as in adults of both sexes. The presence of the \textit{mfs(2)31} transcript in the larvae, pupae and adults was anticipated from the previous genetic characterization of the locus since zygotic \textit{mfs(2)31} functions have been demonstrated during all of these stages of the \textit{Drosophila} life cycle (Chapter 3). In contrast, the presence of an embryonic \textit{mfs(2)31} transcript is somewhat surprising since the larval stage is the earliest period for which we have demonstrated an essential function for the locus. It is possible that the gene product does not have an essential function during embryonic development or alternatively, the embryonic function may be under maternal control. This latter possibility is supported by our detection of large amounts of \textit{mfs(2)31} transcripts in 0-2.5 hr embryos, transcripts which are unlikely to have been the product of zygotic transcription (Anderson and Lengyel, 1979; Edgar and Schubiger, 1986).

A comparison of \textit{mfs(2)31} genomic and cDNA sequences indicate that the structure of this locus may be fairly simple. There is a single message detected at this locus and there are no introns in either the protein coding sequences or the 5' untranslated leader. Although the lack of complexity of the \textit{mfs(2)31} locus at the molecular level appears to be inconsistent with the many developmental functions of the gene, it should be noted that the analysis of the four mutant alleles has suggested little, if any,
genetic complexity at this locus (Chapter 3). These mutations fit into a relatively straightforward allelic series of increased severity. Hypomorphic alleles which exhibit bristle and sterility phenotypes, also exhibit the essential larval function as hemizygotes which is observed in the homozygotes of stronger alleles. Thus, while the \textit{mfs(2)31} locus clearly is required for a variety of developmental events, our molecular analysis suggests that these various functions may be mediated by the same gene product.

What are these various functions of the \textit{mfs(2)31} locus? The best described of all its functions is its requirement for the maturation of the adult reproductive systems. Male sterility has been correlated with a number of spermatid defects, all of which can be explained by abnormal centriole behavior (Lindsley \textit{et al}., 1980), while female sterility has been correlated with a delay in the onset and completion of vitellogenesis (Chapter 3). \textit{mfs(2)31} also has a pupal function necessary for bristle elaboration and an essential function during larval development. We have been primarily interested in \textit{mfs(2)31} because of its \textit{su(var)} phenotype. Surviving allelic combinations suppress position-effect variegation in a variety of variegating backgrounds. It has long been thought that genes which can be mutated to modify PEV will encode proteins which are required for the assembly and/or maintenance of chromatin structure.

We have previously proposed that the variety of phenotypes associated with \textit{mfs(2)31} mutations are consistent with a defect in
chromosome behavior, either during or subsequent to cellular division (see Chapter 3). Such a defect could not only interfere with heterochromatin assembly, thus producing a su(var) phenotype, but it could also prevent a cell from achieving the maximum rates of protein synthesis which are required for bristle elaboration, vitellogenesis and embryonic development, since all of these processes depend on an expedient switch from a mitotic to an endomitotic cycle. A defect associated with nuclear architecture would also account for the abnormal nuclear morphology and centriole behavior observed during spermatid development.

The predicted amino acid sequence for the cDNA 6 encoded protein is not particularly informative with regard to specific biochemical activities that the mfs(2)31 product may have. However, it is intriguing that the only meaningful similarity we have discovered for this protein is to the predicted product of the mouse MAP1B gene (Noble et al., 1989). MAP1B encodes a microtubule associated protein which has been identified in mouse brain tissue. Although the function of brain MAPs is essentially unknown, phosphorylated MAP1B may play a role in the cytoskeletal changes that accompany neurite extension. MAP1B is thought to bind to at least two tubulin subunits in the microtubule polymer, and this bridging of subunits might be involved in either nucleating microtubule polymerization or the stabilization of the structure. The region which is responsible for the binding of MAP1B to microtubules is a highly basic sequence with many copies of the motifs KKEE and KKEI/V which are repeated but not at fixed
intervals. This is the region with which the highly basic mfs(2)31 protein product shares its greatest similarity. Although the similarity between MAP1B and mfs(2)31 may be fortuitous, it is tempting to consider that the sequence similarity could be related to the fact that both genes are involved in binding to microtubules, a function which would be consistent with a role for the mfs(2)31 product in the control of chromosomal movement and structure. A possible nuclear function for the mfs(2)31 gene product related to chromosomal behavior is further suggested by a weak homology in the carboxy terminal of the protein with mammalian histone H1, a protein which is required for the condensation of the 10 nm chromatin fiber into the more compact 30 nm structure.

It has been proposed that dominant Su(var) mutations will identify genes which encode structural components of heterochromatin. It would seem apparent that any protein which functions in some aspect of chromosomal behavior could be required for expedient chromatin assembly, and thus could be mutated to modify PEV. Such genes would differ from the more typical modifiers of PEV in that they are unlikely to exhibit dominant phenotypes. We feel that it is possible that the mfs(2)31 locus encodes a protein which associates with and/or controls the behavior of chromosomes during the cell cycle. If so, it may extend the usefulness of PEV from an assay for genetically dissecting chromatin, to an assay for genetically dissecting the nuclear architecture.


Dorbic, T., and B. Wittig (1987). Chromatin from transcribed genes contains HMG17 only downstream from the starting point of transcription. EMBO J. 6, 2393-2399.


Hartmann-Goldstein, I.J. (1967). On the relationship between heterochromatization and variegation in *Drosophila*, with special


184


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187


