This is to certify that the

dissertation entitled

GENETIC CHARACTERIZATION OF THE 27F;28D3-4

REGION OF DROSOPHILA MELANOGASTER

presented by

ROBIN ADELE STEINMAN WAGNER

has been accepted towards fulfillment
of the requirements for

DOCTORAL degree in GENETICS

Major professor

Date 8-8-97

GENETIC CHARACTERIZATION OF THE 27F;28D3-4 REGION OF *DROSOPHILA MELANOGASTER*

By

Robin Adele Steinman Wagner

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Genetics Graduate Program

1997

ABSTRACT

GENETIC CHARACTERIZATION OF THE 27F;28D3-4 REGION OF *DROSOPHILA MELANOGASTER*

By

Robin Adele Steinman Wagner

wingless (wg), a segment polarity gene on the second chromosome of Drosophila melanogaster, codes for a secreted protein with multiple roles throughout development. It has been localized to the cytological region 28A. During complementation analysis among loci that map within the 28A region, a complicated pattern of overlapping noncomplementation has been observed that includes alleles of *wg, Sternopleural (Sp), neither-inactivation-nor-afterpotential C (ninaC),* and *spade (spd)*.

One specific interaction between the dominant bristle mutation *Sp* and wg^{cx4}, an amorphic allele of *wg*, results in crinkled wings. Two hypotheses can be invoked to explain the genetic interaction:

1.) *wg* and *Sp* code for interacting proteins

2.) *wg* and *Sp* are alleles of the same gene

An attempt to isolate a *cis* double mutant to distinguish between these possibilities was forestalled by the additional phenotypes of male and female sterility and reduced viability. The processes of oogenesis and spermatogenesis are markedly disparate in *Drosophila melanogaster*, and mutants that exhibit sterility in both sexes are relatively rare. This novel sterility

phenotype may provide insight into a previously unidentified role for *wg*.

The temperature sensitive allele, wg^{IL114}, when in trans with *Sp*, mimics the crinkled wing phenotype of the wg^{cx4}/Sp heterozygote at restrictive temperature. At permissive temperature the heterozygote retains fertility, and may be useful for generating a cis double mutant.

for Megan, Jerry and the others

ACKNOWLEDGMENTS

I'd like to thank my committee members, namely Dr. Tom Friedman, Dr. Lenny Robbins, Dr. Ellen Swanson, Dr. Larry Snyder and Dr. Wendy Champness. They have passed on to me an appreciation for genetics done well. A special thanks to the late Dr. James H. Asher, Jr., who was always interested in hearing about my flies, even though their hearing was fine. The past members of the Friedman and Robbins lab groups, especially Dr. Jean Burnett, Dr. Lori Wallrath, Dr. Rob Morell, Dr. Peter Crawley, and the soon-to-be Dr. Mark Thompson have provided valuable support and feedback, as have the Genetics Program staff members Barb, Jeannine and Kim.

I'd like to thank the hundreds of students who became a reason to finish.

A special thanks goes to Susan and Jon Stoltzfus, who have been there to aid and support when there was no one else.

Nora and Ed, who took care of Megan so I could take care of this.

The faculty and staff at LCC.

My parents, brother, and other extended family.

And, of course, Megan and Jerry.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

Introduction

Since the early part of this century researchers have been constructing comprehensive genetic maps in a diverse set of diploid organisms by measuring numbers of meiotic crossover events between chromosome markers (Sturtevant, 1913). These linkage maps are useful for determining the linear arrangement and location of genes, although having no basis in a physical entity, such as bands or nucleotides.

Restriction maps based on nucleotide base pairs have a more recent advent, dating to the ability to experimentally manipulate DNA in the laboratory. The use of restriction endonucleases to construct restriction fragment maps (Danna and Nathans 1971, Nathans and Smith 1975), and the technology to sequence stretches of DNA (Maxam and Gilbert 1977, 1980, Sanger et al. 1977, Smith 1980), have collectively enabled the generation of nucleotide maps of small areas. The labor intensive techniques available have limited the size of these maps to a few million base pairs surrounding cloned genes of interest.

Being able to reconcile data from relative genetic and absolute sequence maps in any region has been a desire of researchers working with various model organisms for years. The practical advantage that would be conferred with the ability to translate biological information from one type of map to another has long been recognized, but no generally applicable rules for the conversion exist. Researchers using *Drosophila melanogaster* as a model organism have the enviable advantage that, like many dipterans, *D. melanogaster* are remarkably amenable to cytogenetic analyses. The visible banding pattern of the giant polytene chromosomes has been critical in the formation of a third map in *D. melanogaster*, the cytogenetic map, which has been a useful framework on which to correlate both genetic and physical data.

It was with an eye toward reconciling the genetic, nucleotide, and cytogenetic maps in a small[1], well-characterized region of chromosome 2 that this research project was initiated, with the expectation that any trends uncovered in the short region between 28A1-28D1 might provide insight into the organization of the euchromatic regions of *D. melanogaster*. The specific

[1]The total number of *Drosophila melanogaster* genes is estimated to be between 5,000 and 20,000 (Lefevre and Watkins 1986) distributed along 165 Megabases of DNA, 120 Megabases of which are euchromatic (Rasch et al. 1971). 120 Mbase of euchromatic DNA, distributed between 120 major numbered regions, suggests that each region contains approximately 1 Megabase of DNA. In Region 28, this 1 Megabase is distributed between 6 major bands and the surrounding faint bands and interband regions. Estimates of the mass distribution vary from 74% (Laird 1980, Kress et al. 1985) to 95% (Beermann 1972, Sorsa 1982, Sorsa 1988) of the DNA being contained in major bands.

original goals of the project were to:

1.) Conduct a saturation mutagenesis of the region 28A1-28D1.

2.) Compile a gross restriction map of the same region.

3.) Use *in situ* hybridization to place restriction fragments on the cytological map.

Uncovering a Complex Genetic Region

This investigation of the 28A1-28D1 cytological region of the second chromosome consisted of a formal genetic analysis (with a limited molecular analysis of the region, described in Appendix A, also conducted). The genetic analysis was initiated by an attempt to saturate the region for lethal genes. 99 lethal mutations were isolated within 27F3-4;28D3-4 and overlapping deficiencies were used to delimit 55 of these (set A) to the region 27F3-4;28B, 5 (set B) to 28B;28C, and 36 (set C) to 28C;28D3-4. The A set of mutants consists of 49 alleles of *Sp* (*Sternopleural*), four new alleles of *wg (wingless)*, and a third complementation group with one member. Further investigations of this set disclosed a complex genetic region. An intricate pattern of overlapping non-complementation exists between lethal and morphological alleles of several markers, including *spd (spade), wg*, and *Sp* (Tiong and Nash 1990, Neumann and Cohen 1996). These loci are all tightly linked, and have been cytologically localized to 28A.

Complementation analysis between *Sp* and wg^{cx4}, a null allele of *wg*,

revealed a genetic interaction -- the *trans* heterozygotes have a crinkled wing phenotype not seen in either mutation alone. Experiments aimed toward elucidating the underlying mechanisms of the complicated genetic relations near the *wg* locus included an attempt to isolate a *cis* double mutant which would distinguish whether the new phenotype was a result of an interaction at the nucleotide or protein level. During this analysis a second novel phenotype of the *Sp/wg trans* heterozygote appeared, male and female sterility. Oogenesis and spermatogenesis occur by vastly different mechanisms in *D. melanogaster*, and mutants exhibiting both male and female sterility are rare. This intriguing phenotype may provide an interesting avenue for further exploration.

Background

Cytogenetic Maps

In the larval salivary glands of *Drosophila melanogaster* about ten rounds of chromosome replication, without cytokinesis, result in giant polytenized chromosomes. Each homologous euchromatic pair of arms is held tightly in register, emanating from a chromocenter consisting of the heterochromatic centromeric regions and the Y chromosome. The euchromatic regions are characterized by the appearance of distinctive bands, first identified and described using conventional light microscopy by C. B. Bridges (Bridges 1935, 1938) and refined by P. N. Bridges (Bridges 1939, 1941a, 1941b, 1942).

Chromosome arms X, 2 and 3 have been divided into 20 divisions, and chromosome 4 has three divisions. The most readily apparent bands in each division are lettered, with number and letter designations given to smaller bands for increasingly fine levels of resolution.

In *D. melanogaster* the finely developed cytogenetic map has historically been a useful framework to correlate genetic and physical data. For the former, markers can be placed on the cytogenetic framework by crossing flies carrying the marker to flies whose chromosomes have abnormalities with recognizable breakpoints (typically deficiencies and duplications) and characterizing the phenotype of the resultant F1 progeny. For example, an F1 heterozygote carrying a deficiency on one chromosome, and a recessive mutation that falls within the confines of the breakpoints of that deletion on the homologous chromosome, will display the mutant phenotype. However, a fly carrying the same deletion, in *trans* to a recessive mutant outside its breakpoints, will be wild type (as long as the deletion heterozygote doesn't possess its own mutant phenotype). For physical map data, locations can be reconciled by *in situ* hybridization (Pardue and Dawid 1981). Cloned fragments will hybridize to polytene chromosomes, positioning isolated pieces of DNA on the cytogenetic map. These two types of procedures allow a correlation between the maps generally limited to the 50 kb resolution of each band and surrounding interband space.

There are impediments to developing the cytological map as a genetic

framework for the organism. Not all regions of the salivary gland polytene chromosomes are equally amenable to cytogenetic study -- some regions simply don't contain a suitable number or arrangement of recognizable banding patterns. A second obstacle to obtaining a serviceable map is that cytological techniques are based on the visual interpretation of the cytologist and, being subjective, may vary from laboratory to laboratory.

Molecular Maps

During the last decade a major collaborative enterprise among researchers studying *Escherichia coli, Saccharomyces cerevisiae, Caenorhabditis elegans, Drosophila melanogaster, Mus musculus*, and our own species was initiated, centered around constructing genome maps, nucleotide-level frameworks spanning the entire genome. The goal of genome mapping is two-fold -- one is to reconcile the genetic and physical maps, allowing researchers in the selected organisms the ability to move between maps with a high degree of facility; the second is the genome maps serve as the first step toward genome sequencing. Although being able to correlate genetic and physical maps on a common framework is not new to *D. melanogaster*, genome maps still offer a higher level of resolution (to the nucleotide level) than has ever been achievable, as well as removing the ambiguities caused by individual interpretations of demanding cytological regions. In addition, the methodology used to construct genome maps is widely applicable, making it

particularly attractive for model organisms that lack refined cytogenetics.

The task of developing a molecular framework for an entire genome is daunting, even in an organism as genetically compact as *Drosophila melanogaster*. Several general strategies have been conceived and implemented. One approach is to work bottom up (Coulson et al. 1986, Olson et al. 1986), starting with small fragments of about 40 kilobase pairs in cosmid vectors, randomly construct contigs (contiguous regions of the chromosome represented by a few demonstrably overlapping clones) and work toward assembling the contigs into wide coverage of the genome (Collins and Hohn 1978, Hohn and Collins 1980, Evans et al. 1989). The advantage of this approach is the stability and ease of working with cosmid vectors, but the disadvantage is the enormous number of clones that need to be analyzed to achieve full coverage (Palazzolo et al. 1991). The alternate top down approach uses very large genome fragments, typically in yeast artificial chromosomes (YACs) which hold up to 1.5 Mb of DNA, or bacterial artificial chromosomes (BACs), achieving wide coverage of the genome, and then working down toward full coverage by discovering overlaps within YACs (Burke et al. 1987). The advantage to this strategy is that fewer clones need to be analyzed to provide a complete map. Unfortunately YACs have posed several unique practical difficulties; these include low transformation efficiency into the host, a large proportion of chimeric clones containing two or more pieces of noncontiguous chromosome (10% reported for some YAC libraries - Merriam et al. 1991), a

propensity of the vectors to undergo rearrangements that forfeit the insert during growth, and an inherent difficulty in separating YAC DNA away from yeast chromosomes. Recently P1 vectors have been developed which purport to retain the advantages of both YAC and cosmid systems, while minimizing their disadvantages (Sternberg 1990, Pierce et al. 1992). The P1 system is designed to package headfuls of genomic DNA from 75-100 kilobase pairs into P1 phage, using *Escherichia coli* as a host at a high transformation efficiency. The vector is then maintained at a single copy, but a repressor under *lac* control may be de-repressed to allow amplification prior to isolation of phage.

A key aspect that ensures the wide and immediate value of the genome maps is their basis on sequence tagged sites (STSs). The term STS refers to the use of regularly interspersed, previously identified, unique sequences that are used as anchors on the support structure from which the genome map emanates (Olson et al. 1989). Such sequences in *Drosophila melanogaster* could be derived from previously cloned and sequenced genes, cDNAs, and P element insertions (Ajioka et al. 1991, Spradling et. al. 1995). A 100 base pair unique sequence stretch is of sufficient length to be amplified by a polymerase chain reaction (PCR), allowing the framework map to be based on STSs without requirement for the cloned DNA. The advantage of separating the genome data from dependence on the limitations of the library it was derived from has two important consequences. The first is that genome maps constructed using different vectors can be instantly correlated. This has been an important feature

in constructing the *D. melanogaster* genome map as several strategies, both top down and bottom up, using YAC, P1 and cosmid vectors, are simultaneously being ventured (Garza et al. 1989, Ajioka et al. 1991, Smoller et al. 1991, Hartl et al. 1994, Kafatos et al. 1991, Sidén-Kiamos et al. 1990). Secondly, the sequence data can be immediately available in a constantly updated form, via electronic databank, for the laboratory needs of the research community, without need for disseminating clones from archives.

In *D. melanogaster*, where the highly refined cytological map has served as a valuable reference during genome map construction, efforts in several laboratories have promulgated success. A P1-based physical map covering 110 Megabase pairs of the euchromatic genome has been recently completed, containing 649 contigs with 2,397 STS, spaced approximately 50 kilobase pairs apart (Smoller et al. 1991, Kimmerly et al. 1996). Randomly selected clones from this library have been *in situ* hybridized to polytene chromosomes, anchoring the P1 genome map in the existing cytological map (Hartl et al. 1994). A YAC genome map consisting of 1,200 clones, with an average insert size of 200 kb and comprising 150 contigs, covers approximately 100 Mb of the euchromatic genome (Ajioka et al. 1991, Garza et al. 1989, Lozovshaya et al. 1993.) *In situ* hybridizations have also been carried out concurrent with map construction, grounding the YACs in the cytogenetic framework (Kimmerly et al. 1996). Lastly, cosmid contigs are being used to generate yet a third physical map of the genome (Sidén-Kiamos et al. 1990, Kafatos et al. 1991).

Collaborations among groups mapping with YAC, cosmid and P1 vectors are ongoing, in an attempt to consolidate the data and provide the most complete and accurate *D. melanogaster* genome map possible.

The recently developed technology for widescale genome mapping efforts have resulted in enormous success in mapping euchromatin, although none of the strategies have been effective in mapping heterochromatic regions. Progress in genome mapping has effectively obviated the need to undergo a more traditional limited molecular analysis of a small region, as was undertaken in the 28A1-28D1 region. The focus of my project has accordingly evolved to concentrate solely on refining the genetic map and unraveling the complicated genetic interactions in the region.

Genetic Maps and Analyzing Complex Regions

The unit of recombination is a relative term. Meiotic exchange rates differ between species, vary between the sexes within most species (Moriwaki 1937, Rhoades 1941, Slizynski 1960, Dunn and Bennett 1967), and may also vary depending on which chromosome and which segment of that chromosome is being investigated (Weinstein 1918, Muller 1916, Offermann and Muller 1932), the age of the organism (Bridges 1915) and environmental factors, such as temperature (Plough 1917, 1921).

The classic definition of a gene is based on function, and is reflected in an organism's phenotype. Two recessive mutants in separate genes will

generally complement one another's functions in a *trans* heterozygote, resulting in a wild type phenotype. Allelic mutants generally fail to complement in *trans*. Noncomplementation of function is typically interpreted as evidence that two individually arising mutants are in the same gene (Lewis 1965). However, cellular interactions can effectively lower function beneath the threshold needed for a wild type phenotype, leading to misclassification of separate genes as allelic. Interpretations of noncomplementation should always carry the caveat that functionally related genes can be indistinguishable by this analysis.

Clusters of functionally related, closely linked genes have been labeled pseudoallelic series (Brink 1932, McClintock 1944). *D. melanogaster* is replete with such regions, notably surrounding *Antennapedia, Bithorax,* and *Star*, which have proven to be remarkably complex. These loci wreak havoc on interpretations of complementation patterns, with the only way to unambiguously separate functional units being observation of the phenotype of mutants in *cis* (Lewis 1965) -- not always practical or possible within the linkage restraints of the complexes. When classical complementation analysis fails to successfully resolve the structure of these regions, extensive labor-intensive molecular analyses, incorporating cloning, sequencing, and transcriptional analyses of megabase segments of DNA, are often the sole means of resolving tightly clustered genes.

The protracted journey toward resolution of the Bithorax complex (BX-C) in *Drosophila melanogaster,* one of the best characterized examples of a

complicated eukaryotic locus, has illustrated the types of problems inherent in analyzing a pseudoallelic series. The recessive mutation *bx*^{1} (*bithorax*), transforming a haltere to a wing, was first described in 1923, and classified as a homeotic mutation (Bridges and Morgan 1923). As other mutant alleles, including the closely linked dominant *Ubx* (*Ultrabithorax*) mutation (Bridges and Brehme 1944), were identified in the region, they appeared to fall into distinct classes, all of which characteristically disrupted aspects of development in specific embryonic thoracic and abdominal metameres. E. B. Lewis embarked upon extensive analyses of BX-C, as a model system to understand embryonic development, noting that BX-C mutations fell within as many as eight functional categories, although the complicated noncomplementation patterns between the different classes made it impossible to ascertain the number of genes (Lewis 1954, 1955, 1963, 1978, 1985). Curiously, the proximo-distal map order of BX-C mutants corresponds with an anterio-posterior gradient of their phenotypic effects in the developing embryo, hypothesized to be caused by a gradient of *cis*-regulatory affinities for an unlinked repressor protein (Lewis 1978). Analogues of BX-C genes have been retained in this same intact structure in other species, including mice and humans (McGinnis et al. 1984, Scott and Weiner 1984, Bachiller et al. 1994, Graham et al. 1989). Function is retained in *D. melanogaster* when the region is divided in half using transpositions (Struhl 1984). Thus, the significance of conservation of gene order is perplexing.

A formal saturation mutagenesis of the BX-C region and subsequent

complementation analysis indicated that only three complementation groups exist -- although the analysis did not consider the effect interactions between functionally related genes might have on interpretations of complementation (Sánchez-Herrero et al. 1985a,b, Lewis 1985). Cloning and sequencing of the entire BX-C region has confirmed the existence of three coding regions (Bender et al. 1983). Subsequent molecular analysis has revealed that the combination of several *cis*-regulatory regions, termed iabs, coupled with alternative splicing and polyadenylation sites for each coding region, are responsible for the enormously complicated pattern of expression at BX-C (Bender et al. 1985, Hogness et al. 1985, Akam et al. 1985, Sanchez-Herrero et al. 1985).

The *wingless* Gene

The *wg* locus was first described in 1973 as an adult viable recessive mutation, wg^1. It was isolated during an EMS screen of the X, but was localized to the second chromosome (Sharma 1973). The wing phenotype of wg^1 is incompletely penetrant, so that adults with two, one or no wings are produced. Wings that do develop are morphologically normal. The gene was cloned independently by two separate approaches, with the first clone isolated from a genomic lambda library prepared with DNA from the P-element tagged *wg* stock, wg^{CP1} (Baker 1987). The second clone was isolated when a molecular analysis of an obscure group of oncogenes, called the *int* family, was extended to the genetically well characterized *D. melanogaster* model system, in the hope of

uncovering an orthologue which would be suitable for studies directed at discovering a function for the enigmatic gene family (Nusse and Varmus 1982). Cross-hybridization of a mouse mammary tumor virus *int-1* probe uncovered a *Drosophila* clone residing at 28A, determined to be identical in sequence to the *wg* clone previously recovered. The int family was subsequently renamed the wnt family (wingless and int) (Rijsewijk et al. 1987). Wnt genes sharing sequence identity at the amino acid level have been cloned from a variety of species, including *Drosophila*, human, mouse, *Xenopus, C. elegans*, and zebrafish.

Several criteria have demonstrated that the wingless protein is a secreted cell signaling factor. Non-autonomy is exhibited in mosaics produced through mitotic recombination, as *wg* cells very close to wild type cells behave normally (Morata and Lawrence 1977). DNA sequence analysis shows conserved cysteines and sites for N-linked glycosylations that are characteristic of secreted proteins (Baker 1987, Rijsewijk et al. 1987). *In situ* labeling of whole embryos using Wg antibodies demonstrated that Wg protein is secreted from the *wg* expressing cells into the extracellular matrix, and then endocytosed into neighboring cells (van den Heuvel et al. 1989, Papkoff et al. 1987). Mutants of *wg* that separate signal transduction and endocytosis indicate that neither function is required for the other. Receptor binding is sufficient for signal induction, and endocytosis is required as part of the signaling pathway in order for Wg to move from cells producing the protein, through the neighboring single

layer of cells, and into the next cell layer within each segment (Bejsovec and Wieschaus 1995). *frizzled,* has recently been implicated as a putative receptor for the wingless ligand (Bhanot et al. 1996, Jones et al. 1996).

D. melanogaster, with the advantages of a small genome, well characterized genetics, and receptiveness to exhaustive mutational analysis, has been a particularly suitable model organism for decoding players and interactions in developmental biology, with notable success in uncovering genes important in setting up basic body polarity. It appears that many of the genes involved in orchestrating early events in *Drosophila melanogaster* have been conserved, with similar or identical roles in vertebrates. The multiple lines of investigation demonstrating that the Wg protein product acts as a soluble signaling factor suggest an *in vivo* role for *wg* and other Wnt orthologues in cell fate determination. A wide variety of genetic and cell biological investigations have been aimed at elucidating in what capacity *wg* directs developmental decisions, and the current understanding of these roles throughout the complicated processes in *D. melanogaster* is summarized here.

Role of *wingless* in Metameric Patterning

Embryogenesis in *Drosophila melanogaster* is a 24 hour process which proceeds in a predictable and sequential fashion, culminating by hatching of larvae with characteristic metameric (segmental) body patterns. The developmental tasks of pattern formation are accomplished by an intricate

spatial and temporal network of interactions among a large set of maternal and zygotic gene products. The initial coordinates of the anterio-posterior and dorso-ventral poles are set up in the egg, prior to fertilization, by gradients of maternal proteins. The first small set of zygotic genes are expressed in response to positional information supplied by the maternal protein gradients. The first (or cardinal) zygotic genes in turn prompt expression of other sets of genes, setting in motion complicated developmental cascades that ultimately result in the characteristic segmental body plan of *D. melanogaster*. Visible indentations separating parasegments first appear at 4 ½ hours after egg laying (AEL), disappearing at 7 hours AEL when a new pattern of grooves, out of register with the first, appears and persists through the larval stages. The final larval pattern consists of morphologically visible indentations separating 14 segments, and the secretion of cuticle by dorsal epidermal cells, with denticles (hooked bristles) on the anterior, but not the posterior, cells of each segment.

In 1980 a systematic attempt to saturate the genome for mutants involved in embryonic pattern formation revealed a large set of embryonic lethal mutations, all of which were morphologically distinguishable into three phenotypic categories (Jürgens et al. 1984, Nüsslein-Volhard et al. 1984, Wieschaus et al. 1984, Nüsslein-Volhard and Wieschaus 1980). Two categories produce the wrong number of segments, either missing large sequential groups of segments (gap) or missing alternating segments (pair-rule). The third group exhibit a segment polarity phenotype; they have the correct

segment numbers, but disrupted patterning within the segments. *wg* is a member of the segment polarity group, with lethal mutants exhibiting a characteristic mirror-image disruption of the embryonic denticle belt pattern. This is seen as a denticle lawn (Nüsslein-Volhard and Wieschaus 1980).

For most gap, pair-rule and segment polarity genes, expression levels rise and fall under very tight temporal and spatial restrictions, with clear indications that a wide variety of complex interactions, many including *wg*, occur throughout embryogenesis. As the genes have been cloned and sequenced the overriding theme has been that gap and pair-rule genes are general transcription factors with DNA and RNA binding properties, exerting their effects during blastulation within the syncytium. Segment polarity genes, in contrast, act during gastrulation, exert their effects through cellular fields, and rely on complex cell signaling pathways. Most have been determined to be ligands, receptors, and protein kinases (Martinez-Arias 1993). It has become clear that these mutants, originally isolated because of an effect on cuticle patterning of epidermal cells, are vital for many other embryonic processes in the nervous, respiratory, and other developing systems. For many of the genetic interactions, overlapping and sequential expression patterns allow inferences to be drawn about corresponding protein interactions, but relatively little experimental evidence yet exists of the mechanistic details of, and of the direct consequences of most interactions. The interplay between *wg* and *en*, the details of which have been painstakingly dissected through genetic and cell biological means,

has turned out to be a key determinant in the organization of metameric patterning.

Several gap and pair-rule genes have been found to be responsible for initiating *wg* and *en* expression between 3 and 4 hours AEL, shortly following the onset of gastrulation. These include *even-skipped (eve), fushi tarazu (ftz), paired (prd)* and *odd-paired (opa)*. (Weir and Kornberg 1985, Ingham 1988, Martinez-Arias et al. 1988). *wg* and *en* are expressed in single side by side cell layers in regular repeats from the anterior to posterior, with 3-4 layers of cells separating each "stripe" (Martinez-Arias et al. 1988). The *wg* row represents the posterior edge, and the *en* row represents the abutting anterior edge of adjacent parasegmental primordia. This expression pattern is stabilized and refined at 4 hours AEL by segment polarity genes, including *wg* and *en* themselves, in a complex and dynamic pattern whose details have not been fully elucidated. Early on, *wg* is necessary for *en* expression; separately for initiation and then again for maintenance (DiNardo et al. 1988, Bejsovec and Martinez-Arias 1991, Gonzalez et al. 1991, Kassis 1990). Between 4.5 and 7 hours AEL, following parasegment formation, *en* becomes independent of *wg*, and both *wg* and *en* expression become dependent on *en*, (Bate and Martinez-Arias 1991, Heemskerk et al. 1991, Weir and Kornberg 1985, Bejsovec and Martinez-Arias 1991). The stripe of *wg-en* expression at the parasegmental boundary, a vital key for future developmental events, is thought to represent the organizing center of the segments that begin to appear as grooves at 7 hours AEL, out of

register with the parasegment grooves that are simultaneously disappearing (Martinez-Arias 1993). At 7-13 hours, the *wg* stripe directs production of Wg protein gradients, which anteriorly suppresses denticles on the cuticle of the posterior portion of segments, and posteriorly restricts the *en* directed deposition of cuticle with denticles (Dougan and DiNardo 1992, Bejsovec and Matinez-Arias 1991, Gonzalez et al. 1991).

Role of *wingless* in Imaginal Disc Development

Following embryogenesis, the larvae hatch and undergo a rapid growth phase, separated into three instar stages by two molts. The larvae then pupuriate, and nearly total reorganization of body tissues takes place preceding emergence of the adult (imago). Some of the adult structures develop from special sacs of cells, called imaginal discs, that were determined and set aside during embryogenesis, most likely using the same protein gradient cues that determine the larval metameres (Bate and Martinez-Arias 1991, Cohen 1993, Simcox et al. 1989, Cohen 1990, Cohen et al. 1993). The imaginal discs are much more refractory to study than the larval cuticle, with corresponding meager headway made in understanding their mechanisms of development. *wg* has been implicated with two roles; it is required for the determination of imaginal primordia, and also has a role in establishing the proximo-distal organization in developing leg and wing discs.

During embryogenesis some segments set aside a pair of ventral discs,

destined to form adult appendages including legs, labia, eye and antennae, and genital organs, with three thoracic segments setting aside an additional pair of dorsal discs, for wings, halteres, and prothoracic spiracles. Anatomical and mosaic evidence indicates the dorsal and ventral discs are of common origin, separating after primordial determination (Steiner 1976, Wieschaus and Gehring 1976, Lawrence and Morata 1977, Anderson 1963, Kukalova-Peck 1978). At 4 ½ hours AEL, an anterio-posterior stripe of *decapentaplegic (dpp)* expression develops down the AP midline that is essential for setting up dorso-ventral polarity in the embryo (Irish and Gelbart 1987, Fergusen and Anderson 1992a, b, Wharton et al. 1993). The organizing centers for the discs coincide with the intersection of the *dpp* anterio-posterior stripe and the *wg* dorso-ventral stripes. Expression of *dpp* at *wg-en* parasegment boundaries is shortly followed by two circles of *Distal-less (Dll)* expression in imaginal founder cells (Steiner 1976, Weischaus and Gehring 1976, Lawrence and Morata 1977, St. Johnston and Gelbert 1987, Blackman et al. 1991, Cohen et al. 1993). Estimates of the last embryonic period in which cell fate determination of discs is made is 9 ½ hours AEL (Hartenstein and Jan 1992, Whiteley et al. 1992, Williams et al. 1991, Cohen 1993), which coincides in thoracic segments with the dorsal migration of a subset of cells to form the wing, haltere, and prothoracic discs. At that time *vestigial (vg)* expression replaces *Dll*. At 12 ½ hours AEL the epidermal cells invaginate to form a three-dimensional disc that is retained, undisturbed by the larval tissue, through the larval stages.

A boundary is established in the growing wing disc coincident with the *wg-en* parasegmental AP boundary. This partitions the anterior and posterior primordia, and compartmentalizes the later actions of *en* and *Ubx* in determining cell fates. (Morata and Ripoll 1975, Garcia-Bellido and Santamaria 1972, Garcia-Bellido 1975). Morphogenesis of the discs retains the partitioning, with the new ventral compartment restricted to *wg* expression and a dorsal compartment with *apterous (ap)* expression. (Couso et al. 1993). The dorso-ventral compartmentalization of *ap* and *wg* expression is necessary for subsequent proximo-distal patterning by *scalloped (sd)* and *vestigial (vg)*, suggesting that the *ap, wg, Dll* intersection is the organizing center for proximo-distal polarity of future appendages. In legs, mosaics demonstrate that localized ectopic *wg* expression spatially correlates with the subsequent development in the adult of supernumerary appendages, although the same demonstration has not been attempted for wings. (Struhl and Basler 1993).

Summary

Genetic investigations of the 27F3-4;28D3-4 cytological region have revealed that loci at 28A comprise a complex set of interacting markers. These interactions center around the segment polarity gene, *wg*, which codes for a secreted cell protein with multiple roles during *D. melanogaster* development. Complementation analysis between *wg* and the closely linked dominant marker *Sp* has identified novel phenotypes for the *wg* gene. Further exploration of these phenotypes is necessary to fully elucidate the intricacies of the relationship.

References

Ajioka, J. W., Smoller, D. A., Jones, R. W., Carull, J. P., Vellek, A. E. C., Garza, D., Link, A. J., Duncan, I. W. and D. L. Hartl, 1991 *Drosophila* genome project: One-hit coverage in yeast artificial chromosomes. Chromosoma. **100**: 495-509.

Akam, M. E., Martinez-Arias, A., Weinzierl, R. and C. D. Wilde, 1985 Function and expression of Ultrabithorax in the Drosophila embryo. Cold Spring Harbor Symp. Quant. Biol. **50:** 195-200.

Anderson, D. T., 1963 The embryology of *Dacu tryoni* 2. Development of imaginal discs in the embryo. J. Embyol. Exp. Morphol. **11**: 339-351.

Bachiller, D., Macias, A., Duboule, D. and G. Morata, 1994 Conservation of a functional hierarchy between mammalian and insect Hox/HOM genes. EMBO. **13**: 1930-1941.

Baker, N. E., 1987 Molecular cloning of *wingless*, a segment polarity gene in *Drosophila*: The spatial distribution of a transcript in embryos. EMBO J. **6**: 1765-1773.

Bate, C. M. and A. Martinez-Arias, 1991 The embryonic origin of the imaginal discs. Development **112**: 755-761.

Bejsovec, A. and A. Martinez-Arias, 1991 Roles of *wingless* in patterning the larval epidermis of *Drosophila*. Development **113**: 471-485.

Bejsovec, A. and E. Wieschaus, 1995 Signaling activities of the *Drosophila wingless* gene are separately mutable and appear to be transduced at the cell surface. Genetics **139**: 309-320.

Beermann, W., 1972 Chromomeres and Genes. In:Developmental studies on giant chromosomes, Volume 4, pp.1-33. W. Beermann (ed.) Springer-Verlag, New York.

Bender, W., Spierer, P. and D. S. Hogness, 1983 Chromosome walking and jumping to isolate DNA from the *Ace* and *rosy* loci and the Bithorax complex in *Drosophila melanogaster*. J. Mol. Biol. **168**: 17-33.

Bender, W., Weiffenbach, B., Karch, F. and M. Peifer, 1985 Domains of *cis*-interaction in the bithorax complex. Cold Spring Harbor Symp. Quant. Biol. **50**: 173-180.

Bhanot, P., Brink, M., Samos, C. H., Hsieh, J.-C., Wang, Y., Macke, J. P., Andrew, D., Nathans, J. and R Nusse, 1996 A new member of the *frizzled* family from *Drosophila* functions as a Wingless receptor. Nature **382**: 225-230.

Blackman, R. K., Sanicola, M., Raftery, L. A., Gillevet, T. and W. M. Gelbart, 1991 An extensive 3' *cis*-regulatory region directs the imaginal disc expression of *decapentaplegic*, a member of the TGF-β family in *Drosophila*. Development **111**: 657-665.

Bridges, C. B., 1915 A linkage variation in *Drosophila*. Jour. Exp. Zool. **19**: 1-21.

Bridges, C. B., 1935 Salivary chromosome maps. J. Hered. **26**: 60-64.

Bridges, C. B., 1938 A revised map of the salivary gland X-chromosomes. J. Hered. **29**: 11-13.

Bridges, C. B. and K. Brehme, 1944 The mutants of *Drosophila melanogaster*. Publ. Carnegie Inst. Washington **552**: 1-257.

Bridges, C. B. and P. N. Bridges, 1939 A new map of the second chromosome: A revised map of the right limb of the second chromosome of *Drosophila melanogaster*. J. Hered. **30**: 475-476.

Bridges, C. and T. H. Morgan, 1923 The third chromosome group of mutant characters of *Drosophila melanogaster*. Carnegie Inst. Wash. Publ. **278:** 123-304.

Bridges, P. N., 1941a A revised map of the left limb of the third chromosome of *Drosophila melanogaster*. J. Hered. **32**: 64-65.

Bridges, P. N., 1941b A revision of the salivary gland 3R-chromosome map of *Drosophila melanogaster*. J. Hered. **32**: 299-300.

Bridges, P. N., 1942 A new map of the salivary gland 2L-chromosome of *Drosophila melanogaster*. J. Hered. **33**: 403-40.

Brink, R. A., 1932 Are the chromosomes aggregates of groups of physiologically interdependent genes? Amer. Natur. **66**: 444-451.

Burke, D. T., Carle, G. F. and M. V. Olson, 1987 Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors. Science **236:** 806-812.

Cohen, B., Simcox, A. and S. Cohen, 1993 Allocation of the thoracic imaginal primordia in the *Drosophila* embryo. Development **117**: 597-608.

Cohen, S. M., 1990 Specification of limb development in the *Drosophila* embryo by positional cues from segmentation genes. Nature **343**: 173-177.

Cohen, S. M., 1993 Imaginal disc development. In: The Development of *Drosophila melanogaster.* Bate, M. and Martinez Arias, A., eds. pp. 747-841.

Collins, J. and B. Hohn, 1978 Cosmids: Type of plasmid gene cloning vector that is packageable *in vitro* in bacteriophage lambda heads. Proc. Natl. Acad. Sci. USA. **75**: 4242-4246.

Coulson, A. R., Sulston, J., Brenner, S. and J. Karn, 1986 Toward a physical map of the genome of the nematode *Caenorhabditis elegans.* Proc. Natl. Acad. Sci. USA. **83**: 7821-7825.

Couso, J. P., Bate, M. and A. Martinez-Arias, 1993 A *wingless*-dependent polar coordinate system in the imaginal discs of *Drosophila.* Science **259**: 484-489.

Danna, K. J. and Nathans, D., 1971 Specific cleavage of simian virus 40 DNA by restriction endonuclease of *Hemophilus influenzae*. Proc. Nat. Acad. Sci. USA. **68**: 2913-2917.

DiNardo, S., Sher, E., Heemskerk, J. J., Kassis, J. A. and P. H. O'Farrell, 1988 Two-tiered regulation of spatially patterned *engrailed* gene expression during *Drosophila* embryogenesis. Nature **332**: 604-609.

Dougan, S. and S. DiNardo, 1992 *Drosophila wingless* generates cell type diversity using *engrailed* expressing cells. Nature **360**: 347-349.

Dunn, L. C. and D. Bennett, 1967 Sex differences in recombinations of linked genes in animals. Genet. Res. **9**: 211-221.

Evans, G. A., Lewis, K. and B. E. Rothenberg, 1989 High efficiency vectors for cosmid microcloning and genomic analysis. Gene **79**: 9-20.

Fergusen, E. and K. Anderson, 1992a Localized enhancement and repression of the activity of the TGF-β family member, *decapentaplegic*, is necessary for dorsal-ventral pattern in the *Drosophila* embryo. Development **114**: 583-597.

Fergusen, E. and K. Anderson, 1992b *decapentaplegic* acts as a morphogen to organize dorsal-ventral pattern in the *Drosophila* embryo. Cell **71**: 451-461.

Garcia-Bellido, A. and P. Santamaria, 1972 Developmental analysis of the wing disc in the mutant *engrailed* of *Drosophila melanogaster*. Genetics **72**: 87-104.

Garcia-Bellido, A., 1975 Genetic control of wing disc development in *Drosophila*. Ciba Found. Symp. **29**: 161-182.

Garza, D., Ajioka, J. W., Burke, D. T. and D. L. Hartl, 1989 Mapping the *Drosophila* genome with yeast artificial chromosomes. Science **246:** 641-646.

Gonzalez, F., Swales, L., Bejsovec, A., Skaer, H. and A. Martinez Arias, 1991 Secretion and movement of the *wingless* protein in the *Drosophila* embryo. Mech. Dev. **35**: 43-54.

Graham, A., Papalopulu, N. and R. Krumlauf, 1989 The murine and *Drosophila* homeobox gene complexes have common features of organization and expression. Cell **57**: 367-378.

Hartl, D. L., Nurminsky, D. I., Jones, R. W. and E. R. Lozovskaya, 1994 Genome structure and evolution in *Drosophila*: Applications of the framework P1 map. Proc. Natl. Acad. Sci. USA. **91**: 6824-6829.

Hartenstein, V. and Y. N. Jan, 1992 Studying *Drosophila* embryogenesis with P-lacZ enhancer trap lines. Roux's Arch. Dev. Biol. **201**: 194-220.

Heemskerk, J., DiNardo, S., Kostriken, R. and P. O'Farrell, 1991 Multiple modes of *engrailed* regulation in the progression towards cell fate determination. Nature **352**: 404-410.

Hogness, D. S., Lipshitz, H. D., Beachy, P. A., Peattie, D. A., Saint, R. B., Goldschmidt-Clermont, M., Harte, P. J., Gavis, E. R. and S. L. Helfand, 1985 Regulation and products of the Ubx domain of the Bithorax Complex. Cold Spring Harbor Symp. Quant. Biol. **50**: 181-194.

Hohn, B. and J. Collins, 1980 A small cosmid for efficient cloning of large DNA fragments. Gene **11**: 291-298.

Ingham, P. W., 1988 The molecular genetics of embryonic pattern formation in *Drosophila*. Nature **335**: 25-34.

Irish, V. and W. M. Gelbart, 1987 The *decapentaplegic* gene is required for dorsal-ventral patterning of the *Drosophila* embryo. Genes Dev. **1**: 868-879.

Jones, K. H., Liu, J. and P. N. Adler, 1996 Molecular analysis of EMS-induced *frizzled* mutations in *Drosophila melanogaster*. Genetics **142**: 205-215.

Jürgens, G, Weischaus, E., Nüsslein-Volhard, C. and H. Klüding, 1984 Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. II. Zygotic loci on the third chromosome. Wilhelm Roux's Arch. Dev. Biol. **196**: 141-157.

Kafatos, F. C., Louis, C., Savakis, C., Glover, D. M., Ashburner, M., Link, A. J., Sidén-Kiamos, I. and R. D. C. Saunders, 1991 Integrated maps of the *Drosophila* genome: Progress and prospects. Trends Genet. **7**: 155-161.

Kassis, J., 1990 Spatial and temporal control elements of the *Drosophila engrailed* gene. Genes Dev. **4**: 433-443.

Kimmerly, W. J., Stultz, K., Lewis, S., Lewis, K., Lustre, V., Romero, R., Benke, J., Sun, D., Shirley, G., Martin, C. and M. J. Palazzolo, 1996 A P1-based physical map of the *Drosophila* euchromatic genome. Gen. Res. **6**: 414-430.

Kress, H., Meyerowitz, E. M. and N. Davidson, 1985 High resolution mapping of *in situ* hybridized biotinylated DNA to surface-spread *Drosophila* chromosomes. Chromosoma **93**: 113-122.

Kukalova-Peck, J., 1978 Origin and evolution of insect wings and their relation to metamorphosis, as documented from the fossil record. J. Morphol. **156:** 53-126.

Laird, C. D., 1980 Structural paradox of polytene chromosomes. Cell **22**: 869-874.

Lawrence, P. A. and G. Morata, 1977 The early development of mesothoracic compartments in *Drosophila*. An analysis of cell lineage and fate mapping and an assessment of methods. Dev. Biol. **56**: 40-51.

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

Lewis, E. B.,1954 The theory and application of a new method of detecting chromosomal rearrangements in *Drosophila melanogaster*. Am. Nat. **88**: 226-239.

Lewis, E. B., 1955 Some aspects of position pseudoallelism. Am. Nat. **89**: 73-89.

Lewis, E. B., 1963 Genes and developmental pathways. Am. Zool. **3:** 33-56.

Lewis, E. B., 1965 Genes and gene complexes. In: Heritage from Mendel: Proceedings of the Mendel Centennial Symposium. Chapter 3 pp. 17-47. Eds. Brink, R. A. and E. D. Styles. University of Wisconsin Press, Madison, Wisconsin.

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

Lewis, E. B., 1985 Regulation of the genes of the *Bithorax* Complex in *Drosophila.* Cold Spring Harbor Symp. Quant. Biol. **50**: 155-164.

Lozovskaya, E. R., Petrov, D. A. and D. L. Hartl, 1993 A combined molecular and cytogenetic approach to genome evolution in Drosophila using large-fragment DNA cloning. Chromosoma **102**: 253-266.

Martinez-Arias A., Baker, N. E. and P. W. Ingham, 1988 Role of segment polarity genes in the definition and maintenance of cell states in the *Drosophila* embryo. Development **103:** 157-170.

Martinez-Arias, A., 1993 Development and patterning of the larval epidermis of *Drosophila.* In: The Development of *Drosophila melanogaster.* Bate, M. and Martinez-Arias, A., eds. pp. 517-608.

McGinnis W., Levine, M. S., Hafen, E., Kuroiwa, A. and W. J. Gehring, 1984 A conserved DNA sequence in homeotic genes of the *Drosophila Antennapedia* and *Bithorax* complexes. Nature **308**: 428-433.

Maxam, A. M. and W. Gilbert, 1977 A new method for sequencing DNA. Proc. Natl. Acad. Sci. USA. **74**: 560-564.

Maxam, A. M. and W. Gilbert, 1980 Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enz. **65(part 1)**: 499-560.

McClintock, B., 1944 The relation of homozygous deficiencies to mutation and allelic series in maize. Genetics **29**: 478-502.

Merriam, J., Ashburner, M., Hartl, D. L. and F. C. Kafatos, 1991 Toward cloning and mapping the genome of *Drosophila*. Science **254**: 221-225.

Morata, G. and P. Lawrence, 1977 The development of *wingless*, a homeotic mutation of *Drosophila*. Dev. Biol. **56**: 227-240.

Morata, G. and P. Ripoll, 1975 *Minutes*: Mutants of *Drosophila* autonomously affecting cell division rate. Dev. Biol. **42**: 211-221.

Moriwaki, D., 1937 A high ratio of crossing over in Drosophila ananassae. Z. Ind. Abst. Vererb. **74**: 17-23.

Muller, H. J., 1916 The mechanism of crossing over. Am. Nat. **50**: 193-221.

Nathans, D. and H. O. Smith, 1975 Restriction endonucleases in the analysis and restructuring of DNA molecules. Ann. Rev. Biochem. **44**: 273-293.

Neumann, C. J. and S. Cohen, 1996 *Sternopleural* is a regulatory mutation of *wingless* with both dominant and recessive effects on larval development of *Drosophila melanogaster*. Genetics **142**: 1147-1155.

Nusse, R. and H. Varmus, 1982 Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell **31**: 99-109.

Nüsslein-Volhard, C., Wieschaus, E. and H. Kluding, 1984 Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. I. Zygotic loci on the second chromosome. Wilhelm Roux's Arch. Dev. Biol. **193**: 267-282.

Nüsslein-Volhard, C. and E. Wieschaus, 1980 Mutations affecting segment number and polarity in *Drosophila*. Nature **287**: 795-801.

Offermann, J. and H. J. Muller, 1932 Regional differences in crossing over as a function of the chromosome structure. Proc. Sixth Int. Cong. Genetics **2**: 143-145.

Olson, M. V., Dutchik, J. E., Graham, M. Y., Brodeur, G. M., Helms, C., Frank, M., MacCollin, M., Scheinman, R. and T. Frank, 1986 Random-clone strategy for genomic restriction mapping in yeast. Proc. Natl. Acad. Sci. USA. **83:** 7826-7830.

Olson, M., Hood, L., Cantor, C. and D. Botstein, 1989 A common language for physical mapping of the human genome. Science **245**: 1434-1435.

Palazzolo, M. J., Sawyer, S. A., Martin, C. H., Smoller, D. A. and D. L. Hartl, 1991 Optimized strategies for sequence-tagged-site selection in genome mapping. Proc. Natl. Acad. Sci. USA. **88**: 8034-8038.

Papkoff, J., Brown, A. M. C. and H. E. Varmus, 1987 The *int-1* proto-oncogene products are glycoproteins that appear to enter the secretory pathway. Mol. Cell. Biol. **7**: 3978-3984.

Pardue, M. L. and I. B. Dawid, 1981 Chromosomal location of two DNA segments that flank ribosomal insertional-like sequences in *Drosophila*: Flanking sequences are mobile elements. Chromosoma **83**: 29-43.

Pierce, J. C., Sauer, B. and N. Sternberg, 1992 A positive selection vector for cloning high molecular weight DNA by the bacteriophage P1 system: Improved cloning efficacy. Proc. Natl. Acad. Sci. USA. **89**: 2056-2060.

Plough, H. H., 1917 The effect of temperature on crossing over in *Drosophila.* J. Exp. Zool. **24**: 147-200.

Plough, H. H., 1921 Further studies on the effect of temperature on crossing over. J. Exp. Zool. **32**: 187-202.

Rasch, E. M., Barr, H. J. and R. W. Rasch, 1971 The DNA content of sperm of *Drosophila melanogaster.* Chromosoma **33** :1-18.

Rhoades, M. M., 1941 Different rates of crossing over in male and female gametes of maize. J. Am. Sac. Agron. **33**: 603-615.

Rijsewijk, F., Schuermann, M., Wagenaar, E., Parren, P., Weigel, D. and R. Nusse, 1987 The *Drosophila* homologue of the mouse mammary oncogene int-1 is identical to the segment polarity gene *wingless*. Cell **50**: 649-657.

Sanchez-Herrero, E., Casanova, J., Kerridge, S. and G. Morata, 1985a Anatomy and function of the *Bithorax Complex* of *Drosophila.* Cold Spring Harbor Symp. Quant. Biol. **50**: 165-172.

Sanchez-Herrero, E., Vernos, I., Marco, R. and G. Morata, 1985b Genetic organization of *Drosophila* bithorax complex. Nature **313**: 108-113.

Sanger, R., Nichlen, S. and A. R. Coulson, 1977 DNA sequencing with chain inhibitors. Proc. Natl. Acad. Sci. USA. **74**: 5463-5467.

Scott, M. P. and A. J. Weiner, 1984 Structural relationships among genes that control development: sequence homology between the *Antennapedia, Ultrabithorax*, and *fushi tarazu* loci of *Drosophila*. Proc. Natl. Acad. Sci. USA. **81**: 4115-4119.

Sharma, R. P., 1973 *wingless*, a new mutant in *Drosophila melanogaster*. Dros. Inf. Ser. **50**:134.

Sidén-Kiamos, I., Saunders, R. D. C., Spanos, L., Majerus, T., Treanear, J., Savakis, C., Louis, C., Glover, D. M., Ashburner, M. and F. C. Kafatos, 1990 Towards a physical map of the *Drosophila melanogaster* genome: Mapping of clones within defined genomic divisions. Nucl. Acids Res. **18**: 6261-6270.

Simcox, A. A., Roberts, I. J. H., Hersperger, E., Gribbin, M. C., Shearn, A. and J. R. S. Whittle, 1989 Imaginal discs can be recovered from cultured embryos mutant for the segment polarity genes *engrailed, naked* and *patched* but not from *wingless*. Development **107**: 715-722.

Slizynski, B. M., 1960 Sexual dimorphism in mouse gametogenesis. Genet. Res. **1** :477-486.

Smith, A. J. H., 1980 DNA sequence analysis by primed synthesis. Methods Enz. **65(part 1)**: 560-580.

Smoller, D. A., Petrov, D. and D. L. Hartl, 1991 Characterization of bacteriophage P1 library containing inserts of *Drosophila* DNA of 75-100 kilobase pairs. Chromosoma **100**: 487-494.

Sorsa, V., 1982 An attempt to estimate DNA content and distribution in the zeste-white region of the X chromosome of *Drosophila melanogaster.* Biol. Zbl. **101**: 81-95.

Sorsa, V., 1988 Chromosome maps of *Drosophila* Volume I and Volume II. CRC Press, Florida.

Spradling, A. C., Stern, D., Kiss, I., Roote, J. and G. M. Rubin, 1995 Gene disruption using P transposable elements: An integral component of the *Drosophila* genome project. Proc. Natl. Acad. Sci. USA. **92**: 10824-10830.

St. Johnston, D. and W. Gelbert, 1987 *decapentaplegic* transcripts are localized along the dorsal ventral axis of the *Drosophila* embryo. EMBO J. **6**: 2785-2791.

Steiner, E., 1976 Establishment of compartments in the developing leg imaginal discs of *Drosophila melanogaster*. Wilhelm Roux's Arch. Dev. Biol. **180**: 9-30.

Sternberg, N., 1990 Bacteriophage P1 cloning system for the isolation, amplification, and recovery of DNA fragments as large as 100 kilobase pairs. Proc. Natl. Acad. Sci. USA. **87**: 103-107.

Struhl, G., 1984 Splitting the *bithorax* complex of *Drosophila*. Nature **308**: 455-457.

Struhl, G. and K. Basler, 1993 Organizing activity of *wingless* protein in *Drosophila*. Cell **72**: 527-540.

Sturtevant, A. H., 1913 The linear arrangement of six sex-linked factors in *Drosophila*, as shown by their mode of association. J. Exp. Zool. **14**: 43-59.

Tiong, S. and D. Nash, 1990 Genetic analysis of the *adenosine3 (Gart)* region of the second chromosome of *Drosophila melanogaster*. Genetics **124**: 889-897.

van den Heuvel, M., Nusse, R., Johnston, P. and P. A. Lawrence, 1989 Distribution of the *wingless* gene product in *Drosophila* embryos: A protein involved in cell-cell communication. Cell **59**: 739-749.

Weinstein, A., 1918 Coincidence of crossing over in *Drosophila melanogaster (Ampelophila)*. Genetics **3**: 135-159.

Weir, M. and T. Kornberg, 1985 Patterns of *engrailed* and *fushi tarazu* transcripts reveal novel intermediary stages in *Drosophila* segmentation. Nature **318**: 433-439.

Wharton, K. A., Ray, R. and W. Gelbart, 1993 An activity gradient of *decapentaplegic* is necessary for the specification of the dorsal pattern elements in the *Drosophila* embryo. Development **117**: 807-822.

Whiteley, M., Noguchi, P. D., Sensabaugh, S. M., Odenwald, W. F. and J. Kassis, 1992 The *Drosophila* gene *escargot* encodes a zinc finger motif found in *snail* related genes. Mech. Dev. **36**: 117-127.

Wieschaus, E., Nüsslein-Volhard, C. and G. Jürgens, 1984 Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. I. Zygotic loci on the X chromosome and the fourth chromosome. Wilhelm Roux's Arch. Dev. Biol. **193**: 267-282.

Wieschaus, E. and W. J. Gehring, 1976 Clonal analysis of primordial disc cells in the early embryo of *Drosophila melanogaster*. Dev. Biol. **50**: 249-263.

Williams, J. A., Bell, J. B. and S. B. Carroll, 1991 Control of *Drosophila* wing and haltere development by the nuclear *vestigial* gene product. Genes Dev. **5:** 2481-2495.

Chapter 2

Genetic Characterization of 27F3-4;28D3-4

A formal genetic analysis was implemented with the goal of saturating the 28A-28D cytological region for lethal and visible complementation groups. In previous saturation screens conducted in *Drosophila melanogaster*, the number of lethal complementation groups in large discrete regions of the chromosome has roughly corresponded to the number of cytologically visible bands within that same region (Judd et al. 1972, Lefevre and Green 1972, Rayle, 1972, Lim and Snyder 1974, Gvozdez et al. 1975, Liu and Lim 1975, Gausz et al. 1979, 1986, Woodruff and Ashburner 1979, Hilliker et al. 1980, Zhimulev et al. 1981), although detailed analyses of small portions of the chromosomes reveal marked variation in the number and arrangement of lethal complementation groups (Young and Judd 1978, Lefevre 1981). Based on these precedents, the 28A-28D region, which contains ten major bands, is predicted to hold ten lethal complementation groups. If the complementation groups fall in a Poisson distribution, it is necessary to isolate 100 individually arising mutants to have a

99.996% confidence level of obtaining at least one mutant representing each complementation group[1].

Ethyl methanesulphonate (EMS) was chosen from many possible chemical mutagens as the vehicle for obtaining point mutations, chiefly for reasons of convenience. EMS has been widely used in mutagenesis studies of *Drosophila* since 1965 (Alderson 1965). Genetic analysis of EMS induced mutations of *Drosophila melanogaster* indicate that ≥ 80% are within single cistrons, with sequence analysis indicating that 76% are G:C→A:T transitions (Lim and Snyder 1968, Lifschytz and Falk 1969, Lim and Snyder 1974, Pastink et al. 1991, Nivard et al. 1992). A relatively safe and simple adult feeding procedure has been devised for EMS mutagenesis (Lewis and Bacher 1968).

Strategy for Genetic Characterization

To isolate lethal mutations in the 28A-28D cytological region, the strategy of screening for lethality over a deficiency was pursued. At the onset of the mutagenesis all deficiencies available had breakpoints near the 28B/C region, with none extending to 28D, so initial efforts were necessarily directed toward isolating a deficiency that spanned the region of interest. Several unsuccessful attempts to generate a suitable deficiency with γ-rays, using spanning recessive

[1]The Poisson distribution predicts that, for any recoverable complementation group, the number of alleles will follow the function $f(x) = e^{-m} m^x/x!$, where x = mutant alleles and m = average alleles per gene. For 10 genes with 100 lethal alleles, $f(0) = e^{-10} \times 10^0/0! = e^{-10} = .99996$ will be the probability of not obtaining an allele of a gene. If the complementation group estimate is off 2-fold, for 20 genes m=5 and P is still ≥99%

markers, the dominant marker *Sp*, and a P element located near 28B will be described. Eventually a deletion was obtained from an external source that had been generated through mobilization of a transposable element (Wustmann et al. 1989). An F_2 screen to isolate mutants following treatment of males with EMS was carried out, and 99 lethals were generated. Initial analysis of the mutants consisted of using four overlapping deficiencies to subdivide the lethals into three cytological regions. Complementation analysis was then carried out between lethals within each region, determining how many individual loci the mutant sets comprised. In one region, 27F3-4;28B, a particularly complex complementation pattern was uncovered, and further complementation analyses of some of these alleles with previously isolated alleles of *wg, spd,* and *Sp* were conducted.

Genetic and Molecular Markers at 28A1-28D1

In 1990 the 28A-28D cytological region already contained several well characterized markers, and during the tenure of this research project genetic and molecular markers have continued to be mapped to the region. *wg*, positioned at 28A, is by far the most extensively studied gene in Division 28. The 28A region also includes the markers *Sp* and *spd*, and these three markers interact in a complex pattern of complementation (Tiong and Nash 1990). Description of the markers assigned to this cytological expanse follows, with the exception that discussion of *wg, Sp* and *spd* is deferred until Chapter 3.

ninaC is a gene that was isolated as a vision-defective mutant, and cytologically localized to 28A1-3 (Stephenson et al. 1983). *ninaC* homozygotes have rhabdomeres, the microvillar structures in the *Drosophila* retinal photoreceptor cells, that are reduced in diameter, with a resultant reduction in rhodopsin (Matsumoto et al. 1987). The coding region of the *ninaC* gene was cloned, and found to be carried on two overlapping mRNAs representing 174 and 132 kilodalton proteins, both of whose predicted cytoskeletal products contain a kinase domain attached to a myosin domain (Montell and Rubin 1988). Calmodulin, which mediates a variety of Ca^{2+}- dependent signaling pathways, is concentrated in the rhabdomeres, with smaller amounts located in the sub-rhabdomeral cytoplasm. *ninaC* is required for correct calmodulin localization. Transgenic p174 deletion mutants have no rhabdomeral calmodulin and p132 deletion mutants have no cytoplasmic (Porter et al. 1993). Two Ca^{2+} dependent calmodulin binding sites, C1 and C2, have been uncovered by *in vitro* binding assays. Both proteins have a C1 domain, while C2 is unique to p174. Experiments with deletion transgenics have demonstrated that correct spatial calmodulin localization is dependent on the presence of both C1 and C2. A deletion for either C1 or C2 also gives a defective photoresponse, suggesting an *in vivo* role for *ninaC* and calmodulin in phototransduction termination (Porter et al. 1995). Curiously, alleles of *ninaC* fail to complement the lethal phenotype of *Sp*, although they lack the dominant bristle phenotype (Matsumoto et al. 1987). This genetic characteristic mimics the complementation pattern of some lethal

alleles of *wg* with *Sp,* and further complicates the interpretation of data for these loci.

An EMS mutagenic screen in 1990 to isolate lethal complementation groups extended from the *adenosine-3 (Gart)* locus at 27D (Tiong and Nash 1990). Although the primary focus was to uncover new *Gart* mutants, the mutagenic screen extended to 28B, and six complementation groups were isolated in the 28A1;28B3-4 region, with a complicated pattern of overlapping non-complementation reported.

Several recent specialized screens for behavioral, sterile, and enhancer phenotypes have also revealed complementation groups in this region. A P element insertional mutagenesis experiment designed to recover male sterile mutations isolated two mutants that have been assigned by *in situ* hybridization to the region. *cuckold*, at 28A, is a behavioral mutant that has a failure to court phenotype, and *gelding* at 28D is deficient in post meiotic differentiation of spermatocytes (Castrillon et al. 1993). A second P-element insertional analysis, exploiting the propensity of a mobilized P-element from the *polyhomeotic* gene to preferentially insert near polyhomeotic and polycomb binding sites, recovered *Enhancer of polycomb [E(Pc)28B]*, assigned to the 28B1-4 region (Fauvarque and Dura 1993). A P-element neurological screen, designed to recover mutants with *Shaker*-like phenotypes, uncovered a gene called *TATA binding protein related factor*, at 28D (Crowley et al. 1993). *midline uncoordinated*, a neural circuitry mutant with defective grooming behavior maps to 28A1-28A6 (Phillis et

al. 1993). Lastly, an enhancer of variegation, *E(var)28AB*, maps within *Df(2L)TE62x2*, but not in *Df(2L)TE62x1*, limiting its location to the 27E5-28B1 region (Wustmann et al. 1989).

Figure 1 summarizes the molecular (panel a) and genetic (panel b) maps of the 28A-28D region. In addition to the genetic markers described here, eleven DNA clones have been assigned to the region by *in situ* hybridization. These molecular markers are listed in Table 1.

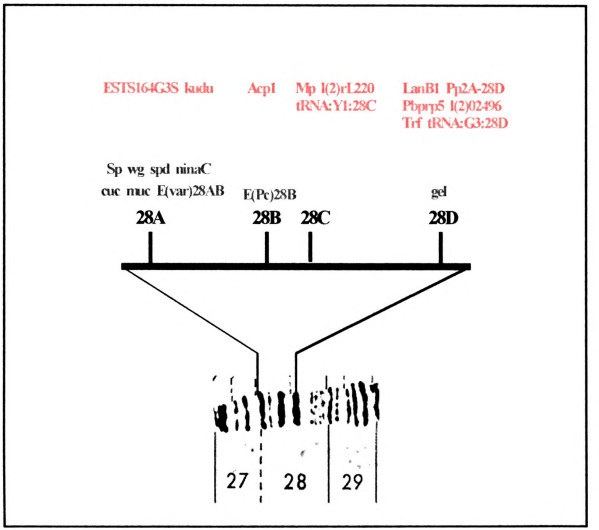


Figure 1 Molecular and Genetic Maps of Cytological Region 28A-28D

Table 1 Molecular Clones in the 27F3-4;28D3-4 Cytological Region

Laminin, B1 subunit Montell and Goodman 1988 basement membrane glycoprotein complex distributed around the developing nervous system	28D
ESTS 164 G3S European Drosophila Genome Mapping Project contains a zinc finger domain match	27F-28A
l(2)rL220 Berkeley Drosophila Genome Project LacZ enhancer-trap salivary gland specific staining	28C4-28C6
l(2)02496 Berkeley Drosophila Genome Project LacZ enhancer-trap brain specific staining	28D1-28D2
tRNA:gly3:28D Hayashi et al. 1980 Hayashi et al. 1981	28D
tRNA:tyr1:28C Dudler et al. 1981	28C
kudu Berg and Spradling 1989 female-sterile eggs have fused or thin membranes	28A
Membrane protein Kubli 1991 distributed in mitochondrial plasma membrane	28C
protein phosphatase 2A Orgad et al. 1990 Mayer-Jaekel et al. 1992 cDNAs isolated from human and rabbit probes	28D2
adult cuticle protein 1 Qui and Hardin 1995	28B3-28C2
Pheromone-binding protein-related protein 5 Pikielny et al. 1994 isolated from an antennal cDNA library with head cDNAs subtracted out	28D

Methods And Results

Fly maintenance and strains

Drosophila melanogaster were maintained on either sucrose/cornmeal or molasses/cornmeal standard food at 21°C. Flies carrying the *Sp* marker were frequently maintained at 27°C to enhance the penetrance and expressivity of the bristle phenotype. Markers are described more fully in Lindsley and Zimm (Lindsley and Zimm 1992), and in FlyBase (http://flybase.bio.indiana.edu:82).

Three common second chromosome balancers are available, namely *SM1=In(2LR)SM1, al^2 Cy cn^2 sp^2; SM5=In(2LR)SM5, al^2 Cy lt^v sn^2 sp^2;* and *CyO=In(2LR)O, Cy dp^{lvl} pr cn^2* . All three carry the dominant Cy marker. Stocks obtained from other laboratories have been assumed to carry the designated balancer; no attempt was made to confirm the identity of the chromosome by cytological examination of the inversion breakpoints.

Single fly crosses were carried out in shell vials with one male and female, and mass matings were carried out in 1/4-pint milk bottles using 20 males and 20 females. In crosses involving flies with deficiencies or lethals over balancer chromosomes, the F_1 heterozygote was deemed inviable if no *trans* heterozygotes (the Cy^+ phenotypic class) were detected among 50 progeny. Inviability of *trans* heterozygotes was interpreted as evidence that two deficiencies overlapped, a mutant fell within deficiency breakpoints, or that two mutants were allelic.

Attempts to Obtain Deletions...

A screen for recessive mutations in a specific region of an autosome requires a deletion spanning the region. Two γ-irradiation mutagenesis screens were devised to isolate a deletion spanning the region of interest; one taking advantage of the recessive mutations *spd* and *cui* flanking the region, and the second exploiting a P-element in a neighboring region.

The cytological location of *spd* has been reported to be 27D-E on the basis of the deletion *Df(2L)spd*=Df(2L)27D-E;28C and the transposition T(Y;2)A171=T(Y;2)27C9-27E (Kotarski et al. 1983). However, it falls within Df(2L)J136-H52, reported to have breakpoints at 27C2-9;28B3-4 (Tiong and Nash, 1990). This is inconsistent with the reported breakpoint of 27E for T(Y:2)A171 (Lindsley et al. 1972), and it seems likely that the position of T(Y:2)A171 was initially incorrectly determined. The genetic location of *spd* is 2-21.9, to the right of *dumpy* (Doane 1961). spd^{flg}, the allele used for this analysis, may be a regulatory allele of *wg* that exerts its mutant effect during the third instar (Buratovich et al. 1996). The spd^{flg} phenotype is a rounded and shortened paddle-shaped wing (Doane 1960). *Sp* resides at the genetic map position 2-22.0, and falls within 28A1-28B4. *Sp* was a spontaneously generated dominant mutation, causing an increase in size and number of the sternopleural bristles, with a recessive lethal phenotype (Mann 1923). The cytological location of *cui* has not been determined, but its genetic position is 2-23.4 (1.4 cM to the right of *Sp*). The next proximal cytologically characterized marker on the

genetic map is l(2)34Da, at 34A, corresponding to the genetic map position 2-48.6, leaving a wide expanse of possible cytogenetic locations for *cui* (Nicoletti 1957). The *cui* phenotype is a slight, but easily visible, upturn to the wing tips. *Sp*, *spd*^{flg}, and *cui* are all easily distinguishable in the presence of one another.

...spanning recessive markers

To increase the odds of detecting a deficiency covering the 28A - 28D region, an attempt was made to produce a chromosome with recessive markers *spd* and *cui*, that also carried the dominant marker *Sp*. The rationale for devising such a chromosome was that one could screen for the appearance of the recessive *spd* and *cui* phenotypes, while simultaneously monitoring for the loss of the dominant *Sp* phenotype. The following cross was designed to obtain an *Sp spd cui* crossover chromosome, with the predicted F2 offspring tabulated in Table 2. (As the location of *Sp* and *spd* relative to one another is not known, it wasn't evident whether an *Sp spd cui* chromosome would be a single or double crossover product, although the cross is diagramed with the assumption that *Sp* is distal to *spd*.).

spd cui	***X***	***Sp cui***
spd cui		***SM1***
	//	
Sp cui	***X***	***spd cui***
spd cui		***spd cui***

Table 2 Predicted F_2 Offspring From *Sp cui* X *spd cui* Cross

	spd cui	PHENOTYPE
NCO	spd cui	spd cui
	Sp cui	Sp cui
CO	spd cui	spd cui
	Sp cui	Sp cui
	Sp spd	Sp spd
	Sp spd cui	spd cui
	+	+
	spd	spd
	cui	cui
	Sp	Sp

25,000 chromosomes were screened, with no triple mutant chromosomes recovered. In addition, there was a failure to recover any *Sp spd* crossover chromosomes. indicating a 99% probability that the two markers reside within 0.02 cM of one another.

Lacking an *Sp spd cui* chromosome, a mutagenesis screen was designed to recover a deletion in an *Sp* chromosome that spanned the recessive mutations *spd* and *cui*.

(γ irradiated) ♂	***Sp lys d*** ***SM1***	***X***	***spd cui*** ***spd cui***	
		⇓		
		deletion ***spd cui***	***screen for spd, cui, loss of Sp***	
	deletion ***spd cui***	***X***	***spd cui*** ***spd cui***	***confirm heritability***
		⇓		
	deletion ***spd cui***	***X***	***Pm*** ***SM1***	***balance***
		⇓		
		deletion ***SM1***	***stock ♂ and ♀***	

288 male flies were γ-irradiated using a sealed Co source for 180 seconds at Flux 5, approximately 2800 Rads. Following mass mating to *spd cui* females, 52,228 Cy^+ progeny flies were screened for gain of *spd* or *cui*, or loss

of *Sp* phenotypes. A single *cui* mutation was recovered, *cui*^{G1}, that has not been cytologically examined. When crossed to *Df(2L)TE128x11* and *Df(2L)TE128x14*, deficiencies which both uncover the *cui* phenotype (see Table 17), viable *cui*^{G1}*/* deficiency heterozygotes are recovered, indicating that *cui*^{G1} is most likely not a large deficiency. No *spd cui* double mutations were recovered.

...by Loss of P element White Phenotype

P[A1-2-29] was obtained from Rolf Bodmer (University of Michigan). The stock contains an insertion of the P element CasPer near the cytological region 28A. The CasPer vector contains a truncated version of the white gene. In a white deficient background, flies with one copy of the vector have light apricot colored eyes, while two copies give a darker orange eye phenotype (Pirotta 1988).

In situ hybridization of a labelled CasPer probe to P[A1-2-29] chromosomes was carried out to confirm the location of the P element insert (Pardue and Dawid 1987, Hayashi et al. 1978, Pardue et al. 1987). Salivary glands were squashed in 45% acetic acid and frozen on dry ice. Cover slips were flipped, the preparations were dehydrated in 95% ethanol and air dried. The slides were heated at 70°C for 30 minutes, dehydrated with successive rinses in 70% and 95% ethanol, and air dried. The preparations were treated with 1 mg/ml RNaseA in 2x SSC (0.3M NaCl, 0.03 M sodium citrate, pH 7.0) at room temperature for 2 hours. Acetylation of samples was carried out in 0.5 ml

acetic anhydride in 200 ml triethanolamine, pH 8.0 for 10 minutes at room temperature, followed by washing in 2X SSC, and dehydration in 70% ethanol, 95% ethanol, and air drying. The DNA was denatured in 0.07 N NaOH for three minutes, followed by three washes each in 70% and 95% ethanol, and air drying. A biotinylated CasPer DNA probe was obtained from T. Friedman. Hybridization took place in 2X TNS (0.3M NaCl, 0.02 M Tris, pH 6.8) for 16 hours at 67°C. Slides were rinsed three times in 2x SSC at 60°C for 10 minutes each rinse, treated for 1 hour at 37°C with 20 ug/ml RNaseA in 2x SSC to remove non-specifically bound RNA, rinsed again in 2x SSC, dehydrated in 70% and 95% ethanol, and dried. Samples were observed under 630 X magnification on an Olympus AH-2 phase contrast microscope. The P element probe hybridized to the 28B/C region of Oregon-R (data not shown).

The following mutagenesis screen was devised to screen for loss of the apricot eye phenotype conferred by The CasPer Vector.

γ irradiated ***w; P[A1-2-29]*** ***X*** ***w; Sp lys d***
Y P[A1-2-29] ***w SM1***

//

w,w; deletion ***screen for***
Y w SM1 ***white eyes***

38,296 chromosomes were screened. No white-eyed flies were seen, but seven putative mutants with lighter apricot eyes were recovered. These were stocked and salivary gland polytene squashes were carried out. For this, the mutations/deficiencies were outcrossed to Oregon-R. The salivary glands of the

progeny were hand dissected from third instar larvae into Ringer's solution. The glands were incubated in 45% acetic acid for one minute, and fat deposits were removed. The glands were stained for one minute with aceto-orcein stain, squashed under a glass cover slip, and observed using an Olympus AH-2 phase contrast microscope. Cytological examination of each of the mutants revealed no gross chromosomal abnormalities, indicating that each had probably arisen from small abnormalities in the vector's white gene, rather than from deletion of CasPer.

...Externally

The deficiency strain *Df(2L)TE62x2*=Df(2L)27F3-4;28D3-4 was obtained from G. Reuter (Wustmann et al. 1989). The cytological endpoints of the deficiency were confirmed by salivary polytene squash as described above, and no further attempts to generate another deficiency spanning the 28A-28D region were undertaken.

Characterization of Externally Available Deficiencies in the 28A-28D Region

Table 3 lists the available deficiencies of the 28A1-28D4 region. Crosses of deletion strains to one another, and to markers in the region were carried out to genetically characterize the extent of each deficiency. Viability and phenotypes (Sp=sternopleural, cui=curvi, wg=wingless, " -"=lethal, N. D.=not determined) of viable offspring are reported for each combination in

Table 17 (Appendix B).

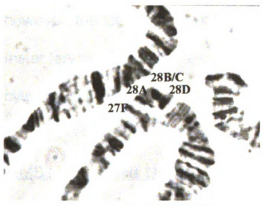
In addition, salivary gland polytene squashes confirming the reported breakpoints of the deficiency strains were prepared as described above (page 49). Ten or more individual preparations were inspected from each deficiency strain, and representative polytene squashes are shown in Figure 2.

Cytological observation of *Df(2L)TE62x2* and *Df(2L)TE62x1* (Figure 2, panels a and b) confirmed their reported breakpoints of 27E5-F1;28D3-4 and 27E5-F1;28C, respectively. *Df(2L)TE62x2, Df(2L)TE62x1, Df(2L)SS1* (panel c) and *Df(2L)Az* (panel d) all appear to have a distal breakpoint near 27F. No visual difference is readily detectable by cytological inspection, although the 27F band appears fuzzier in *Az* than in *SS1,* perhaps indicating that the *Az* aberration extends into the 27F band, while *SS1* does not. In addition, *Df(2L)ade3*^{27}=Df 27C6;27F is lethal in *trans* to *TE62x1, TE62x2* and *Az*, but not *SS1*, confirming that the *SS1* breakpoint is most likely more proximal than the others. *Df(2L)spd*^{x4} is a rather large deletion that breaks at 28B3-4 and extends distally past the 27F band (data not shown). *J136-H52* was a synthetic deficiency produced from the T(Y:2) transpositions *J136* and *H52* (Lindsley et al. 1972), and also extends distally from 28B/C past 27F into 27C2-9. Its breakpoints have been reported as 27C2-9;28B3-4 (Tiong and Nash 1990), differing slightly from the breakpoints originally reported for the transpositions (Lindsley et al. 1972). The proximal breakpoints of *Df(2L)spdA2, Df(2L)spd*^{x4}, *Df(2L)Az* and *Df(2L)SS1* all fall at 28B3-4.

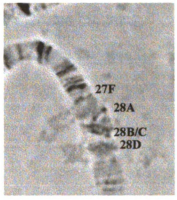
Table 3 Deficiencies in the 27F;28D Cytological Region

Df(2L)TE62x1	27E5-F1;28C	Wustmann et al. 1989
Df(2L)TE62x2	27E5-F1;28D3-4	Wustmann et al. 1989
Df(2L)spdA2	27F1;28B3-4	
Df(2L)spd^{x4}		
Df2L)SS1	27F1;28B3-4	
Df(2)J136-H52	27C2-9;28B3-4	Tiong and Nash 1990
Df(2)ade3^{27}	27C6;27F-28A1	Tiong and Nash 1990
Df(2L)TE80x1		Wustmann et al. 1989
Df(2L)TE128x11		Wustmann et al. 1989
Df(2L)TE128x14		Wustmann et al. 1989

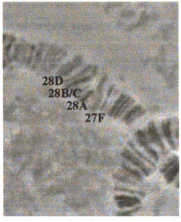
Figure 2 Polytene Chromosome Squashes of Deficiencies



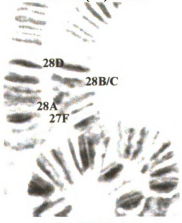
A. Df(2L) TE62x2



B. Df(2L) TE62x1



Df(2L) SS1



D. Df(2L) Az

For some deficiencies, inspection of individual preparations did not reveal which breakpoint of a pair was most centromere distal. In an attempt to localize breakpoints with respect to one another, deficiency strains were crossed to one another with the hope that deficiency heterozygotes would survive to the third instar larval stage for salivary gland preparation. For the strains tested, however, the lethal phase of the deficiency heterozygote preceded the third instar larvae. Figure 3 summarizes the deficiency map of the 28F3-4;28D3-4 cytological region.

EMS Mutagenesis Using *Df(2L)TE62x2*

Stocks homozygous for a single second chromosome from Oregon-R (wild-type) flies were generated, as an identical chromosome set in the parents should facilitate subsequent molecular analyses of the mutants.

	Oregon-R 1 ***Oregon-R 2***	***X***	***Pm*** ***SM1***	
	⇓			
	Oregon-R 1 ***SM1***	***X***	***Pm*** ***SM1***	***single fly***
	⇓			
Inter se	***Oregon-R 1*** ***SM1***	***X***	***Oregon-R 1*** ***SM1***	***stock***

The deficiency *Df(2L)TE62x2* = Df(2L)27F-28D, obtained from Guenther Reuter

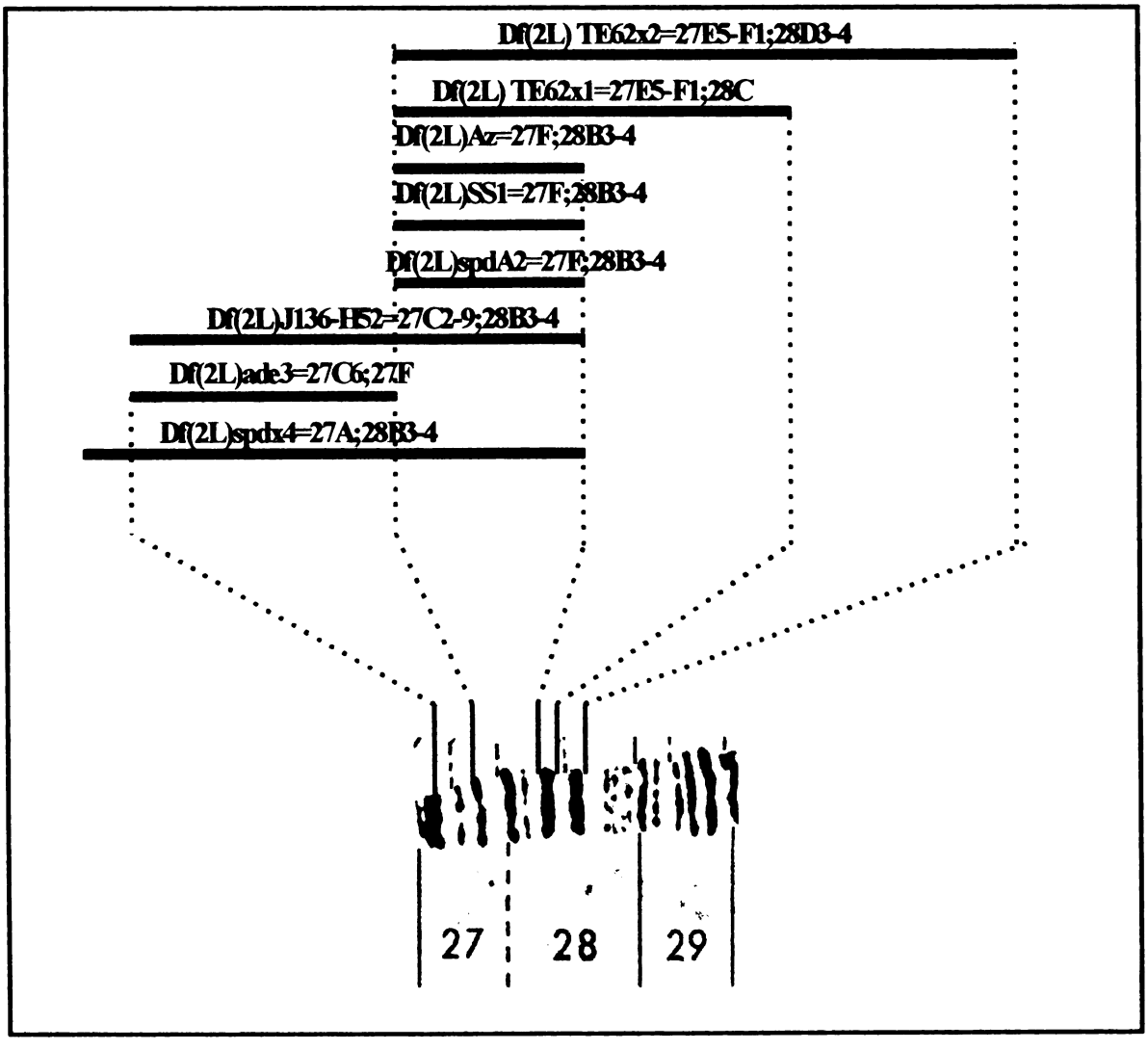
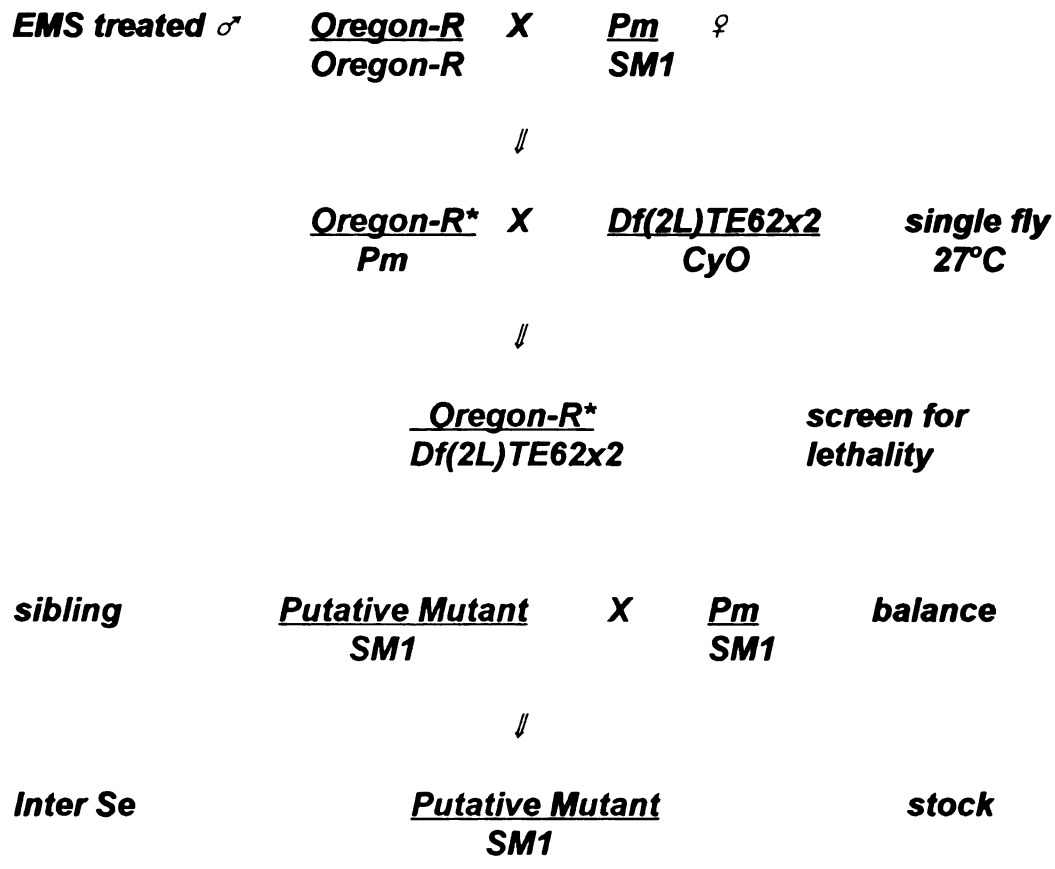


Figure 3 Deficiency Map of Cytological Region 27F3-4;28D3-4

(Wustmann et al. 1989), was used to recover recessive lethal and visible mutations in the region, using the following scheme:



EMS fed Oregon-R males were mass mated to *Pm/SM1* females, and 1,096 Oregon-R* / *Pm* male progeny were individually crossed to females of the deficiency stock. Progeny heterozygous for the deficiency and mutagenized chromosome are a phenotypically distinct class, Pm^+ Cy^+. The absence of this phenotypic class in progeny from single fly matings was an indication that a lethal recessive mutation within the cytological bounds of *Df(2L)TE 62x2* had been generated. Pm^+ Cy flies from these crosses, representing chromosomes carrying putative lethal mutations over a balancer, were used to generate

balanced stocks which were maintained and crossed to the deficiency to confirm the mutation.

A total of 99 individual lethals, 10% of the chromosomes screened, and no visible mutations, were recovered. When tested, none of the mutations demonstrated an increase in viability at 21°C. Three of the lethal mutation stocks were lost before being fully characterized.

Deletion Analysis of Lethal Mutations

Crosses to the overlapping deficiencies *Df(2L)TE62x1, Df(2L)Az, Df(2L)spd*^{rk}, and *Df(2L)SS1* were undertaken to delimit the region within which a particular recessive resided.

$$\frac{\textit{lethal}}{\textit{SM1}} \quad X \quad \frac{\textit{Deficiency}}{\textit{SM1}}$$

$$\Downarrow$$

$$\frac{\textit{lethal}}{\textit{SM1}} \; ; \quad \frac{\textit{Deficiency}}{\textit{SM1}} ; \quad \frac{\textit{SM1}}{\textit{SM1}} ; \quad \frac{\textit{lethal}}{\textit{Deficiency}}$$

Balanced lethal flies were mated to flies from the deficiency stock, and the progeny were observed. As outlined above, the lethal/deficiency heterozygotes comprise the only Cy^+ phenotypic class. Absence of Cy^+ offspring from the cross indicates the heterozygote is lethal, with the most likely explanation that the lethal falls within the limits of the deficiency. Figure 4 is a map of the overlapping deficiencies used, and the three cytological regions, A, B and C, that they delimit. Table 17 (See Appendix B) summarizes the results of the

deficiency analysis.

The 99 lethals fall within three groups. 54 are within A (27E5-F1;28B3-4), five are within B (28B3-4;28C), and 37 are within C (28C;28D3-4).

Complementation Analysis

Complementation analysis was carried out among lethals within each cytological region.

$$\frac{\textit{lethal 1}}{\textit{SM1}} \quad X \quad \frac{\textit{lethal 2}}{\textit{SM1}}$$

$$\Downarrow$$

$$\frac{\textit{lethal 1; lethal 2}}{\textit{lethal 2}} \quad \frac{\textit{lethal 1;}}{\textit{SM1}} \quad \frac{\textit{lethal 2;}}{\textit{SM1}} \quad \frac{\textit{SM1}}{\textit{SM1}}$$

The *trans* heterozygote of the two mutants would be Cy^+. Absence of Cy^+ progeny among 50 progeny was interpreted as failure of the mutants to complement, and the lethals were classified as allelic. (If the two lethals are not allelic, the probability of obtaining a Cy^+ offspring is .33. The probability of a brood of 50 with no Cy^+ offspring is $1-(1/3)^{50} \leq 0.0005$.)

Group B and C Mutants

Complementation analysis performed among the five Group B mutants yielded three complementation groups within the 28B3-4;28C cytological region.

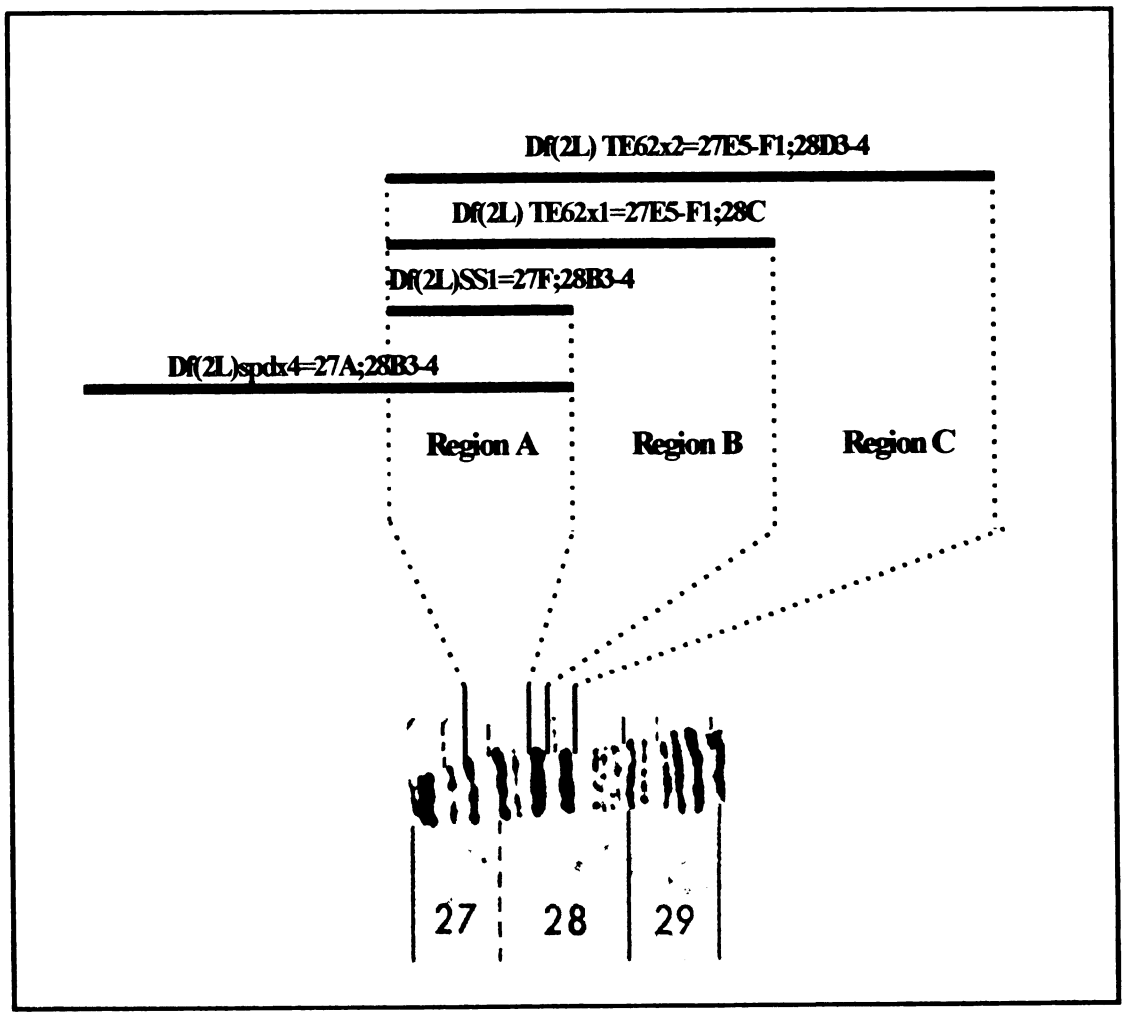


Figure 4 Overlapping Deficiencies

Table 4 Group B Complementation Groups

Group B Mutations	
Complementation Group 1	l(2)13 and l(2)69
Complementation Group 2	l(2)16 and l(2)83
Complementation Group 3	l(2) 61

Complementation analysis performed among the 27 Group C mutants yielded at least six complementation groups within the 28C;28D3-4 cytological region.

Table 5 Group C Complementation Groups

Group C Mutations	
Complementation Group 1	l(2)8, l(2)25, l(2)34, l(2)45, l(2)46
Complementation Group 2	l(2)23, l(2)32, l(2)47, l(2)79
Complementation Group 3	l(2)20, l(2)21, l(2)49
Complementation Group 4	l(2)24, l(2)80, l(2)82
Complementation Group 5	l(2)18, l(2)84
Complementation Group 6	l(2)57, l(2)65

Eight other mutants (lethals 26, 39, 48, 51, 56, 73, 87, 95) complemented all other Group C mutations. Presumably they each represent separate loci within the 28C;28D3-4 cytological region. However, there were several mutants among the Group C mutations that could not be recrossed to *Df(2L)TE62x2* after the initial isolation in the mutagenic screen. Loss of the deficiency occurred in

this laboratory, and efforts to regain the stock from the labs of Guenther Reuter, Trudi Schüpbach, and others to whom the stock had been released were unsuccessful, as none of the labs carrying this stock had managed to maintain it.

Group A Mutants

Among group A mutations, 48 exhibited the dominant *Sp* phenotype, and were designated as Complementation Group 1A. The alleles that exhibit the *Sp* phenotype are:

l(2)3, l(2)4, l(2)5, l(2)6, l(2)9, l(2)11, l(2)12, l(2)14, l(2)15, l(2)17, l(2)19, l(2)22, l(2)27, l(2)28, l(2)29, l(2)33, l(2)35, l(2)36, l(2)37, l(2)38, l(2)40, l(2)42, l(2)43, l(2)44, l(2)52, l(2)53, l(2)54, l(2)55, l(2)58, l(2)59, l(2)60, l(2)62, l(2)64, l(2)66, l(2)67, l(2)68, l(2)70, l(2)74, l(2)75, l(2)76, l(2)77, l(2)78, l(2)81, l(2)85, l(2)90, l(2)91, l(2)94 and *l(2)96.*

Four mutants, *l(2)1, l(2)7, l(2)10* and *l(2)71*, failed to complement the recessive lethality of *Sp* but lacked the dominant bristle phenotype; these were determined to be alleles of *wg*, based on the presence of an adult viable wingless phenotype when in *trans* with wg^1, and were designated as Complementation Group 2A. A third group, Complementation Group 3A, consisted of a single mutation, *l(2)31*.

Further Complementation Analysis of the *wg* region

The recovery of 48 alleles of the dominant marker *Sp* in the mutagenesis was unexpected. Failure of complementation between *Sp* and *wg* alleles confirmed the complicated pattern of overlapping non-complementation uncovered during a previously reported genetic analysis of this region (Tiong and Nash 1990). A more extensive complementation analysis of the region, summarized in Table 6, using other available markers, was undertaken in an attempt to further characterize the region.

Genetic interactions between *Sp* and other markers near 28A became apparent during complementation analysis on Group A mutants. As illustrated in Figure 5, spd^{flg} homozygotes have a rounded paddle-shaped wing (panel b), when compared to wild type (panel a). spd^{flg}/Sp flies manifest a narrowed wing phenotype (panel c), that differs from the spd^{flg} homozygotes. $l(2)^{Wg}$, isolated during the *Gart* mutagenesis (Tiong and Nash 1990), has a nicked wing phenotype in *trans* with alleles of *Sp*. $l(2)71^{wg}$, isolated in this mutagenesis, is mostly lethal over *Sp*, but one rare escaper had the same nicked wing phenotype. When in *trans*, *Sp* and wg^{cx4} cause a crinkled effect on the fly wing that isn't observed with either mutant alone (panel d). Experiments designed to more fully characterize this particular interaction are described in Chapter 3.

Table 6 Complementation Analysis of 28A Region Mutants

	allele	phenotype	source
1	wg1	weak wingless	A. Bejsovec
2	wg-cx4	lethal wingless	A. Bejsovec
3	l(2)1	lethal wingless	R. Wagner
4	l(2)7	lethal wingless	R. Wagner
5	l(2)10	lethal wingless	R. Wagner
6	l(2)71	lethal wingless	R. Wagner
7	l(2)H	lethal wingless	D. Nash
8	spd cui	spade curvi	T. Friedman
9	spd-flg Sp	Sternopleural	T. Friedman
10	l(2)4	Sternopleural	R. Wagner
11	l(2)28	Sternopleural	R. Wagner
12	l(2)29	Sternopleural	R. Wagner
13	ade3-27	deficiency	D. Nash

	1	2	3	4	5	6	7	8	9	1	11	12	13
1	wg												
2	wg	††											
3	wg	††	††										
4	wg	††	††	††									
5	wg	††	††	††	††								
6	wg	††	††	††	††	††							
7	wg	††	††	††	††	††	††						
8	+	+	+	spd	spd	+	+	flg					
9	Sp	cp	cp	+	††	N-1	N	spd	††				
10	+	cp	††	††	††	††	N	spd	††	†			
11	Sp	cp	††	††	††	††	N	spd	††	†	††		
12	Sp	cp	††	††	††	††	N	spd	††	†	††	††	
13	+	+	+	††	††	+	+	+	+	+	+	+	††

cp = crinkled wing phenotype
spd = narrowed spade wing
†† = lethal

N= notched wing
+ = wild type
Sp = Sternopleural

N-1=lethal with notched escapers
wg=adult viable wingless
flg=spd^{flg}

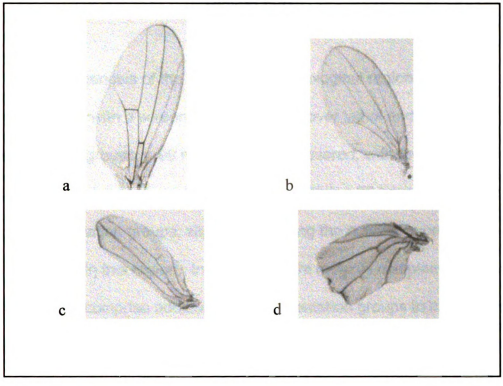


Figure 5 Wing Phenotypes

Allelism with the *Gart* set of mutants

Five lethal EMS-induced mutations in polytene section 27F-28B3-4 were generated in a screen for *Gart* mutants by S. Tiong and D. Nash (Tiong and Nash 1990). These are named *H, I, J, K* and *L*, with *l(2)H* corresponding to an allele of *wg*. Complementation analysis of each of these mutants with *l(2)31, l(2)61, l(2)69* and *l(2)83* revealed that all of the Nash mutants complement *l(2)31* and the Group B mutants. The Nash and Tiong mutants were recovered as lethals over *Df(2L)J136-H52*, which has its proximal breakpoint at 28B3-4, and so are unlikely to be allelic with Group C mutants.

Discussion

An EMS mutagenesis of the 27F1;28D3-4 cytological region of chromosome 2 was undertaken with a screen designed to uncover lethal and visible complementation groups. 99 mutants were recovered, with three being lost before being completely characterized. The remaining 96 have been assigned to 12 complementation groups, slightly exceeding the number expected based on the ten bands in this region. In addition, eight mutants complement all other groups, and may comprise additional complementation groups in the region, although the unfortunate loss of *Df(2L)TE62x2* makes it difficult to confirm that these lethal loci reside within the cytological region.

Saturating the 27F3-4;28D3-4 Region

Based on a Poisson distribution, by isolating 96 mutants I have exceeded a 99% probability of uncovering at least one mutation in each recoverable gene in the region. However, an assumption inherent in this calculation is equal mutability for each potentially lethal locus, and there is strong evidence that EMS, like other alkylating agents, mutates in a non-random fashion (Benzer 1959, 1961, Singer and Grunberger 1983). Careful and repeated attempts to isolate EMS-induced lethal alleles of some genes, notably *gooseberry (gsb)* and *decapentaplegic (dpp)*, have demonstrated that some genes are refractory to the mutagenic effects of EMS, although lethal alleles are recoverable with other

mutagens (Nüsslein-Volhard et al. 1984, Spencer et al. 1982). The unexpectedly large number of *Sp* alleles that were isolated further complicates the interpretation of saturation. Disregarding the 49 alleles of *Sp*, only 51 individually arising mutants, comprising at least 11 complementation groups, were isolated.

Even in the event that saturation for mutable lethals was attained, there remain many genes that are refractory to lethal analysis. Supressors, enhancers, and female and male sterile mutants are often not uncovered by lethal screens. There are also a wide range of behavioral and anatomical mutants that have required sophisticated screening analyses to recover, including larval morphology mutants (Wieschaus and Nüsslein-Volhard 1980), neurological mutants (Trout and Kaplan 1969, Wu and Ganetzky 1980, Wu et al. 1978), vision mutants (Meyerowitz et al. 1980), and mutants for grooming behavior and memory (Gailey et al. 1984). Lastly, there are documented examples of visible loci for which lethal alleles simply aren't recoverable, even after repeated attempts with a wide variety of mutagens (Perrimon et al. 1989)

Characterization of Mutants

The mutagenesis screen in the 27F;28D3-4 cytological region has uncovered at least 12 complementation groups, some of which correspond to previously identified genes in the region. Crosses with the overlapping deficiencies *TE62x2* (27E5-F1;28D3-4), *TE62x1* (27E5-F1;28C) and *SS1* (27F;28B3-4) were

used to delimit the cytological limits of the complementation groups.

Group A Mutants -- Polytene section 27F;28B3-4

The Group A mutants are lethal over *Df(2L)TE62x2, Df(2L)TE62x1* and *Df(2L)SS1*, limiting their cytological location to 27F-28B3-4. Four mutants fail to complement the lethality of *Sp*, although they lack the dominant bristle phenotype. These mutants also fail to complement wg^{1}, a weak wingless allele, and therefore comprise new *wg* alleles. Cuticle preps of these indicate that $l(2)wg^{71}$ has a weak wg phenotype similar to some previously identified *wg* alleles and $l(2)wg^{10}$ looks like a null allele. This allele is currently undergoing sequence analysis to identify the molecular lesion (A. Bejsovec, pers. comm.). During complementation analysis, $l(2)wg^{7}$ failed to complement a large number of separate loci, indicating that it is most likely a deficiency. $l(2)wg^{1}$ and $l(2)wg^{71}$ complement spd^{flg}, described as a regulatory mutant of *wg*. $l(2)wg^{10}$ does not complement spd^{flg}. All four new alleles of *wg* fail to complement the lethality of *Sp*. 49 alleles of *Sp* have been isolated. A single mutant, *l(2)31*, comprises the third complementation group in this region. Crosses with mutants *G, H, I, J, K* and *L* from the Nash mutagenesis indicates that *l(2)31* complements all of these.

Complementation analysis of *Sp* alleles with previously isolated alleles of *wg* and *spd* confirm that this is a complex genetic region. The alleles of *wg* generated in this mutagenesis fail to complement the recessive lethality of *Sp*, and the null allele wg^{cx4} has a novel crinkled wing phenotype when in *trans* with *Sp*. However, the weak allele wg^{1} complements *Sp*. Failure of complementation

is generally interpreted as evidence of allelism, but the overlapping pattern of complementation and noncomplementation makes discerning whether *Sp* and *wg* are allelic difficult.

Group B mutants -- Polytene section 28B3-4;28C

This region is uncovered by the overlapping deficiencies *Df(2L)TE62x2* and *Df(2L)TE62x1*, but is outside of *Df(2L)SS1*. Three complementation groups were assigned to this region, with one, two and two alleles, respectively. Complementation analysis has demonstrated that none of these are allelic with the mutations *G, H, I, J, K* or *L* reported by Nash (Tiong and Nash 1990).

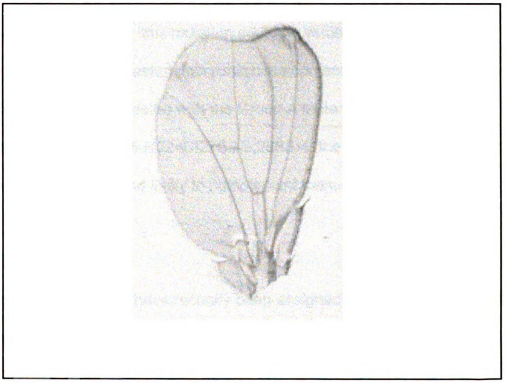


Figure 6 l(2)24 Homozygous Survivor

Group C mutants -- Polytene section 28C;28D3-4

The mutants in this region are uncovered by *Df(2L)TE62x2*, but neither *Df(2L)TE62x1* nor *Df(2L)SS1*. There are six complementation groups that fall within this region, with five, four, three, three, two and two alleles. In addition, eight mutants failed to complement all others, although it was not possible to confirm that they actually fall within this region due to loss of *Df(2L)TE62x2*. *l(2)24*, a member of Complementation Group C4, shows rare homozygous survivors with a clipped wing phenotype, and could represent a hypomorphic allele of that gene (Figure 7). No wing mutations have previously been reported to map in the C region. As many wing mutations turn out to be genes involved in developmental cascades, this mutation could be valuable in the ongoing genetic dissection of *D. melanogaster* embryonic development. Allelism with the Nash mutations has not been tested with the Group A mutants, which are outside the boundaries of *Df(2L)J136-H52*=Df27C2-9;28B3-4, the deficiency used to recover Nash's set of mutants, and likely to complement them.

Reconciling maps

Genetic markers that have recently been assigned to the Group A, B and C cytological regions include *cuckold, gelding, Enhancer of polycomb, TATA binding protein related factor, midline uncoordinated*, and *Enhancer of variegation 28AB*. None of these have been tested for allelism with this EMS generated set of mutants. In addition, the possibility that *l(2)31* is allelic to *ninaC*

has not yet been tested.

The majority of the markers in the 28A-28D region are molecular, with no known corresponding mutants. Reconciliation of molecular and genetic maps is challenging as typically no prediction of the type of phenotype to be expected can be made based on sequence data. If PCR primers can be designed for the molecular markers of interest, DNA isolated from flies from the Oregon-R parental stock and mutants potentially corresponding to the gene can be amplified and sequenced. Such an undertaking would be labor-intensive, and in addition may not be informative, as finding a correlative discrepancy is not sufficient evidence to demonstrate that a phenotype is a direct result of a molecular lesion. In human gene analysis, highly polymorphic sites surrounding genes often confuse and confound efforts to demonstrate relationships between phenotypes and molecular markers. Even when the link between gene and phenotype is already documented, as in the case of many of the hemoglobinopathies, demonstrating that a particular sequence discrepancy causes a phenotype, and is not just a circumstantial polymorphism, can be problematic. Although an effort was made in generating these mutants to start with molecularly identical chromosome sets, no guarantees can be made that the accumulative effect of random mutation during passage of the stocks has not resulted in the generation of polymorphisms.

Complex Nature of the 28A Region

A formal genetic analysis of the 27F1;28D3-4 cytological region has been carried out (with a concurrent limited molecular examination of the same region, as described in Appendix A). The initial purpose of these investigations, to characterize the number and arrangement of genes in this polytene section, has not been fulfilled. However, the genetic analysis has confirmed the complex nature of the 28A region that has previously been described (Tiong and Nash 1990, Matsumoto et al. 1987). A complicated pattern of overlapping non-complementation between alleles of *spd, wg, Sp*, and *ninaC* make it impossible to ascertain by classical genetic means how many genes are in this region. This is a particularly intriguing aspect of the biology of this polytene section, and further exploration into some of these interactions are described in Chapter 3.

References

Alderson, T., 1965 Chemically induced delayed germinal mutation in *Drosophila*. Nature **207**: 164-169.

Benzer, S., 1959 On the topology of the genetic fine structure. Proc. Natl. Acad. Sci. USA. **45**: 1607-1620.

Benzer, S., 1961 On the topography of the genetic fine structure. Proc. Natl. Acad. Sci. USA. **47**: 403-415.

Berg and A. Spradling, 1989 *kudu* mutants produce eggs with fused or thin filaments. A. Conf. Dros. Res. **30:** 11.

Buratovich, M. Phillips, R. and J. Whittle, 1996 Genetic analysis suggests that *spade* is a regulatory allele of *wingless* and that *Sternopleural* is an independent adjacent gene with synergistic effects. A. Conf. Dros. Res. **37**: 286.

Castrillon, D. H., Gönczy, P., Alexander, S., Rawson, R., Eberhat, C. G., Viswanathan, S., DiNardo, S., and S. A. Wasserman, 1993 Toward a molecular genetic analysis of spermatogenesis in *Drosophila melanogaster*: Characterization of male-sterile mutants generated by single P-element mutagenesis. Genetics **135**: 489-505.

Crowley, T. E., Hoey, T., Liu, J.-K., Jan, Y. N., Jan, L. Y. and R. Tjian, 1993 A new factor related to TATA-binding protein has highly restricted expression patterns in *Drosophila*. Nature **361**: 557-561.

Doane, W. W., 1960 Report. Dros. Inf. Serv. **34**: 25.

Doane, W. W., 1961 Report. Dros. Inf. Serv. **35**: 45-46.

Dudler, T., Schmidt, T., Bienz, M. and E. Kubli, 1981 The genes coding for tRNA:tyrosine of *Drosophila melanogaster*: Localization and determination of the gene numbers. Chromosoma **84**: 49-60.

Fauvarque, M. O. and J. M. Dura, 1993 *polyhomeotic* regulatory sequences induce developmental regulator-dependent variegation and targeted P-element insertion in *Drosophila*. Genes Dev. **7**: 1508-1520.

Gailey, D. A., Jackson, F. R. and R. W. Siegel, 1984 Conditioning mutations in *Drosophila melanogaster* affect an experience-dependent behavioral modification in courting males. Genetics **106**: 613-623.

Gausz, J., Bencze, G., Byurkovics, H., Ashburner, M., Ish-Horowicz, D. and J. J. Holden, 1979 Genetic characterization of the 87C region of the third chromosome of *Drosophila melanogaster*. Genetics **93**: 917-934.

Gausz, J., Hall, L. M. C., Spierer, A. and P. Spierer, 1986 Molecular genetics of the *rosy-Ace* region of *Drosophila melanogaster*. Genetics **112**: 43-64.

Gvozdev, V. A., Gotimsky, S. A., Gerasimova, T. I., Drubovskaya, E. S. and O. Y. Braslavskaya, 1975 Fine genetic structure of the 2D3-2F5 region of the X-chromosome of *Drosophila melanogaster*. Mol. Gen. Genet. **141**: 269-275.

Hayashi, S., Gillam, I. C., Delaney, A. D. and G. M. Tener, 1978 Acetylation of chromosome squashes of *Drosophila melanogaster* decreases the background in autoradiograms from hybridization with [^{125}-I]-labeled RNA. J. Histochem. Cytochem. **26**: 677-679.

Hayashi, S., Gillam, I. C., Delaney, A. D., Dunn, R., Tener, G. M., Grigliatti, T. A. and D. T. Suzuki, 1980 Hybridization of tRNAs of *Drosophila melanogaster* to polytene chromosomes. Chromosoma **76**: 65-84.

Hayashi, S., Addison, W. R., Gillam, I. C., Grigliatti, T. A. and G. M. Tener, 1981 Hybridization of tRNAs of *Drosophila melanogaster* to the region of the 5S RNA genes of the polytene chromosomes. Chromosoma **82**: 385-387.

Hilliker, A. J., Clark, S. H., Chovnick, A. and W. M. Gelbart, 1980 Cytogenetic analysis of the chromosomal region immediately adjacent to the *rosy* locus in *Drosophila melanogaster.* Genetics **95**: 95-110.

Judd, B. H., Shen, M. W. and T. C. Kaufman 1972 The anatomy and function of a segment of the X chromosome in *Drosophila melanogaster*. Genetics **71**: 139-156.

Kotarski, M. A., Pickert, S. and R. J. MacIntyre, 1983 A cytogenetic analysis of the chromosomal region surrounding the α-glycerophosphate dehydrogenase mutants in *Drosophila melanogaster*. Genetics **105**: 371-386.

Kaplan, W. D. and W. E. Trout, 1969 The behavior of four neurological mutants of *Drosophila*. Genetics **61**: 399-409.

Kubli, E., 1991 Mitochondrial fusion protein. Europ. Dros. Res. Conf. **12**: 109.

Lefevre, G., Jr., 1981 The distribution of randomly recovered X-ray-induced sex-linked genetic effects in *Drosophila melanogaster*. Genetics **99**: 461-480.

Lefevre, G., Jr. and M. M. Breen, 1972 Genetic duplication in the *white-split* interval of the X chromosome in *Drosophila melanogaster*. Chromosoma **36**: 391-412.

Lewis, E. B. and F. Bacher, 1968 Method of feeding ethyl methane sulfonate (EMS) to *Drosophila* males. Dros. Inf. Serv. **43**: 193.

Lifschytz, E. and R. Falk, 1969 Fine structure analysis of a chromosome segment in *Drosophila melanogaster*. Analysis of ethyl methanesulphonate-induced lethals. Mut. Res. **8**: 147-155

Lim, J. K. and L. A. Snyder, 1968 The mutagenic effects of two monofunctional alkylating chemicals on mature spermatazoa of *Drosophila*. Mut. Res. **6**: 129-137.

Lim, J. K. and L. A. Snyder, 1974 Cytogenetic and complementation analyses of recessive lethal mutations induced in the X chromosome of *Drosophila* by three alkylating agents. Genet. Res. **24**: 1-10.

Lindsley, D.L., Sandler, L., Baker, B., Carpenter, A. T. C., Denell, R. E., Hall, J. C., Jacobs, P. A., Miklos, G. L. G., Davis, B. K., Gethmann, R. C., Hardy, R. W., Hessler, A., Miller, S. M., Nozawa, H., Parry, D. M. and M. Gould-Somero, 1972 Segmental aneuploidy and the genetic gross structure of the *Drosophila* genome. Genetics **71:** 157-184.

Lindsley, D. L. and G. G. Zimm, 1992 The genome of Drosophila melanogaster. Academic Press, Inc. San Diego, California.

Liu, C. P. and J. K. Lim, 1975 Complementation analysis of methyl methanesulphonate-induced recessive lethal mutations in the *zeste-white* region of the X chromosome of *Drosophila melanogaster*. Genetics **79**: 601-611.

Mann, M., 1923 The occurrence and hereditary behavior of two new dominant mutations in an inbred strain of *Drosophila melanogaster*. Genetics **8**: 27-36.

Matsumoto, H., Isono, K., Pye, Q., and W. L. Pak, 1987 Gene encoding cytoskeletal proteins in *Drosophila* rhabdomeres. Proc. Natl. Acad. Sci. USA. **84**: 985-989.

Mayer-Jaekel, R. E., Baumgartner, S., Bilbe, G., Ohkura, H., Glover, D. M. and B. A. Hemmings, 1992 Molecular cloning and developmental expression of the catalytic and 65-kDA regulatory subunits of protein phosphatase 2A in *Drosophila*. Molec. Biol. Cell. **3:** 287-298.

Meyerowitz, E. and D. Kankel, 1978 A genetic analysis of visual system development in *Drosophila melanogaster*. Dev. Biol. **62**: 112-142.

Montell, C. and G. M. Rubin, 1988 The *Drosophila ninaC* locus encodes two photoreceptor cell specific proteins with domains homologous to protein kinases and the myosin heavy chain head. Cell **52**: 757-772.

Montell, D. J., and C. S. Goodman, 1988 *Drosophila* substrate adhesion molecule: sequence of Laminin B1 chain reveals domains of homology with mouse. Cell **53**: 463-473.

Nicoletti, M, 1957 Report. Dros. Inf. Ser. **31**: 84.

Nivard, M. J. M., Pastik, A. and E. W. Vogel, 1992 Molecular analysis of mutations induced in the vermilion gene of *Drosophila melanogaster* by ethyl methanesulfonate. Genetics **131**: 673-682.

Nüsslein-Volhard, C. and E. Wieschaus, 1980 Mutations affecting segment number and polarity in *Drosophila*. Nature **287**: 795-801.

Nüsslein-Volhard, C., Wieschaus, E. and H. Kluding, 1984 Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. I. Zygotic loci on the second chromosome. Roux's Arch. Dev. Biol. **193**: 267-282.

Orgad, S., Brevis, N. D., Alphey, L., Axton, J. M., Dudai, Y. and P. T. W. Cohen, 1990 The structure of protein phosphatase 2A is as highly conserved as that of protein phosphatase. FEBS. **275**: 44-48.

Pardue, M. L. and I. B. Dawid, 1981 Chromosomal location of two DNA segments that flank ribosomal insertional-like sequences in *Drosophila*: Flanking sequences are mobile elements. Chromosoma **83**: 29-43.

Pardue, M. L., Lowenhaupt, K. Rich, A. and A. Nordheim, 1987 $(dC\text{-}dA)_n$-$(dG\text{-}dT)_n$ sequences have evolutionarily conserved chromosome locations in *Drosophila* with implications for roles in chromosome structure and function. EMBO. **6**: 1781-1789.

Pastink, A. Heemskerk, E., Nivard, M. J. M., van Vliet, C. J. and E. W. Vogel, 1991 Mutational specificity of ethyl methanesulphonate in excision-repair-proficient and -deficient strains of *Drosophila melanogaster*. Mol. Gen. Genet. **229**: 213-218.

Perrimon, N., Smouse, D. and G. L. G. Miklos, 1989 Developmental genetics of loci at the base of the X chromosome of *Drosophila melanogaster*. Genetics **121**: 313-331.

Phillis, R. W., Bramlage, A. T., Wotus, C., Whittaker, A, Gramates, L. S., Seppala, D., Farahanchi, R., Caruccio, P. and R. K. Murphey, 1993 Isolation of a mutation affecting neural circuitry required for grooming behavior in *Drosophila melanogaster.* Genetics **133**: 581-592.

Pikielny, C. W., Hasan, G., Rouyer, F. and M. Rosbash, 1994 Members of a family of *Drosophila* putative odorant-binding proteins are expressed in different subsets of olfactory hairs. Neuron **12**: 35-49.

Pirrotta, V., 1988 Vectors for P-mediated transformation in *Drosophila*. In: Vectors: A survey of molecular cloning vectors and their uses. eds. Rodriguez, R. L. and D. T. Denhardt (Buttersorths, Boston) pp. 437-456.

Porter, J. A., Minke, B. and C. Montell, 1995 Calmodulin binding to *Drosophila ninaC* required for termination of phototransduction. EMBO. **14**: 4450-4459.

Porter, J. A., Yu, M., Doberstein, S. K., Pollard, T. D. and C. Montell, 1993 Dependence of calmodulin localization in the retina on the *ninaC* unconventional myosin. Science **262**: 1038-1042.

Qui, J. and P. E. Hardin, 1995 Temporal and spatial expression of an adult cuticle protein gene from *Drosophila* suggests that its protein product may impart some specialized cuticle function. Dev. Biol. **167**: 416-425.

Rayle, R. E., 1972 Genetic analysis of a short X-chromosomal region in *Drosophila melanogaster.* Genetics **71(Suppl.)**: s50.

Singer, B. and D. Grunberger, 1983 Molecular Biology of Mutagens and Carcinogens, Plenum Press, New York.

Spencer, F. A., Hoffmann, F. M. and W. M. Gelbart, 1982 *Decapentaplegic*: A gene complex affecting morphogenesis in *Drosophila melanogaster.* Cell **28**: 451-461.

Stephenson, R. S., O'Tousa, J. E., Scavarda, N. J., Randall, L. L. and W. L. Pak, 1983 *Drosophila* mutants with reduced rhodopsin content. In: Biology of Photoreceptors, D. Cosens and C. Vince-Prue, eds. Cambridge: Cambridge University Press. pp. 471-495.

Tiong, S. and D. Nash, 1990 Genetic analysis of the *adenosine3 (Gart)* region of the second chromosome of *Drosophila melanogaster*. Genetics **124**: 889-897.

Whittinghill, M., 1950 Two crossover-selector systems: New tools in genetics. Science **111**: 377-378.

Woodruff, R. C. and M. Ashburner, 1979 The genetics of a small autosomal region of *Drosophila melanogaster* containing the structural gene for *alcohol dehydrogenase*. II. Lethal mutations in the region. Genetics **92**: 133-149.

Wu, C. F. and B. Ganetzky, 1980 Genetic alteration of nerve excitability in temperature-sensitive paralytic mutants of *Drosophila melanogaster*. Nature **286**: 814-816.

Wu, C. F., Ganetzky, B., Jan, L. Y., Jan, Y. N. and S. Benzer, 1978 A *Drosophila* mutant with a temperature-sensitive block in nerve conduction. Proc. Natl. Acad. Sci. USA. **75**: 4047-4051.

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

Young, M. W. and B. H. Judd, 1978 Nonessential sequences, genes, and the polytene chromosome bands of *Drosophila melanogaster*. Genetics **88**: 723-742.

Zhimulev, I. F., Pokholkova, G. V., Bgatov, A. V., Semeshin, V. F. and E. S. Belyaeva, 1981 Fine cytogenetical analysis of the band 10A1-2 and the adjoining regions in the *Drosophila melanogaster* X chromosome. II. Genetical analysis. Chromosoma **82**: 25-40.

Chapter 3

The *Sp / wg* Interaction

During complementation analysis among lethals in the 27F;28B3-4 polytene section, a complicated pattern of overlapping non-complementation between alleles of *spade (spd), wingless (wg)*, and *Sternopleural (Sp)* was revealed, confirming literature reports of the same (Tiong and Nash 1990). *Sp* is a recessive lethal with a dominant bristle phenotype manifesting as thickened and excess sternopleural setae, *wg* is a recessive gene with many alleles, ranging in phenotype from incompletely penetrant wing loss to lethality, and *spd* is a recessive gene, that when mutant gives rise to shortened paddle-shaped wings (Mann 1923, Sharma 1973, Babu 1977, Doane 1960). Based on similar phenotypes, close linkage and failure of some alleles to complement, *spd* and *wg* have recently been reported to be allelic (Neumann and Cohen 1996). Complementation analysis of three new *wg* alleles isolated in this mutagenesis indicate that $l(2)1^{wg}$ and $l(2)71^{wg}$ complement spd^{flg}, while $l(2)10^{wg}$ does not. *Trans* heterozygotes for *Sp* and spd^{flg} have a spade-like narrowed wing,

intermediate in phenotype between wild type and paddle-shaped wings. *ninaC*, a visual mutant that is located at 28A, also fails to complement the lethality of *Sp* (Matsumoto et al. 1987). In addition, wg^{cx4}, an amorphic *wg* allele missing a large portion of the 5' coding region, fails to complement *Sp*. The wg^{cx4}*/Sp trans* heterozygotes have been reported to be missing dorsocentral setae, a novel bristle phenotype not seen in either mutant alone (Neumann and Cohen 1996). In my hands I have not observed that *Sp/*wg^{cx4} *trans* heterozygous flies experience loss of dorsocentral setae. Instead they have the dominant sternopleural bristle phenotype and present with severely reduced and shriveled wings. These complicated complementation patterns are reminiscent of data uncovered during genetic analyses of *Ultrabithorax, Antennapedia* and *Star*, and make it impossible to ascertain by classical means how many genes reside in the region (Lewis 1945, 1951, 1955, Kaufman et al. 1990).

Out of these many complex genetic interactions at 28A, I chose to focus particularly on the wing effect in the *Sp/*wg^{cx4} heterozygote, and undertook further investigations into the genetic interaction resulting in the shriveled wing phenotype. Noncomplementation of *trans* mutants is generally interpreted as evidence of allelism. However, separate, functionally related loci may also demonstrate noncomplementation reflective of interactions at the protein level (Lewis 1965).

At least two hypotheses can be invoked to explain the interaction between *Sp* and *wg*:

1. *wg* and *Sp* code for interacting proteins

   e.g. *Sp* could be part of a multiprotein receptor complex, or another gene involved in the signalling pathway

2. *wg* and *Sp* are the same gene

In order to elucidate which circumstance is responsible for the shriveled wing phenotype, an attempt was initiated to recover a rare *cis* double mutant recombinant of *Sp* and wg^{cx4}. The phenotype of the heterozygous double mutant should reveal the nature of the relationship.

1. If *wg* and *Sp* code for interacting proteins, no difference in phenotype is expected whether the mutants are in *cis* or *trans*.
2. If *wg* and *Sp* are the same gene, the double mutant in *cis* should lack the shriveled phenotype.

A mating strategy for isolating a rare *cis* double mutant between *Sp* and wg^{cx4} was devised, with screening facilitated by flanking lethal markers that eliminate non-recombinant progeny. Five new chromosomes were necessary to implement the strategy, and their construction is described. Unexpectedly, recovering a *Sp* wg^{cx4} *cis* double mutant proved to be intractable, due to sterility of *Sp* / wg^{cx4} *trans* heterozygotes. Although delaying the generation of the *cis*

double mutant that would effectively distinguish the *Sp* and wg^{cx4} interaction, the male and female sterile phenotype provided an intriguing glimpse into a putative role for the Wg ligand during oogenesis and spermatogenesis. Characterization was conducted of the fertility phenotype that serves as a first step toward elucidating what this role might be.

In light of the inability to generate a *Sp*-wg^{cx4} double mutant, experiments were carried out using the well characterized temperature sensitive allele wg^{IL114}. At the restrictive temperature of 27°C, the *trans* heterozygotes mimic the sterility and shriveled wing phenotype seen with wg^{cx4}/*Sp*. However, this *wg* allele is fertile in *trans* with *Sp* at permissive temperatures, which may allow the generation of a *cis* double mutant whose phenotype will reveal whether the interaction between *Sp* and *wg* is at the nucleotide or protein level.

Approximately 30 well characterized alleles of wg have been reported in the literature. The alleles that are relevant to my work are listed in Table 7.

Table 7 *wingless* Alleles

Allele	Generation	Notes
wg^{1}	EMS	original allele, adult viable, variable penetrance (Sharma 1973)
wg^{CP1}	hybrid dysgenesis	P-element tagged allele used to isolate lambda clones of wg, embryonic lethal (Baker 1987)
wg^{cx4}	X-ray	null embryonic lethal, small deletion that extends into the 5' coding region, no RNA or protein detectable in homozygous embryos (Baker 1987)
wg^{IL114}	EMS	temperature sensitive, homozygous adult viable at permissive temperature of 16°C, lethal at restrictive temperature of 18°C, Cys104→Ser, no protein export at restrictive temperature (Nüsslein-Volhard et al. 1984)

Methods And Results

Recovery of *Sp-wg* Recombinant

During previous fine structure analyses in *Drosophila,* flanking lethal markers have been used to eliminate non-recombinant progeny, simplifying the screening process (Whittinghill 1950, Chovnick et al. 1962, Green and Green 1949, 1956, 1961). Mimicking this strategy, I used two recessive lethal mutations, *l(2)G* at 27E1-2;27E3-F (Tiong and Nash 1990) and *l(2)23* at 28C;28D3-4, isolated in my EMS analysis. The flanking lethals were crossed onto *Sp* and wg^{cx4} chromosomes.

The following scheme was designed to recover a *Sp-wg* recombinant

$$\frac{l(2)23\ wg^{cx4}}{SM1} \quad X \quad \frac{Sp\ l(2)G}{SM1}$$

$$\Downarrow$$

$$\frac{l(2)23\ wg^{cx4}}{Sp\ l(2)G} \quad X \quad \frac{l(2)23\ l(2)G}{SM1}$$

Table 8 Punnett Square representing the offspring from the I(2)23 Sp / wg^{cx4} I(2)G X I(2)23 I(2)G / SM1 cross.

		I(2)23 G	SM1 (Cy)
Non Crossover	I(2)23 wg-cx4	††	Curly
	Sp G	††	Curly Sternopleural
Crossover	I(2)23 wg-cx4 G	††	Curly
	Sp	Sternopleural	Curly Sternopleural
	I(2)23 Sp G	††	Curly Sternopleural
	wg-cx4	wild type	Curly
	I(2)23 G	††	Curly
	Sp wg-cx4	Sternopleural	Curly Sternopleural

There are three viable crossover products. Two of these are Cy^+ Sp; one is the *Sp - wg^{cx4} cis* recombinant, the other is only Sp. Individual *Cy^+ Sp* flies would be mated to G wg^{cx4}/SM1 to test for the presence of the recessive wg marker.

$$\frac{Sp\ wg^{cx4}}{I(2)G\ I(2)23} \quad X \quad \frac{I(2)G\ wg^{cx4}}{SM1}$$

$$\Downarrow$$

$$\frac{Sp\ wg^{cx4}}{I(2)G\ wg^{cx4}}\dagger\dagger; \quad \frac{I(2)23\ I(2)G}{wg^{cx4}}\dagger\dagger; \quad \frac{Sp\ wg^{cx4}}{SM1}; \quad \frac{I(2)23\ I(2)G}{SM1}$$

$$\Downarrow$$

$$\sigma^{\prime}\ and\ \varphi \quad \frac{Sp\ wg^{cx4}}{SM1} \quad stock$$

If wg^{cx4} is not present, there will be a non-Curly Sternopleural progeny class, and these flies can be discarded. If wg^{cx4} is on the *Sp* chromosome, only Curly progeny emerge in the F1. The *Cy Sp* phenotypic class has the desired crossover chromosome balanced against SM1, and brother-sister matings will produce a stock. In the mutant Cy^{+} Sp, there is no balancer and there exists a low, but finite, probability of a crossover removing *wg*. To guard against the possibility of a second *Sp*-wg^{cx4} crossover event, and loss of wg^{cx4}, several F1 matings should be set up.

Although genetic evidence indicates that *Sp* should be distal to *wg*, prudence dictated that the screen to obtain a recombinant be carried out simultaneously for both possible orientations.

Chromosome Construction

It was necessary to construct five new chromosomes carrying flanking lethal markers for the screen to recover a *Sp-wg* recombinant. The construction strategy for each is outlined below. The presence of the dominant marker bw^{D} on each of the recessive marker chromosomes facilitated crossing the lethal markers onto the chromosomes by allowing testing for the presence of recessive markers and establishment of a balanced stock in a single step.

G-wg^{cx4} Chromosome

$$\frac{l(2)G}{SM1} \quad X \quad \frac{wg^{cx4}}{SM1}$$

$$\Downarrow$$

$$\frac{l(2)G}{wg^{cx4}} \quad X \quad \frac{l(2)G}{SM1}$$

$$\Downarrow$$

$$\frac{l(2)G\dagger\dagger}{l(2)G}; \quad \frac{l(2)G}{SM1}; \quad \frac{l(2)G}{wg^{cx4}}; \quad \frac{wg^{cx4}}{SM1}$$

$$\frac{+}{l(2)G}; \quad \frac{+}{SM1}; \quad \frac{l(2)G\ wg^{cx4}}{SM1}; \quad \frac{l(2)G\ wg^{cx4}\dagger\dagger}{l(2)G}$$

The desired crossover chromosome is scored as Curly progeny. To identify them, individual male Curly flies were mated to *l(2)G* bw^D/SM1 and wg^{cx4}-bw^D/SM1 female flies simultaneously.

$$\frac{l(2)G\ wg^{cx4}}{SM1} \quad X \quad \frac{l(2)G\ bw^D}{SM1}; \ \frac{wg^{cx4}\ bw^D}{SM1}$$

$$\Downarrow$$

$$\frac{l(2)G\ wg^{cx4}}{SM1}; \quad \frac{l(2)G\ wg^{cx4}\dagger\dagger}{l(2)G\ bw^D}; \quad \frac{SM1\dagger\dagger}{SM1}$$

$$\frac{l(2)G\ wg^{cx4}}{SM1}; \quad \frac{l(2)G\ wg^{cx4}\dagger\dagger}{wg^{cx4}\ bw^D}; \quad \frac{SM1\dagger\dagger}{SM1}$$

Those vials giving Cy^+ offspring were discarded, as the paternal chromosome in those crosses lacked either wg^{cx4} or the flanking lethal, or both. In vials with only Curly progeny, the bw^{D+} offspring were crossed, *inter se*, to produce a

putative *G-wg*^{cx4} stock balanced against SM1, which was subsequently confirmed by crossing individually to both *l(2)G* and *wg*^{cx4}. Observing lack of *Cy*$^{+}$ progeny in these crosses confirmed the presence of the recessive lethals on the chromosome.

wg^{cx4}*-23* Chromosome

This chromosome was constructed in an identical manner to the *G-wg*^{cx4} chromosome, except that *l(2)23* was used in place of *l(2)G*.

G-Sp Chromosome

$$\frac{l(2)G}{SM1} \times \frac{Sp}{SM1}$$

$$\Downarrow$$

$$\frac{l(2)G}{Sp} \times \frac{l(2)G}{SM1}$$

$$\Downarrow$$

$$\frac{l(2)G\,tt;}{l(2)G} \quad \frac{l(2)G;}{SM1} \quad \frac{l(2)G;}{Sp} \quad \frac{Sp}{SM1}$$

$$\frac{+\;;}{l(2)G} \quad \frac{+\;;}{SM1} \quad \frac{l(2)G\ Sp\,tt;}{l(2)G} \quad \frac{l(2)G\ Sp}{SM1}$$

Sp Cy flies were mated to *G bw^D / SM1* females

$$\frac{\textbf{l(2)G Sp}}{\textbf{SM1}} \quad \textbf{X} \quad \frac{\textbf{l(2)G bw}^D}{\textbf{SM1}}$$

$$\Downarrow$$

$$\frac{\textbf{l(2)G Sp††;}}{\textbf{l(2)G bw}^D} \qquad \frac{\textbf{l(2)G Sp;}}{\textbf{SM1}} \qquad \frac{\textbf{SM1††}}{\textbf{SM1}}$$

Those vials giving *Cy$^+$* offspring were discarded, as the paternal chromosome in those crosses lacked *l(2)G.* In vials with only Curly progeny, the offspring were mated, inter se, to produce a putative *G-Sp* stock balanced against SM1, which was subsequently confirmed by crossing to *l(2)G* and observing lack of *Cy$^+$* progeny.

Sp-23 Chromosome

This chromosome was constructed in an identical manner to *G-Sp*, except that *l(2)23* was used in place of *l(2)G.*

G-23 Chromosome

$$\frac{l(2)23\ bw^D}{SM1} \quad X \quad \frac{l(2)G}{SM1}$$

$$\Downarrow$$

$$\frac{l(2)G}{l(2)23\ bw^D} \quad X \quad \frac{l(2)G}{SM1}$$

$$\Downarrow$$

$$\frac{l(2)G\dagger\dagger}{l(2)G}; \quad \frac{l(2)G}{SM1}; \quad \frac{l(2)23\ bw^D}{l(2)G}; \quad \frac{l(2)23\ bw^D}{SM1}$$

$$\frac{+}{l(2)G}; \quad \frac{+}{SM1}; \quad \frac{bw^D}{l(2)G}; \quad \frac{bw^D}{SM1}$$

$$\frac{l(2)23}{l(2)G}; \quad \frac{l(2)23}{SM1}; \quad \frac{l(2)G\ l(2)23\ bw^D\dagger\dagger}{l(2)G}; \quad \frac{l(2)G\ l(2)23\ bw^D}{SM1}$$

Cy bw^D offspring were putative crossovers between *l(2)G* and *l(2)23*. Male progeny of this phenotype were mated to *l(2)G/SM1* and *l(2)23/SM1* females simultaneously.

$$\frac{l(2)G\ l(2)23\ bw^D}{SM1} \quad X \quad \frac{l(2)G}{SM1}; \ \frac{l(2)23}{SM1}$$

$$\Downarrow$$

$$\frac{l(2)G\ l(2)23\ bw^D}{SM1}; \quad \frac{l(2)G\ l(2)23\ bw^D\dagger\dagger}{l(2)G}; \quad \frac{l(2)G\ l(2)23\ bw^D}{l(2)23}$$

Vials with Cy^+ offspring lacked either *l(2)G* or *l(2)23* or both, and were discarded. In vials with only Curly offspring, Cy progeny were crossed, *inter se*, to produce a stock, and the putative *l(2)G-l(2)23* chromosome was confirmed by crossing to *l(2)G/SM1* and *l(2)23/SM1*.

Map Distance Between *l(2)G* and *l(2)23*

A rough measure of map distance between the flanking lethals, *l(2)G* and *l(2)23* was generated during construction of the crossover chromosomes. For the cross

$$\frac{l(2)G}{SM1} \times \frac{wg^{cx4}}{SM1}$$

$$\Downarrow$$

$$\frac{l(2)G}{wg^{cx4}} \times \frac{l(2)G}{SM1}$$

134 Curly male progeny were tested for the presence of the two recessive markers by crossing to wg^{cx4} and *l(2)G* females. 133 gave Cy^{+} progeny, indicating that both markers were not present, and 1 gave only *Cy* progeny, indicating that it was a crossover. Map distance is therefore calculated as 1/134 = .00746 = .746 cM.

For the corresponding crosses with *l(2)23* and wg^{cx4}, 392 Cy^{+} male progeny were tested by mating to *l(2)23* and wg^{cx4} females. One male gave only *Cy* progeny, indicating that it was a crossover. Map distance is therefore calculated as 1/392 = .0025 = .25 cM.

Fertility

It became immediately apparent, upon embarking on the mating scheme to obtain a *Sp-*wg^{cx4} crossover, that the female *trans* heterozygous flies were

sterile. In addition, when compared to the expected ratio of that phenotypic class among offspring, they suffered reduced viability. Crosses designed to gain insight into the character of the fertility and viability of the *trans* heterozygotes were done. *Trans* heterozygotes were generated at three temperatures, 18°C, 21°C, and 27°C, from reciprocal crosses, with and without lethal flanking markers. Viability of the F. progeny was tabulated, and, to test fertility, sets of male and female heterozygotes obtained at 18°C, 21°C, and 27°C were incubated at 18°C, 21°C, and 27°C, with 2-3 Oregon-R (wild-type) flies of the opposite sex for the duration of their lifespan. Tables 9, 10 and 11 diagram the outcome of these crosses. Viabilities for reciprocal crosses are recorded as number of heterozygotes / total offspring. Fertilities are recorded as number of heterozgyotes producing offspring / total number of heterozygotes.

Table 9 Viability and Fertility of *Sp / wg^{cx4}* and *wg^{cx4} / Sp* Heterozygotes

GENOTYPE	GENERATION TEMPERATURE	VIABILITY	TEMPERATURE OF CROSS		
			18	21	27
Sp-29 / / wg-cx4 female	18	50/1090	O / 10	O / 11	O / 10
	21	66/1237	O / 10	O / 10	O / 12
	27	38 / 964	O / 11	O / 11	O / 11
Sp-29 / / wg-cx4 male	18		0 / 10	O / 9	O / 12
	21		O / 10	O / 10	O / 10
	27		O / 11	O / 10	O / 12
wg-cx4 / / Sp-29 female	19	45 / 948	0 / 10	0 / 10	0 / 11
	21	57 / 1399	O / 10	O / 11	O / 15
	27	51/1141	O / 13	O / 14	O / 14
wg-cx4 / / Sp-29 male	19		0 / 10	O / 14	0 / 10
	21		O / 10	O / 10	O / 10
	27		1 / 14	O / 10	O / 10

Table 10 Viability and Fertility of *l(2)23 Sp / wg^{cx4} l(2)G* and *wg^{cx4} l(2)G / l(2)23 Sp* Heterozygotes

GENOTYPE	GENERATION TEMPERATURE	VIABILITY	TEMPERATURE OF CROSS		
			18	21	27
23 Sp-29 / / G wg-cx4 female	18	77 / 1566	O / 11	O / 13	O / 10
	21	60/1137	O / 10	O / 12	O / 10
	27	67 / 1521	O / 10	O / 12	O / 15
23 Sp-29 / / G wg-cx4 male	18		O / 10	O / 12	O / 15
	21		O / 12	O / 11	O / 12
	27		O / 14	O / 10	O / 11
G wg-cx4 / / 23 Sp-29 female	18	51 / 1050	O / 10	O / 10	O / 12
	21	49 / 1083	O / 10	O / 10	O / 10
	27	82 / 1625	O / 12	O / 10	O / 10
G wg-cx4 / / 23 Sp-29 male	19		O / 10	O / 12	O / 14
	21		O / 6	O / 10	O / 10
	27		O / 10	O / 10	O / 13

Table 11 Viability and Fertility of *l(2)G Sp / wg^{cx4} l(2)23* and *wg^{cx4} l(2)23 / l(2)G Sp* Heterozygotes

GENOTYPE	GENERATION TEMPERATURE	VIABILITY	TEMPERATURE OF CROSS		
			18	21	27
G Sp-29 / 23 wg-cx4 female	18	62 / 1313	O / 12	O / 13	O / 11
	21	68 / 1298	O / 10	O / 11	O / 12
	27	58 / 1190	O / 10	O / 11	O / 13
G Sp-29 / / 23 wg-cx4 male	18		O / 11	O / 9	O / 10
	21		O / 12	O / 10	O / 11
	27		O / 11	O / 11	O / 11
23 wg-cx4 / / G Sp-29 female	18	78 / 1422	O / 10	O / 11	O / 14
	21	87/ 2080	O / 17	O / 14	O / 14
	27	87 / 2191	O / 15	O / 14	O / 13
23 wg-cx4 / / G Sp-29 male	18		O / 10	O / 10	O / 11
	21		O / 11	O / 11	O / 11
	27		O / 11	O / 15	O / 10

The fertility results from this experiment obviously are a homogeneous data set as all flies, excepting one, produced no progeny. A total of 626 females and 589 males were sterile, and one wg^{cx4}/Sp^{29} male generated at 27°C and mated at 18°C produced offspring. As the offspring from this cross were not saved, the possibility that the male was incubated with a previously inseminated Oregon-R female cannot be ruled out. The *trans* heterozygous progeny are expected to be 1/3 of the offspring, with Curly flies being the remaining 2/3. 1,133 heterozygotes were observed, only 9.8% of the 11,560 predicted based on the observation of 23,120 Cy progeny. The contingency test (Table 12) indicates that temperature, maternal effect, and the presence of the flanking lethal markers do not affect viability.

The Sterility Phenotype

The Sp/wg^{cx4} females were inspected to determine whether an obvious cause for sterility was apparent. Representative one day-old females were dissected to remove their ovaries. Each female examined had two large and well-formed ovaries, with no obvious deformities present. The ovaries appeared to contain eggs, although inspection of vials from crosses with Oregon-R males revealed that the eggs were retained.

Males were not inspected for presence of external genitalia. However, Oregon-R females lay eggs when incubated with the *trans* heterozygous males,

Table 12 Contingency Chi Square Analysis of Viabilities of *Trans* Heterozygotes

	heterozygotes					Cy						
	obs	exp	diff	squ	/	obs	exp	diff	squ	/	totals	
18C	363	345.1	17.84	318.4	0.92	7026	7043.8	-17.8	318.4	0.045	7389	
21C	387	384.6	2.37	5.63	0.01	7847	7849.3	-2.37	5.63	0.001	8234	
27C	383	403.2	-20.21	408.7	1.01	8249	8228.7	20.2	408.7	0.049	8632	
	1133	0.046				23122	0.95				24255	
					1.95					0.095	chisquar	2.0463
											dfreedom	2
											prob	~0.4

	heterozygotes					Cy						
	obs	exp	diff	squ	/	obs	exp	diff	squ	/	totals	
Sp mom	625	619.7	5.26	27.71	0.04	13324	13329.2	-5.26	27.7	0.002	13949	
Sp dad	692	697.2	-5.26	27.71	0.03	15002	14996.7	5.26	27.7	0.001	15694	
	1317	0.044				28326	0.95				29643	
					0.08					0.003	chisquar	0.088
											dfreedom	1
											prob	~0.95

	heterozygotes					Cy						
	obs	exp	diff	squ	/	obs	exp	diff	squ	/	totals	
with flank	826	816.3	9.66	93.33	0.11	16650	16659.6	-9.66	93.3	0.005	17476	
without	307	316.6	-9.61	93.33	0.29	6472	6462.3	9.66	93.3	0.014	6779	
	1133	0.046				23122	0.95				24255	
					0.4					0.02	chisquar	0.429
											dfreedom	1
											prob	~0.6

indicating the ability of the males to copulate and suggesting the presence of functional genitalia.

Characterization of the Temperature Sensitive Mutant *wg^{lL114}*

The temperature sensitive mutant *wg^{lL114}* was obtained in the hope that a *trans* heterozygote of *wg^{lL114}/Sp* generated at permissive temperature would be viable and fertile, and at restrictive temperature might mimic the phenotype of *wg^{cx4}/Sp*.

The *wg^{lL114} cn bw* chromosome is balanced against *CyO=Df(2LR)O, Cy dp^{lvl} pr cn^{2}* at restrictive temperatures. At permissive temperatures, inter se matings result in generation of *wg^{lL114}* homozygous flies. The combination of *cn* and *bw* gives the flies white eyes; they also have an outspread wing phenotype of unknown etiology. Single pair matings of *wg^{lL114}* / CyO*(cn)* were done over a range of temperatures to determine the restrictive limit.

$$\frac{wg^{lL114}\ cn\ bw}{CyO} \quad X \quad \frac{wg^{lL114}\ cn\ bn}{CyO}$$

$$\Downarrow$$

$$\frac{wg^{lL114}\ cn\ bw\ ;}{CyO} \qquad \frac{wg^{lL114}\ cn\ bw}{wg^{lL114}\ cn\ bw}$$

Results are shown in Table 13.

Table 13 Temperature Dependent Viability of wg^{lL114} Homozygotes

Temperature	white ♀	white♂	cn	wg^{lL114} Viability
16 C	114	136	1858	26.91%
18 C	79	81	2197	14.57%
19 C	0	0	2328	0
21 C	0	0	2303	0
25 C	0	0	2039	0
27 C	0	0	2146	0

From the cross wg^{lL114}/CyO X wg^{lL114}/CyO, 1/3 of the zygotes are homozygous for *cn* and *bw*, while 2/3 are wg^{lL114}/CyO, and homozygous only for *cn*. Viability of the wg^{lL114} homozygotes is calculated as (white progeny / 0.5 x cinnabar progeny) X 100%. Viability is zero at 19°C and above. At 18°C viability is 14.57% and reducing the temperature to 16°C increases viability to 26.91%. Males and females are equally affected.

wg^{lL114}/Sp heterozygotes were generated over the same temperature range previously observed with wg^{cx4}. As expected, male and female heterozygotes generated at the restrictive temperatures of 21°C and 27°C were sterile. At 27°C the heterozygotes demonstrate a shriveled wing phenotype, mimicking the wg^{cx4} heterozygote, and at 21°C there is an intermediate shortened wing phenotype. Viability and fertility of the heterozygotes, performed as described previously, is recorded in Table 14.

Table 14 Viability and Fertility of *Sp / wg^{IL114}* and *wg^{IL114} / Sp* Heterozygotes

GENOTYPE	GENERATION TEMPERATURE	VIABILITY	TEMPERATURE OF CROSS		
			18	21	27
Sp-29 / / wg-ILI114 female	18	128 / 2206		5 / 14	
	21	95 / 2649	0 / 21	O / 19	0 / 20
	27	24 / 3202	O / 20	O / 21	0 / 21
Sp-29 / / wg-IL114 male	18			6 / 12	
	21		O / 19	O / 20	O / 20
	27		O / 23	O / 24	O / 21
wg-IL114 / / Sp-29 female	18	215 / 2567		4 / 13	
	21	105 / 2755	O / 20	O / 22	O / 19
	27	13 / 2115	O / 20	O / 20	0 / 19
wg-IL114 / / Sp-29 male	18			5 / 41	
	21		O / 21	O / 25	O / 15
	27		O / 19	O / 20	O / 19

A contingency test (Table 15) indicates that the viability of the wg^{lL114}/Sp *trans* heterozygote at 21°C is different than the viability of the wg^{cx4}/Sp heterozygotes, being slightly lower. The viability of the wg^{lL114}/Sp heterozygotes at 27°C is appreciably lower. Males and females generated at 18°C, the permissive temperature, had no apparent wing phenotype, and were fertile, raising the possibility that wg^{lL114} could be used to generate a *cis* double mutant.

Table 15 Contingency Chi Square Analysis - wg^{cx4} vs. wg^{lL114}

	heterozygotes					Cy					
	obs	exp	diff	squ	/	obs	exp	diff	squ	/	totals
wg-cx4/21C	387	354.4	32.59	1062.5	2.99	7847	7879.5	-32.59	1062.5	0.13	8234
wglL114/21C	200	232.5	-32.59	1062.5	4.56	5204	5171.4	32.59	1062.5	0.2	5404
	587	0.04				13051	0.95				13638
					7.56					0.34	chisquare 7.91
											dfreedom 1
											prob <0.005

Discussion

The observation of a novel mutant phenotype for *Sp/wg^{cx4} trans* heterozygotes during complementation analyses prompted an attempt to recover a rare *Sp-wg^{cx4}* recombinant. Observation of a mutant phenotype in F1 heterozygotes is generally interpreted as non-complementation and evidence of allelism, and on this basis it has been suggested that *Sp* represents a mutant cis-regulatory allele of *wg* (Neumann and Cohen 1996). However, the alternative interpretation that the loci code for separate, but functionally related proteins cannot be dismissed by the evidence available to date. Many examples of pseudoallelic series have been documented, revealing precedent in *Drosophila melanogaster* for this phenomenon (Lewis 1945, 1951, 1965).

Another locus near 28A, *ninaC*, a rhodopsin visual mutant, is closely linked to, and also demonstrates non-complementation with, *Sp* (Matsumoto 1987). This further complication creates another impediment toward distinguishing between several existing possibilities for the underlying genetic organization of the region. Both *ninaC* and *wg* have been cloned, with sequence analyses indicating they are clearly different genes. *Sp* could represent a third gene in the area, coding for a protein that interacts with Wg and NinaC at different stages of development. An alternative possibility is that *Sp* is a regulatory mutant of either *ninaC* or *wg*, and the corresponding gene products possess some functional commonality during aspects of development, leading to a lack of

complementation of *Sp* with both genes. In theory *Sp* could simultaneously act as a regulatory allele of both genes, even if they are absolutely unrelated in function. *ninaC*, an unconventional myosin, is involved in calmodulin localization in the microvilli of rhabdomeres. Wg is known to affect determination of imaginal disc tissue, and in leg and wing discs has a second role governing the proximo-distal axis. Eyes also are formed from imaginal disc tissue, and a second role for Wg in eye discs, although not documented, is possible.

wg/Sp Trans Heterozygotes

The viability of wg^{cx4}/Sp heterozygotes is 9.8%, based on the expected numbers for that phenotypic class in crosses. The viability is not affected by temperature or the presence of flanking lethal markers, and is not the result of a maternal effect.

The temperature sensitive wg^{IL114} allele, in trans with *Sp*, exhibits a graded wing and viability effect at restrictive temperatures, showing more severe phenotypes at 27°C (the same crinkled wing phenotype seen in the wg^{cx4}/Sp heterozygote), than 21°C (a narrowed wing). The viability of the *trans* heterozygotes is 15.4% at 18°C, 7.6% at 21°C, and 1.4% at 27°C. wg^{IL114} is an amino acid substitution, Cys104→Ser, producing a protein that fails to be exported at restrictive temperatures. The graded effect of the wing phenotypes in the heterozygotes suggest that the wg^{IL114} allele might be "leaky", with some protein export at especially low restrictive temperatures. However, *Sp* also

exhibits reduced penetrance at low temperatures that may be responsible for the graded phenotypes observed.

wg Affects Fertility

The observation of a sterile phenotype for wg^{cx4}/*Sp trans* heterozygous flies, although delaying the generation of a *cis* recombinant, provides an opportunity to gain insight into what may be another role of the wingless protein. Particularly intriguing is the observation of sterility in both males and females, as the *wg/Sp* heterozygotes do not demonstrate the type of phenotypic effects previously seen in male/female sterile mutants.

One interpretation of a mutation that affects both male and female fertility is that the gene acts during a common pathway in the early stages of oogenesis and spermatogenesis. Only two such common functions have been found so far in *D. melanogaster*. First, reproductive primordial cells develop from pole cells, and represent one of the earliest migration events in development. Pole cells migrate during blastulation, and the primordial germ cells develop into ovaries or testes. Mutants affecting pole cell migration cause sterility in both males and females as a result of lack of development of the primary sex organs (Schüpbach and Wieschaus 1989). A second commonality is demonstrated by *S352* and *ord*, mutations that mark convergence of the male and female meiotic cycles. The normal proteins function to allow proper disjunction of homologues during meiosis I (Mason 1976. Hawley et al. 1993).

The lack of previous indication that Wg is a signal in early reproductive development, and the observation of well-formed ovaries in wg^{cx4}/Sp females, both suggest that *wg* is not affecting pole cell migration. Although no corresponding observation was made of testes in Sp/wg^{cx4} males, Oregon-R females lay infertile eggs during incubation with the heterozygotes, implying the presence of sex organs and copulating ability in the males. *wg* also seems an unlikely candidate to affect meiotic processes, as its only known function to date has been to direct cell fate decisions. If *wg* has a direct effect on fertility, it is likely that the effect is manifested separately in males and females in later stages of oogenesis and spermatogenesis. This seems particularly feasible for a gene like *wg* that is already known to participate in a plethora of cell fate decisions during *Drosophila* development.

Effect on Oogenesis

Accumulation of information pertaining to oogenesis in *Drosophila melanogaster* has been steady and comprehensive for decades. Ovaries in *Drosophila melanogaster* are composed of approximately 16 ovarioles, which form anterior to posterior assembly lines of egg production. Within each ovariole there are 6-7 developing egg chambers, or follicles, that form at the anterior end, or germarium, of the ovary. These follicles are formed by unequal division of a germ cell into a cystoblast and replacement stem cell, followed by four successive divisions to form 16 cells. One cell continues through meiosis

and forms the egg, while the other 15 leave the meiotic cycle to form nurse cells. As the follicles develop they become surrounded by approximately 1,000 somatically derived follicle cells, that eventually will be responsible for secreting a protective cuticle around the developing egg. Subpopulations of follicle cells are additionally responsible for secretion of two anterior respiratory structures, called filaments, and a ridged collar surrounding the micropyle that will allow sperm entry into the egg.

A link between female sterility and embryonic lethality has been anticipated (Perrimon 1986, 1989, Shüpbach and Wieschaus 1989, 1991). Complementation analysis between independently isolated sets of X-linked female sterile mutants revealed that these mutants fall into two broad groups -- a set of loci for which both screens isolated many alleles, and a set of mutants for which only a single allele has been isolated among the screens (Gans 1975, Mohler 1977, Perrimon 1986). The interpretation of this observation is that the first group represents genes whose developmental role is exclusively in oogenesis. The second group represents genes that have a role in oogenesis, but additionally affect other aspects of embryonic development. Most alleles of the second group, being embryonic lethals, would never be isolated in screens to uncover sterility, and the single female sterile alleles that have been successfully isolated are representative hypomorphs (Perrimon 1986, Schüpbach and Weischaus 1989).

One likely candidate for the *wg* effect on oogenesis is the follicle cell. Wg

gradients have been shown to direct the type of cuticle secreted in larvae, and might similarly affect the deposition of cuticle in eggs. For instance, Wg might affect the migration patterns of follicle cells, disrupting their normal spatial localization and consequently disrupt distribution of cuticle. Alternatively, Wg secreting cells might exert influence over cuticle deposition in follicle cells after migration and egg enclosure.

A logical first step in examining the effect of *Sp/wg*^{cx4} on oogenesis would be to carefully examine the phenotype of the ovaries and eggs produced. Extensive genetic screens have generated a large number of female sterile mutants that have been categorized into phenotypic classes reflecting the timing of oogenic events (Schüpbach and Weischaus 1989, 1990). Some of the classes are visible abnormalities of cuticle and interference with follicle cell migration patterns, such as open-ended chorion, dorso-ventral patterning, formation of dorsal appendages, and abnormal egg shell structure. Observation of the ovaries of the *trans* heterozygous females, and comparison with the phenotypic classes previously identified, may lend insight into the type of fertility effect exerted by *wg*.

Effect on Spermatogenesis

In marked contrast to oogenesis, information about the process of spermatogenesis in *Drosophila melanogaster* is parse. Like oogenesis, spermatogenesis begins with division of a cyst progenitor cell into a replacement

stem cell and a primary spermatogonial cell, which undergoes four mitotic divisions to become a 16-cell cyst. Each cell undergoes meiosis, accompanied by many morphological changes in the organelles, and the final result is a 64-cell cyst. Individualization of the sperm occurs in a progressive wave through the cyst, with cytoplasm expelled and collected for disposal. The sperm is then coiled into a spermatid bundle, and becomes surrounded by two somatically derived cyst cells during the differentiation process (Tokuyasu 1972a, 1972b). During insemination, the sperm is passed to the female, and stored in the seminal receptacles. With a paucity of details about the genetic interactions that control spermatogenesis, it is difficult to entertain even a preliminary guess of how *wg* could affect the process. As in oogenesis, the surrounding somatic cells seem the most likely target for Wg signalling, although how that might happen is a mystery. A recent P-element screen for male sterile mutations has uncovered several classes of phenotypes, and examination of the mutants is ongoing (Castrillon et al. 1996). As more elaborate details become available about the processes of spermatogenesis, a more likely role for *wg* may present itself.

A working hypothesis for how *wg* is associated with fertility suggests that the likely candidates for action are the cells of somatic origin that surround the developing germ cells, namely follicle cells in females, and sheath cells in males. This hypothesis, presently untested, is consistent with the sterility phenotype uncovered and the known roles of the Wg ligand in *D. melanogaster* development. The alternative possibility, that a common aspect of oogenesis

and spermatogenesis, such as pole cell migration or homologue disjunction, is involved is contradictory to the data. A third possibility, that the fertility effect is not a direct result of the action of Wg, has not been excluded. The process of oogenesis is dependent on a readily available food source and the general health of the female. In nonideal conditions, females capable of producing eggs will hold onto them, not allowing them to enter the oviducts. It may be that the infertility phenotype of many embryonic lethals is a result of a general pleiotropic effect on overall viability, rather than loss of specific protein functions. The observation that *wg/Sp*^{cx4} heterozygotes suffer severely reduced viability is consistent with this third interpretation.

Nature of the *Sp* allele

Insight into the complicated underlying genetic structure of the 28A region helps reconcile some superficially inconsistent classical observations of the *Sp* phenotype. The bristle phenotype of *Sp*, extra and thickened bristles, fits the prediction of the appearance of a dominant gain-of-function mutant, or hypermorph, and *Sp* is also a recessive lethal, again consistent with an overdose effect (Muller 1932). The observation that *Sp* is lethal over deletions was originally perplexing, as hypermorphic alleles are predicted to have a severity of phenotype following the order Sp/Sp ⟩ Sp/+ ⟩ Sp/[deletion]. The phenotypic effects seen in the *Sp/wg*^{cx4} heterozygote conforms with *Sp* being a hypomorphic allele of *wg* rather than a gain-of-function, but this explanation still

is not compatible with all of the data. wg^{cx4} is a null allele known to produce no protein or message, and the phenotypes of a hypomorph/amorph and hypomorph/deficiency are predicted to be identical. This is not the case for *Sp*, as *Sp*/wg^{cx4} is viable, but *Sp/deficiency* dies. The behavior of *Sp* with deletions clearly does not fit into the classical morphic descriptions for a single locus, a portent of additional genetic complexity lying within the confines of the deficiencies. The observation that *Sp* fails to complement the lethality of both *ninaC* and *wg* provides an explanation for these apparent dichotomies, as the deficiencies tested are large enough to span all these loci.

Generating a *Cis* Double Mutant With wg^{lL114}

A genetic means to unambiguously differentiate between these possibilities requires comparison of the phenotypes of *cis* and *trans* heterozygotes. This is not possible in the case of the non-complementing alleles *Sp* and *ninaC*, as the lethal phenotype of the *trans* heterozygote precludes generation of a *cis* recombinant. *Sp* and wg^{cx4} generate a viable adult in *trans*, but the additional phenotype of sterility effectively eradicates this experimental approach for these alleles, also. Fortunately, the temperature sensitive wg^{lL114} allele in *trans* with *Sp* mimics the crinkled wing phenotype at the restrictive temperature of 27°C and is fertile at the permissive temperature of 18°C.

If *Sp* is distal to *wg*, the following scheme could yield a recombinant between *Sp* and wg^{lL114}.

$$\frac{wg^{lL114} \quad cn \quad bw}{Sp \quad l(2)23} \quad X \quad \frac{l(2)23}{SM1(cn)}$$

$$\Downarrow$$

$$\frac{Sp \quad wg^{lL114} \quad cn \quad bw}{l(2)23}$$

The map distance between *wg (Sp)* and *l(2)23* is approximately 0.25 cM. Viable Sp Cy^+ progeny represent a crossover between *Sp* and *l(2)23*, and are potential crossovers between *Sp* and *wg*. These putative crossover flies can be balanced against SM1 to establish a stock, and crossed to wg^1 at restrictive temperature to test for the presence of wg^{lL114}.

$$\frac{Sp \quad wg^{lL114} \quad cn \quad bw}{SM1\ (cn)} \quad X \quad \frac{wg^1}{wg^1}$$

wg^1/wg^{lL114} heterozygotes demonstrate an adult-viable weak-wingless phenotype and are white-eyed. Sp/wg^1 has no mutant phenotype. Thus, the presence of wg^{lL114} is distinguishable from its absence despite the fact that we don't know what affect *Sp* might have on this phenotype. A confirmed crossover stock can then be crossed to Oregon-R (wild type) at restrictive temperature for observation of the phenotype in the *cis* heterozygote.

If *wg* is distal to *Sp*, the following scheme could yield a recombinant.

$$\frac{G \quad wg^{IL114} \quad cn \quad bw}{Sp \qquad\quad cn} \quad X \quad \frac{G \quad bw^{D}}{SM1\ (cn)}$$

$$\Downarrow$$

$$\frac{Sp \quad wg^{IL114} \quad cn \quad bw}{G \quad bw^{D}}$$

The map distance between *wg (Sp)* and *l(2)G* is approximately 0.75 cM. Viable Sp Cy+ progeny have a crossover between *Sp* and *G*, and are potential wg^{IL114} *Sp* crossovers. The identical steps as outlined above to test and stock would be performed.

Isolation and testing of 100 crossovers between *l(2)23* and *wg,* and 300 crossovers between *l(2)G* and *wg* would allow resolution between *Sp* and *wg* of 0.005 cM. With an estimate of 1 cM = 1 Mbase, if no crossovers are detected, then *Sp* and wg^{IL114} most likely reside within a 5 kilobase pair span of DNA. As the molecular lesion for wg^{IL114} is an amino acid change at position 104 within a 10 kb gene, this would be a very strong argument that *Sp* and *wg* are allelic. If a crossover is obtained, the phenotype of the *cis* heterozygote will reflect the genetic interaction between *Sp* and *wg*. If *Sp* and *wg* are truly alleles, a wild type phenotype should result. If *Sp* and *wg* code for different proteins, the *cis* and *trans* arrangements would both confer the crinkled wing phenotype.

Future Directions

In summary, analyses of lethal and visible mutations surrounding 28A have revealed a complex pattern of overlapping non-complementation between alleles of *wg, spd, Sp* and *ninaC* (Neumann and Cohen 1996, Matsumoto et al. 1987, Tiong and Nash 1990) that make it difficult to ascertain how many genes exist in this area. Molecular analysis, including mapping, sequencing, and searching for coding regions is an obvious way to unequivocally address this issue, and the success of the genome mapping project has demonstrated the effectiveness of this strategy. Alternatively, genetic analyses of some of these interactions, consisting of observation and comparison of phenotypes in *cis* and *trans* heterozygotes, can be executed. Although not possible for the *Sp-ninaC* interaction, which results in lethality, we now know that this analysis should be feasible for *Sp* and *wg*. wg^{lL114}*/Sp trans* heterozygotes manifest the phenotypes of male and female sterility, reduced viability, and crinkled wings at restrictive temperatures, while at permissive temperature the flies are phenotypically wild type, and at least somewhat fertile. Generating crossover progeny should elucidate whether *Sp* and *wg* represent the same or separate genes. If a crossover is not obtained, then an upper limit for the distance between *Sp* and *wg* will be determined.

The 28A cytological region housing *wg* is a complex genetic segment, reminiscent in organization to previously characterized pseudoallelic series such as *Antennapedia, Ultrabithorax, Star* and *decapentaplegic*. In addition,

reductions of *wg* sufficient to reduce viability and affect wing morphology have consequences for both male and female fertility. Although this may represent a general pleiotropic effect rather than a direct consequence of Wg reduction, this intriguing phenotype could prove to be a worthy avenue for future exploration.

References

Babu, P., 1977 Early developmental subdivisions of the wing disk in *Drosophila*. Mol. Gen. Genet. **151**: 289-294.

Baker, N. E., 1987 Molecular cloning of *wingless*, a segment polarity gene in *Drosophila*: The spatial distribution of a transcript in embryos. EMBO J. **6**: 1765-1773.

Castrillon, D. H., Gönczy, P., Alexander, S., Rawson, R., Eberhart, C. G., Viswanathan, S., DiNardo, S. and S. A. Wasserman, 1993 Toward a molecular genetic analysis of spermatogenesis in *Drosophila melanogaster*: Characterization of male-sterile mutants generated by P element mutagenesis. Genetics **135**: 489-505.

Chovnick, A., Schalet, A., Kernaghen, R. P. and J. Talsma, 1962 The resolving power of genetic fine structure analysis in higher organisms as exemplified by *Drosophila*. Amer. Nat. **96**: 281-296.

Doane, W. W., 1960 Report. Dros. Inf. Serv. **34**: 25.

Gans, M., Audit, C. and M. Masson, 1975 Isolation and characterization of sex-linked female sterile mutants in *Drosophila melanogaster*. Genetics **81**: 683-704.

Green, M. M., 1961 Phenogenetics of the *lozenge* loci in *Drosophila melanogaster*. II Genetics of *lozenge-Krivshenko* (lz^k). Genetics **46**: 1169-1176.

Green, M. M. and K. C. Green, 1949 Crossing over between alleles at the *lozenge* locus in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA. **35:** 586-591.

Green, M. M. and K. C. Green, 1956 A cytogenetic analysis of the *lozenge* pseudoalleles in *Drosophila*. Z. Vererbungslehre **87**: 708-721.

Hawley, R. S., McKim, K. S. and T. Arbel, 1993 Meiotic segregation in *Drosophila melanogaster* females: Molecules, mechanisms, and myths. Annu. Rev. Genet. **27**: 281-317.

Kaufman, T. C., Seeger, M. A. and G. Olsen, 1990 Molecular and genetic organization of the *Antennapedia* gene complex of *Drosophila melanogaster*. In: Advances in Genetics, vol. 27: Genetic Regulatory Hierarchies in Development. Ed., T. R. F. Wright, 309-362.

Lewis, E. B., 1945 The relation of repeats to position effect in *Drosophila melanogaster*. Genetics **30**: 137-166.

Lewis, E. B., 1951 Pseudoallelism and gene evolution. Cold Spring Harbor Harb. Quant. Biol. **16**: 159-174.

Lewis, E. B., 1955 Some aspects of position pseudoallelism. Am. Nat. **89**: 73-89.

Lewis, E. B., 1965 Genes and gene complexes. In: Heritage from Mendel: Proceedings of the Mendel Centennial Symposium. Chapter 3 pp. 17-47. Eds. Brink, R. A. and E. D. Styles. University of Wisconsin Press, Madison, Wisconsin.

Mann, M., 1923 The occurrence and hereditary behavior of two new dominant mutations in an inbred strain of *Drosophila melanogaster*. Genetics **8**: 27-36.

Mason, J. M., 1976 *Orientation disrupter (ord)*: A recombination-defective and disjunction-defective meiotic mutant in *Drosophila melanogaster*. Genetics **84**: 545-572.

Matsumoto, H., Isono, K., Pye, Q., and W. L. Pak, 1987 Gene encoding cytoskeletal proteins in *Drosophila* rhabdomeres. Proc. Natl. Acad. Sci. USA. **84**: 985-989.

Mohler, J. D., 1977 Developmental genetics of the *Drosophila* egg. I. Identification of 50 sex-linked cistrons with maternal effects on embryonic development. Genetics **85**: 259-272.

Muller, J. H., 1932 Further studies on the nature and causes of gene mutations. Proc. Sixth Int. Congr. Genet. **1**: 213-272.

Neumann, C. J. and S. Cohen, 1996 *Sternopleural* is a regulatory mutation of *wingless* with both dominant and recessive effects on larval development of *Drosophila melanogaster*. Genetics **142**: 1147-1155.

Nüsslein-Volhard, C., Wieschaus, E. and H. Kluding, 1984 Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. I. Zygotic loci on the second chromosome. Wilhelm Roux's Arch. Dev. Biol. **193**: 267-282.

Perrimon, N., Engstrom, L. and A. P. Mahowald, 1989 Zygotic lethals with specific maternal effect phenotypes in *Drosophila melanogaster*. I. Loci on the X chromosome. Genetics **121**: 333-352.

Perrimon, N., Mohler, D., Engstrom, L. and A. P. Mahowald, 1986 X-linked female-sterile loci in *Drosophila melanogaster.* Genetics **113**: 695-712.

Schüpbach, T. and E. Wieschaus, 1989 Female sterile mutations on the second chromosome of *Drosophila melanogaster.* I. Maternal Effect Mutations. Genetics **121**: 101-117.

Schüpbach, T. and E. Wieschaus, 1991 Female sterile mutations on the second chromosome of *Drosophila melanogaster.* II. Mutations blocking oogenesis or altering egg morphology. Genetics **129**: 1119-1136.

Sharma, R. P., 1973 *wingless*, a new mutant in *Drosophila melanogaster.* Dros. Inf. Ser. **50**:134.

Tiong, S. and D. Nash, 1990 Genetic analysis of the *adenosine3 (Gart)* region of the second chromosome of *Drosophila melanogaster.* Genetics **124**: 889-897.

Tokuyasu, K. T., Peacock, W. J. and R. W. Hardy, 1972a Dynamics of spermiogenesis in *Drosophila melanogaster.* I. Individualization process. Z. Zellforsch. **124**: 479-506.

Tokuyasu, K. T., Peacock, W. J. and R. W. Hardy, 1972b Dynamics of spermiogenesis in *Drosophila melanogaster.* II. Coiling process. Z. Zellforsch. **127**: 492-525.

Appendix A

Molecular Characterization of the 28A1-28D1 Cytological Region

A molecular characterization of the 28A-28D region was initiated by a chromosome walk, designed to extend the molecular map around the gene *urate oxidase*. Using probes to *urate oxidase*, overlapping clones were recovered from a genomic lambda library, covering 38 kilobase pairs (kb) of DNA surrounding the gene. The entire 38 kb span was divided into 13 genomic restriction fragments, and those fragments used to probe Northern blots (Gal et al. 1983) of poly(A)+ RNA isolated from third instar larvae. The Northern blots revealed that at least six tightly packed transcripts from this temporal period were coded from the 38 kb of DNA. *In situ* hybridization of several of the probes to well stretched salivary gland polytene chromosomes revealed that all six transcripts fell within the cytological region between the major bands 28C1 to 28D1, defined by very faint bands and interband space. No transcripts could be attributed to DNA from either major band (Freidman et al. 1991).

Figure 7, taken from Friedman et al. 1991, depicts the extent of the molecular map surrounding UO at the onset of this research project. Panel a depicts overlapping genomic clones (circle-ended bars), their corresponding restriction map (R=EcoRI, X=XbaI, B=BamHI, S=PstI, H=HindIII), and the six transcripts

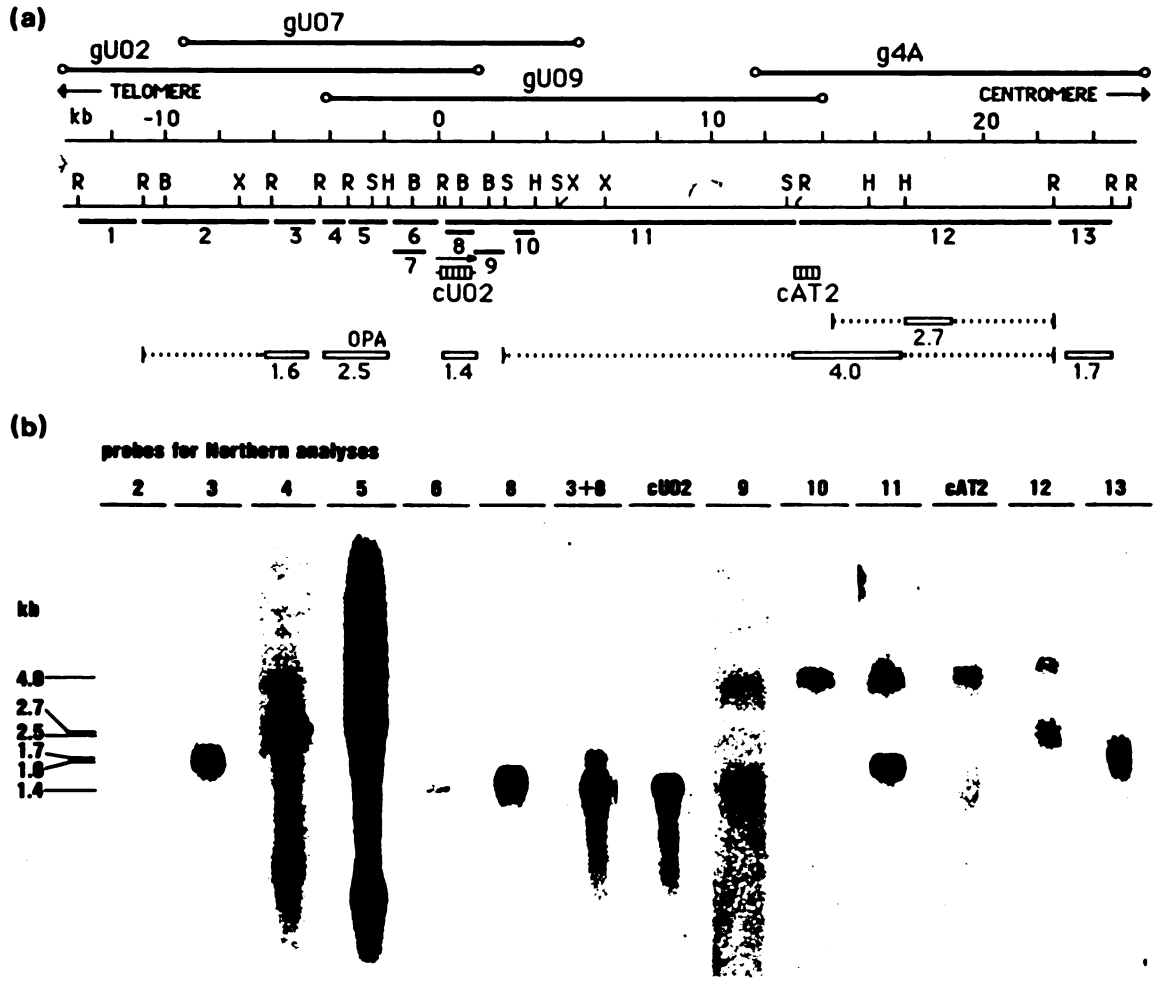


Figure 7 Northern Analysis of 28C2 Region

uncovered by Northern analysis. Panel b shows the Northern blot of third instar larvae. Restriction fragment 5 hybridizes with a pattern indicative of the presence of a repetitive element, and upon further analysis was discovered to contain a $(CAG)_n$ repeat (Wharton et al. 1985). A nonrepetitive segment of this gene was used as a probe to isolate a cDNA clone from a library prepared from third instar larval RNA.

One aspect of my thesis project was to extend the molecular characterization of this chromosomal region to span the cytological endpoints at major bands 28A1 and 28D3-4(-). The immediate goals were, first, to construct a crude restriction enzyme map of the region which could be juxtaposed onto known cytological markers by *in situ* hybridization, thereby reconciling the two maps, and secondly to confirm the amount of DNA contained within the 28A - 28D region, estimated to be 500 kb. During the characterization of this region, the cDNA clone containing the $(CAG)_n$ repeat was sequenced.

The long-term goal of this limited molecular characterization would be to catalog and map transcription units that exist within those cytological confines, thereby determining the number and organization of genes in the region. In addition to the value of characterizing the 28A-28D region, the analysis would also preliminarily address whether the motif of tightly packed transcripts in interband regions, as were uncovered surrounding *urate oxidase*, is an anomaly or a general property of *D. melanogaster* euchromatin.

Over its course, the molecular characterization of the 28A-28D region was

eclipsed, both by the genome mapping project and a desire to focus research efforts on the intriguing genetics of the 28A cytological region. Efficient and successful means of mapping large segments of the chromosome developed during the past decade have allowed the generation of a complex euchromatic map in *D. melanogaster*, and this endeavor includes the 28A region (Kimmerly et al. 1996).

Detection of Genes by Molecular Means

The question of total gene number has challenged researchers for decades (Muller 1928, Hochman 1973, Judd et al. 1972, Lefevre 1981, Lefevre and Watkins 1986). Mutagenesis studies in *D. melanogaster* uncover approximately as many lethal complementation groups per region as there are visible bands, leading to the hypothesis that a band is a physical manifestation of gene structure and provided an estimate of 5,000 total genes (Judd et al. 1972, Hochman 1973, Lim and Snyder 1974, Liu and Lim 1975, Gausz et al. 1986, Woodruff and Ashburner, 1979). However, it is clear that not all genes are identifiable as lethal complementation groups (O'brien 1973, Young and Judd 1978). Some genes whose null mutants are not lethal require sophisticated screens to recover mutants. In addition, molecular analyses of large cloned regions have revealed that the number of open reading frames is in excess of lethal complementation groups, indicating that many genes cannot be isolated by classical screens at all. Extrapolations from the number of open reading

frames in discrete DNA stretches provides an estimate of 15,000 total genes. (Voelker et al. 1985, Gausz et al. 1986).

The gene number issue should ultimately be resolved upon completion of the genome mapping project. Mapping collaborations in *D. melanogaster*, described in Chapter 1 of this thesis, have been enormously successful. Current projections predict that the entire euchromatic genome of *D. melanogaster* will be completely sequenced within a few years. The task of further analyzing the data looms, with almost inconceivable enormity, for the future. Divining the biological significance in seemingly random nucleotide sequence is not a trivial matter.

The prediction that there are 15,000 genes, spread among 120 Megabase pairs (Mb) of euchromatic DNA, with an average gene length of 10 kb, suggests that approximately 10% of the euchromatin can be expected to correspond to coding and control regions (Lefevre and Watkins 1986, Rasch et. al. 1971). Although the remaining chromatin cannot all be biologically insignificant, insight into its functions remains largely obscured. DNA stretches of vital importance to chromosome structure and function, such as chromatin attachment regions and pairing regions, have been identified but no generalized schemes for uncovering biologically significant DNA in the noncoding portion of the euchromatic genome have been advanced (Kellum and Schedl 1991, Kellum and Schedl 1992, Cai and Levine 1995, Hawley 1980). It is not surprising that initial characterization efforts are concentrating on developing strategies to identify sequence that

corresponds to coding regions.

Many approaches, varying in level of sophistication, have been devised to identify sequence that represents coding regions. The initial strategy used to determine transcribed DNA was to cross-hybridize genomic fragments from one species to "zoo" blots, containing genomic DNA from several disparate species. The rationale for the procedure is that coding regions, presumably under strict evolutionary constraints, will have been highly conserved and will bind to one another under stringent conditions that disallow hybridization of more randomized sequence (Rommens et al. 1989, Monaco et al. 1986).

Another detection strategy arose from the observation that vertebrate DNA, normally devoid of CpG dinucleotides, has islands of CpG rich methylated DNA near all housekeeping and about 40% of tissue specific genes (Gardiner-Garden and Frommer 1987, Larsen et al. 1992, Antequera and Bird 1993). Use of rare cutters (restriction enzymes recognizing the CpG dinucleotide) to identify the islands is one method for uncovering coding regions that has developed (Valdes et al. 1994). CpG rich DNAs also harbor a propensity to resist melting in denaturing gradient gels (DGG), and can be isolated from other DNAs with DGG electrophoresis (Shiriasi et al. 1995). The rat chromosomal protein MeCP2 (Lewis et. al. 1992), which binds CpG islands via the methylated DNA, has also been used to purify DNA fragments containing CpG islands with affinity chromatography (Cross et al. 1994).

Another tactic for recognizing mammalian genes has been to capitalize on

the ubiquitous presence of binding sites for the SP1 transcription factor (Briggs et al. 1986). A method called promoter capture uses a recombinant glutathione S-transferase-SP1 fusion protein conjugated to glutathione-agarose beads to isolate promoter regions via their SP1 binding sites (Mortlock et al. 1996).

Exploiting CpG islands and SP1 binding sites have been useful stratagems for mouse and human gene detection, but rely on biological characteristics unique to vertebrates, and are not generally applicable to *D. melanogaster.* Two processes that are available for nonvertebrate gene identification are cDNA selection and exon trapping.

cDNA selection uses a random portion of genomic DNA, such as might be contained in a YAC, as a probe to screen a cDNA library. Subsequent hybridization of cDNAs is employed to reveal the coding regions contained in the YAC that were responsible for their selection (Burke et al. 1987, Elvin et al. 1990, Wallace et al. 1990). The initial procedure proved to be laborious, time consuming, and also to require extensive blocking to prevent non-specific binding to repetitive DNA. Improvements on the scheme, devised to enrich cDNA libraries with an immobilized YAC prior to screening have proven to be more efficient (Lovett et al. 1991, Parimoo et al. 1991). The newest twist in library enrichment is to biotin label random restriction fragments from YACs, allow them to hybridize to an amplified cDNA library, and use streptavidin-bound magnetic beads to efficiently pull the YAC-cDNA complex out of solution (Tagle et al. 1993).

Lastly, exon-trapping strategies have been developed to take advantage of the splice acceptor and donor sites present at intron boundaries in almost all eukaryotic genes. Genomic fragments, shotgun cloned into specially designed vectors, undergo a selection for the subset of vectors whose exon-containing inserts have promulgated a splicing event. One system relies on the loss of a *β-galactosidase* reporter cassette to select potential reading frames (Duyk et al. 1990). As this system requires only a 5' splice site in the genomic insert, cryptic splice sites in the genomic DNA resulted in a high false positive rate. A more advanced system of splicing drastically reduces the false positive rate by requiring both 3' and 5' splice sites (Buckler et al. 1991). The selection relies on the ability of a primer set to initiate successful PCR amplification subsequent to splicing, a process that is intractable with a large unspliced insert.

Many methods are currently available for identifying transcription units within random fragments of genomic DNA. What was once viewed as an impossibly ambitious goal -- to gain a clear understanding of the number and organization of genes in chromatin, is on the brink of realization. Presumably, the next step will be to dissect the structure and function of the non-coding portions of the chromosome, undoubtedly a more challenging puzzle.

Molecular Characterization

In summary, a molecular characterization of the cytological region 28A-28D was undertaken to initialize a delineation of the distribution of genes at this

location. A variety of methods were employed, including using lambda clones, YACs and cosmids, to isolate DNA from the region surrounding *uo*. A restriction map of the isolated DNA was prepared. While a complete assessment would require that one evaluate approximately 500 kb of sequence for the presence of transcription units, the preliminary characterization, including constructing a rough restriction map with concurrent *in situ* hybridization onto the cytological map, would be expected to contribute the necessary prelude for this analysis. In addition, a cDNA clone containing a $(CAG)_n$ repeat was sequenced.

Methods And Results

Standard Buffers and Reaction Conditions (Maniatis, et. al. 1982)

TAE Buffer -- 40 mM Tris-acetate, pH 8.0, 1 mM (ethylenedinitilo)-tetraacetic acid (EDTA)

TBE Buffer -- 89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA, pH 8.0

Selective Yeast Media -- 0.67% Bacto-yeast nitrogen base without amino acids, 2% glucose, 0.2% amino acid mix without adenine and tryptophan

NZCM -- 1% NZ amine, 0.1% casamino acids, 0.5% NaCl, 0.2% $MgSO_4$-$7H_2O$, pH 7.5

Luria Broth -- 1% Bacto-tryptone, 0.5% Bacto-yeast extract, 1% NaCl, pH 7.5

Top agar contains 0.7% Bacto-agar

Plates contain 1.5% Bacto-agar

M9 Complete Salts -- 2 mM $MgSO_4$, 0.2% glucose, 0.1 mM $CaCl_2$, 40mM Na_2HPO_4, 20 mM KH_2PO_4, 10 mM NaCl, 20 mM NH_4Cl, pH 7.4

SM -- 0.1 M NaCl, 5 mM $MgSO_4$, 0.01% gelatin, 50 mM Tris-Cl, pH 7.5

TE Buffer -- 1 mM EDTA, 10 mM Tris-Cl, pH 7.5

Lysozyme Solution -- 50 mM glucose, 10 mM EDTA, 0.5% lysozyme, 25 mM Tris-Cl, pH 8.0

Lysing Buffer -- 0.2 N NaOH, 1% sodium dodecyl sulfate (SDS)

20x SSC -- 0.3 M NaCl, 0.6 M sodium citrate, pH 7.0

Elutip Low Salt Buffer -- 0.2 M NaCl, 1.0 mM EDTA, 20 mM Tris-Cl, pH 7.4

Elutip High Salt Buffer -- 1.0 M NaCl, 1.0 mM EDTA, 1.0 mM Tris-Cl, pH 7.4

Denaturation Solution -- 1.5 M NaCl, 0.5 N NaOH

Neutralization Solution -- 1.5 M NaCl, 1 M Tris-Cl, pH 8.0

Restriction endonuclease digestions of DNA samples were accomplished using manufacturer's supplied buffers, 2 Units/ug DNA restriction enzyme, with distilled water to bring reaction mixes to desired volume, at 37°C for 2 hours.

DNA separations were accomplished in 1% agarose gels in TAE buffer, using 100 Volts, unless otherwise indicated.

Standard Prehybridization Conditions -- 0.1 M NaCl, 0.2 M sodium citrate, 0.5% SDS, 10% denatured salmon sperm DNA, 0.1% Ficoll, 0.1% polyvinyl pyrrolidone-40, 0.1% Pentax Fraction V bovine serum albumin (BSA), 50% formamide. Solutions preheated for 30 minutes to 100°C, cooled on ice 15 minutes, incubated filters overnight at 42°C.

Standard Hybridization Conditions -- Added radioactively labelled probe to Prehybridization Mix, heated to 100°C for 30 minutes, cooled on ice 15 minutes, incubated filters overnight at 42°C.

Confirming Endpoints of Previous Walk

Lambda clones covering the 38 kb span of DNA had been isolated from an overlapping genomic library constructed using sheared genomic DNA ligated to EcoRI linkers and inserted into a lambda Charon 4A vector. The left end (centromere distal) of the walk had been determined through standard restriction

digests, and corresponded to a 2.3 kb fragment "D". The right end of the previous molecular walk had been narrowed to two fragments, of 670 bp and 2.1 kb, whose position relative to one another could not be reconciled by standard restriction mapping. The expectation was that the centromere proximal end fragment was a part of a larger genomic EcoRI fragment, and would therefore hybridize to an intact fragment of larger size.

DNA was extracted from Canton-S (wild-type) *Drosophila melanogaster.* (adapted from Bender, et. al. 1983) Approximately 200 adult flies were ground in 2 ml of 0.1 M NaCl, 0.2 M sucrose, 0.05 M EDTA, 0.5% SDS, 1% Diethyl Pyrocarbonate, 0.1 M Tris-HCl, pH 9.1. The extracts were incubated at 65°C for 30 minutes, brought to 1 M Potassium acetate, incubated at 0°C for 30 minutes and spun for 5 minutes at 10,000g. The supernatant was recovered and mixed with an equal volume of ethanol for 5 minutes at room temperature, and the DNA was centrifuged for 5 minutes at 10,000g, washed with 80% ethanol, dried under vacuum, and resuspended in 1 ml Elu-tip low salt buffer. The DNA was then passed through an equilibrated Elu-tip© (Schleicher and Schuell) column, washed, and the DNA eluted with Elutip high salt buffer. Fractions of genomic DNA were digested with EcoRI (the enzyme that had been used to produce the library) run on 0.8% agarose TAE gels, transferred by Southern blot (Southern, 1975) with 20x SSC onto Immobilon© nylon filters and UV cross-linked using Stratagene's Stratalinker© .

Probes for the two potential end fragments were isolated from agarose gel

slices, following standard restriction digests of their plasmids, with the corresponding fragments being electroeluted into dialysis tubing at 100 V for one hour in TAE buffer. The fragments were labelled to $9x10^8$ counts per ug DNA specific activity using ^{32}P-dATP and Boehringer Mannheim's random oligo-labelling system©. Prehybridization and hybridization of probes to blot was accomplished at 42°C overnight in the standard solutions. The blots were subsequently washed in 2x SSC, 0.05% N-laurylsarkosine, 0.02% sodium pyrophosphate three times at room temperature briefly, then at 50°C for 1 hour each in 0.1x SSC, 0.05% N-laurylsarkosine, 0.02% sodium pyrophosphate. Following exposure overnight to Kodak X-Omat film at -70°C, the banding pattern of the blots revealed that a 670 bp piece of DNA from the right end was intact in the genomic sample, but a 2.1 kb fragment was not visible, and presumably was part of a larger 8.0 kb fragment that did hybridize to the probes.

Extending the Walk Using Lambda Clones

Using the 2.3 kb left and 2.1 kb right fragments as probes corresponding to the end points of the 38 kb walk, the molecular walk was extended. The λ Charon 4A genomic *D. melanogaster* library from which the original 38 kb of DNA had been isolated, obtained from Dr. Tom Maniatis (Maniatis et. al. 1978) and amplified one time, was screened using a mix containing 1.5×10^7 counts of each fragment labelled to a specific activity of 1.3×10^9 counts/ug. $1x10^5$ phage plaques were screened, representing 99% of the total genome. Twenty putative

positive clones were isolated, 12 of which remained after rescreening. Phage were dotted onto a bacterial lawn, transferred to nitrocellulose filters, and hybridized to the individual probes. Nine were positive for hybridization to the D fragment, and none to the 2.1 kb fragment. Four positive clones were randomly chosen for DNA preparation.

Plating bacteria were prepared by growing the *E. coli* strain K802 *(hsdR-, hsdM+, gal-, met-, SupE*) overnight at 37°C in Luria Broth (LB) with aeration, pelleting and then resuspending the bacteria in one-half of the original culture volume in 10 mM $MgSO_4$. High titer lysates of the phage clones were prepared by mixing 100 ul plating bacteria with 100 plaque forming units (pfu) phage in 3 ml LB top agar, plating onto LB plates, and incubating overnight at 37°C to obtain contiguous phage plates. Phage were harvested by scraping top agar into 2 ml of SM with 100 ul chloroform, then pelleting out agarose and debris for 10 minutes at 4000g. The titer for each lysate was determined to be 10^6 pfu/ul by mixing various phage dilutions with 100 ul plating bacteria, incubating overnight, and counting plaques. DNA was prepared by mixing 100 ul plating bacteria with 10^6 pfu phage and plating with 3 ml of top agar onto LB plates, and incubating overnight at 37°C to obtain contiguous phage plates. The plates were overlaid with 6 ml SM buffer. After two hours of gentle rocking the SM was recovered and spun at 8000g for 10 minutes. The phage were precipitated from the supernatant in the presence of 66 ug/ml RNAse, 1ug/ml DNAse, 10% polyethylene glycol 6000, 1M NaCl on ice for one hour. The phage were

pelleted at 10,000g for 10 minutes, and resuspended in 500 ul SM. The phage were lysed in 0.1% SDS, 5 mM EDTA, pH 8.0 for 15 minutes at 68°C. Protein was extracted from the solution with phenol, phenol:chloroform:isoamyl alcohol (25:24:1), and chloroform: isoamyl alcohol (24:1). The DNAs were precipitated in 0.15 M sodium acetate, 50% isopropanol at -20°C for 12 hours. The DNAs were centrifuged for 15 minutes, washed with 70% ethanol, dried and resuspended in 50 ul TE Buffer. The DNA preparations were digested with BamHI under standard conditions, and the restriction patterns revealed that two overlapping clones were represented among the four positive clones.

For further analysis of phage clones 7-2 and 14-7, one liter growths were prepared. 5 X 10^8 phage were mixed with 300 ul of K802 plating bacteria in NZCM media with 0.2% glucose, 0.00167% thiamine and an additional 10mM $MgSO_4$. The bacteria were grown for approximately 6 hours, allowing the phage to lyse, clearing the solution. The phage were pelleted at 10,000g and resuspended in 6 ml SM. Phage DNA was then prepared as outlined above. Restriction enzyme analysis was carried out under standard conditions, using the enzymes HindIII, PstI, and EcoRI. The digests were run on a 1% agarose TAE gel, transferred to Immobilon© by the method of Southern (Southern 1975) in 20X SSC, and probed with the labelled D fragment under standard hybridization conditions. The probe hybridized to a 5.5 kb and 6.7 kb EcoRI band, a 3.7 kb and 3.0 kb EcoR1/HindIII band, and a 3.7 kb and 18.4 (with phage arm) HindIII band, in the 7-2 and 14-7 clones respectively. The restriction

maps of 7-2 and 14-7 were assembled from fragment data in the usual manner. Comparison with the existing map, as well as the hybridization patterns revealed that the lambda clones had extended the walk 14.1 kb.

Extending the Walk Using YACs

Two overlapping yeast artificial chromosome (YAC) clones, R15-17 (28A1 to C8) and DY609 (28C1-2 to D4-5), isolated in the laboratories of Dr. Ian Duncan and Dr. Dan Hartl, were obtained. Cytological evidence indicated the YACs covered the entire region from 28A to 28D. An attempt to isolate *Drosophila* from yeast sequence in the YACs was initiated, in order to have available DNA suitable for creating an overlapping cosmid library of the 28A to 28D region.

To isolate YAC DNA, 5 ml yeast cultures were grown at 30°C in selective yeast media overnight. Pellets were recovered and resuspended in 1M sorbitol, 100 mM sodium citrate, 60 mM EDTA, 5% β-mercaptoethanol, 1% Lyticase, pH 7.0, for 2 hours at room temperature. The solution was then mixed with an equal volume of 1% (w/v) Lo-melt agarose/water and formed into small 1 cm plugs. The yeast were lysed by overlaying plugs with 0.5 M EDTA, 1% sarkosyl, 0.5 mg/ml Proteinase K, 10 mM Tris-HCl, pH 9.0 for 36 hours at 50°C.

CHEF gel electrophoresis (Vollrath and Davis, 1987) of YACs R15-17 and DY609 was carried out in a 1% agarose TBE gel at 4°C, using 150 volts, initially alternating at 5 seconds, ramped to 120 seconds final alternation, for 48 hours. The gels were stained in 0.5 ug/ml ethidium bromide for 30 minutes and

destained for 60 minutes in water. The YAC R15-17, reported to contain 240 kb of *Drosophila* DNA, rides coincident upon electrophoresis with a 225 kb yeast chromosome. Numerous attempts to uncover suitable electrophoretic conditions which would separate the two DNA species were unsuccessful.

YAC DNA was transferred by Southern blotting (Southern 1975) onto Immobilon© nylon membranes, using 10x SSC. The DNA was bound to the filter using Stratagene's Stratalinker©, prehybridized and hybridized under standard conditions using $4x10^8$ counts against either a urate oxidase (**MBUO XIII page** 121 *D melanogaster Urate oxidase* **BamHI** fragment) probe or **pKSII(+)wg**, a probe of the wg gene obtained from Steve DiNardo (The Rockefeller University, New York, New York). Both probes were labelled using Amersham's **Megaprime** labelling system© to $1.3x10^8$ counts/ug specific activity. Neither YAC hybridized to the wg probe, implying that the YAC contigs did not include a complete representation of the 28A-28D region. The difficulty in separation of the R15-17 YAC from the yeast chromosome, coupled with the probability that the entire genomic span of interest was not completely represented in the samples, led to abandonment of the use of YACs as a viable tactic for isolating DNA in the 28A1-28D1 region.

Extending the Walk Using Cosmids

An intact overlapping cosmid library, *D. melanogaster* (iso-1) genomic in a Not-Bam-Not CoSpeR P-element vector (Tamkun et al. 1992) hosted in HB101

(F-, hsdS20 (r_B-, m_B-), recA13, ara-14, proA2, lacY1, galK2, rpsLso(Sm^r), xyl-5, mtl-1, supE44, λ-), of the entire *Drosophila* genome was obtained. Approximately 30,000 cosmids, theoretically >99% of available sequence, were plated onto LB (40ug/ml ampicillin) plates overlaid with Amersham Hybond-N+ filters© which had been prewetted in ethanol and washed in water. After overnight growth at 37°C, a second prewetted filter was placed over the first, and the two filters were rolled together with a pipet to effect transfer of the *E. coli.* The second filter was returned to a fresh LB (40ug/ml ampicillin) plate and incubated at 37°C for six hours and subsequently transferred to a LB (25% glycerol, 40ug/ml ampicillin) plate for four hours of growth. These plates and filters were stored, well wrapped, at -20°C as the masters. The original filter was processed for hybridization by incubating for 7 minutes in Denaturation Solution, 7 minutes in Neutralization Solution, and cross-linked with Stratagene's Stratalinker©. Filters were prewashed, with hand rubbing, in 1 M NaCl, 1 mM EDTA, 0.1% SDS, 0.5 M Tris-HCl, pH 7.5, before prehybridization and hybridization under standard conditions.

Two successive screenings were carried out with the filter set. Hybridization to 4 x 10^8 counts of *wg* DNA labelled to 1.3 x 10^8 counts/ug specific activity uncovered 6 putative wg cosmids. Approximately 500 colonies of each were plated onto LB (40ug/ml ampicillin) plates and incubated overnight at 37°C and processed as described above. One of the six remained positive, and was replated and screened to obtain isolated positive colonies. The 2.1 kb fragment

representing the right end of the λ walk (Rend) was also labelled to 10^9 counts/ug specific activity, hybridized to the library as described above, and uncovered one positive Rend cosmid.

To isolate DNA from the cosmids, one liter M9 complete salts with 40 ug/ml ampicillin were inoculated with 10 ml overnight growths of the cosmid and grown to an OD_{600} of 0.5, at which time 85 mg chloramphenicol was added, and the cultures were grown overnight at 37°C. The cells were harvested at 4000g for 10 minutes, washed in TE Buffer, resuspended in 4 ml lysozyme solution, and incubated at 0°C for 30 minutes. Addition of 8 ml of lysing buffer was followed by 10 minutes of incubation at 0°C to lyse the bacteria. 6 ml of 3M sodium acetate, pH 4.8, was added and the mixture was incubated for one hour at 0°C. Cellular debris was removed by spinning 10 minutes at 10,000g. An equal volume of isopropanol was added to the supernatant, and it was incubated overnight at -20°C. The DNA was pelleted at 12,000g and resuspended in 20 ml TE Buffer. Cesium chloride gradients were prepared according to the procedure of Maniatis (Maniatis et. al., pp 93-94) sealed into 40 ml Beckman Quick Seal Tubes©, and centrifuged 18 hours at 45,000 rpm in a VTi50 rotor. The band representing intact cosmid DNA was removed, extracted extensively with Tris saturated butanol, and ethanol precipitated. Interpretation of standard restriction enzyme digests determined that the cosmids had extended the walk 17.2 kb in the centromere proximal area, as well as provided a noncontiguous map of 27 kb near 28A.

Figure 8 represents the restriction map extending from the UO locus with clones RW14-7, RW7-2 and CscRend, as well as the noncontiguous region represented by CrwWg surrounding the wg gene at 28A.

Characterization of the OPA Repeat

One of the original transcription units isolated in the 38 kb of DNA surrounding *urate oxidase* gave a hybridization pattern characteristic of the presence of a repeated element (Friedman et al. 1991). Hybridization against several known repeats uncovered that the transcribed element contained an OPA $(CAG)_n$ repeat (Wharton et al 1985), and the transcriptional unit was subsequently named Opa. Using a neighboring non-repetitive sequence as a probe, two adjacent cDNA Opa clones, a total of 2.5 kb, were isolated from a third instar larval library. A restriction map was generated of the two clones using standard techniques, and is shown in Figure 9.

Fragments generated with the restriction enzymes HaeIII, ClaI, SmaI, RsaI and PstI were subcloned into the vectors m13mp18 and m13mp19. The inserts were sequenced by the dideoxy method using standard techniques, with the sequence overlaps being hand matched. (Sanger et. al 1977). The sequence, shown in Figure 10, was submitted to GenBank (Accession number BankIt133170 AF018078).

Figure 8 Restriction Map Within 28A-28D

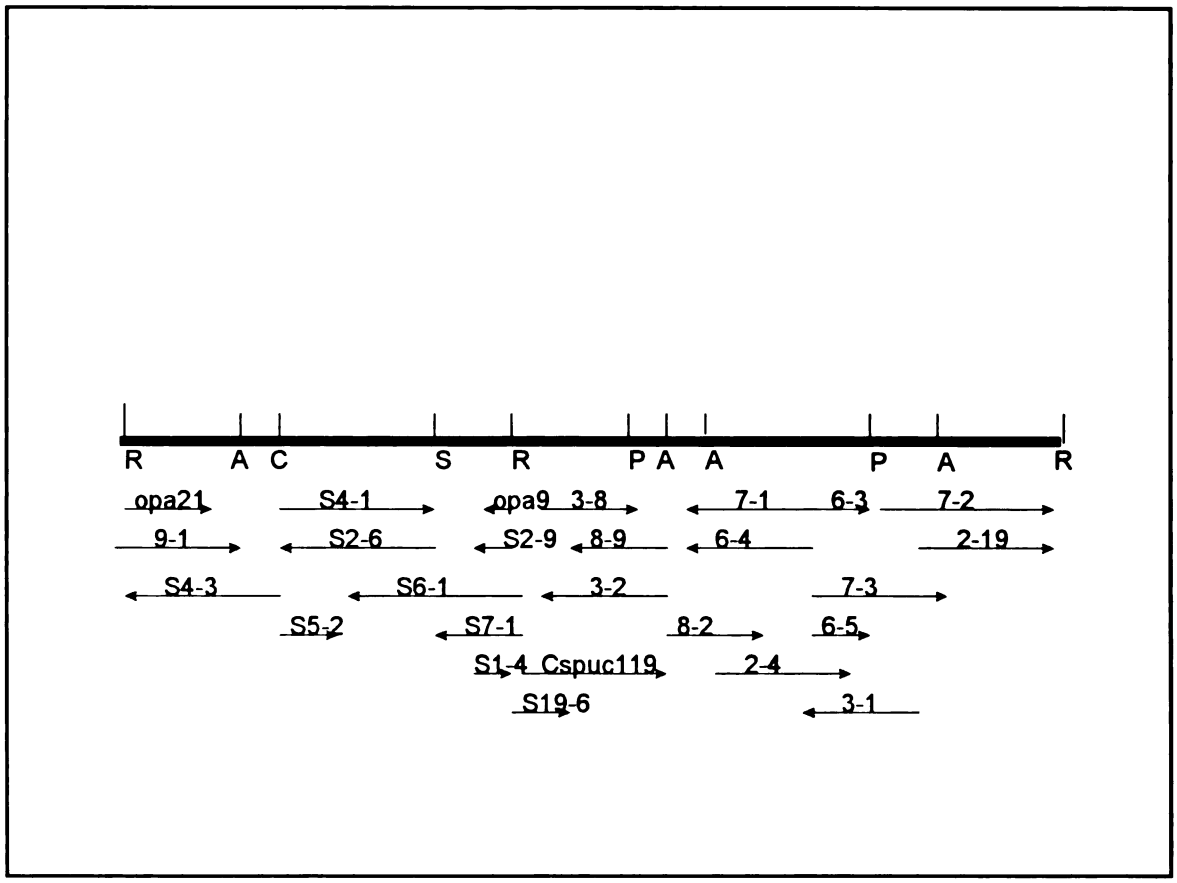


Figure 9 Restriction Map of OPA Clones

```
    10         20         30         40         50         60
CCCCCCCCCC CCCCGAGTTG CGTCNTTTGA GCGCGTTACC AGCGGACGGA CGAACAACGA
        70         80         90        100        110        120
CATGGAGCTG CCAATAGTCC TTCTCTTGGT ACTGAGTGTC ATTCCATTTG AAGCCCAGGG
       130        140        150        160        170        180
AGCTGTGCAC GATGAGCTGC CCCAGTGCGG CTTACATGGT GTTTACAGGC AGCGACAGAG
       190        200        210        220        230        240
TCTCGTCGAA TCGCCCAACT ATCCGGACAA CTATCCGGTG AACACCTGCT GGGATTATGT
       250        260        270        280        290        300
GGTGCGATCT CCCTACCGCT GCCCCACCAA GTTTCACATA CAGTTCTTGG ACTTCAAGCT
       310        320        330        340        350        360
GGAGCTATCG GAGAACTGCA GTCGGGATTA CTTGGCCATT GNTGAACAAC GATGGCGACG
       370        380        390        400        410        420
ACATGGAGGT CTTGTGCGGT CAGGTGCTGG GTATCAAAAA GTATCAACCC CCGATGGAGT
       430        440        450        460        470        480
CCTGCGTCTG CGCTTCCTTA GCGATGATTC GCCTTGGACA ACCAACGGTG GNTTCCGTTT
       490        500        510        520        530        540
GCTCATCACG CGTTTGGCCT GCGAAAAGGA GACTTGTGGC CAGGGTCTGG ACGATGACGA
       550        560        570        580        590        600
GGAGGACGTA CATGGCGACA CGGTGCAGGT GAGCGCCAAG TCGCCTCCGA AGAAGCTGCT
       610        620        630        640        650        660
CACCCAGCAT CACCATCATC ACCTGCCACA GCAAAGCCAT CAGCCGCAAC TGGAGCCAGC
       670        680        690        700        710        720
TTTCAACTAT ACGCAACCAA ATCGGTTGGG GTTTAATTTG GGCTTACAAC CAGGTCTTGA
       730        740        750        760        770        780
TTATCCCGGT GGATTATACC CACCGCCTGC GGGATTACCA CCCCAGTACA CACCGCCATG
       790        800        810        820        830        840
TGCTCCCCAG CAGCAGCAAG TGTTGCAGCA GCAGCAGGAG CAGCAAAGAT TCCTGCAGCA
```

Figure 10 OPA Sequence

850 860 870 880 890 900

ACAGCAGCTG CAACAACAAG TACAAGTTCA ACATCCACAG CAACTTCAAC TTTCCCAGCA

910 920 930 940 950 960

GCGTCAGCAA CTGCAGCAAG AGTTGCCCCT GATTTCCGAT CAATATCAGA CCACATCCTT

970 980 990 1000 1010 1020

TCAGCCACAT GCTGTTACTC TCAAGGATTA CGATGCCCAG ACCTTGCAGC AGTTTGGTGG

1030 1040 1050 1060 1070 1080

TCAGTTGGAT CTCTGCTGTG CCAGCAGCTT CAATCAGAAT CACTTCTATC TCTCCAGTCC

1090 1100 1110 1120 1130 1140

CGGATTTCCA AGGACAGTCT TGAACTATCT GCTACCCAAT CAGCAGAGGG ATTGCGTATT

1150 1160 1170 1180 1190 1200

TTACATCGAG AAGAGCTCAG CAAATGTCTG TCGACTGCGG ATTCAGTTCA AGTTCTTTGA

1210 1220 1230 1240 1250 1260

TTTCGGTCAG AATTCAGGAG GTTCAATTGG AGGCAGCTTT GGTGACGGAT TCGGTGGTGG

1270 1280 1290 1300 1310 1320

AGTGTCCGGT GGATTGGGCG GCGGACATAG TGGCGGTTTT GGAGGCCAAC ATAATTGCAA

1330 1340 1350 1360 1370 1380

TGGTGACTTT CTGGAGCTGG ATGGTCAGCG CTATTGCGGC TGTCCTTCCG GCTATGTCCA

1390 1400 1410 1420 1430 1440

CAAATCGCAT TGGGATCAGG GTCGCAAGGC CTGCGCATGC GCATTGGACA ATCGAGCAGC

1450 1460 1470 1480 1490 1500

ACCACGTCCA ATGGCTTTCT GCTGGAGATA TTCCAGGATC AGGACTCCGA TGGCTGTCGT

1510 1520 1530 1540 1550 1560

CAAGATGCTG GCCAAGGATT TGGTTTGGGC TTGCAACCGC AGCAACCTCA AAGGGGATTG

1570 1580 1590 1600 1610 1620

GGTTTGTGGC CACAGGGGTT ACAGCCTCAG TTGGGCTTGC AACCTCAGTT GGGCTTGCAA

1630 1640 1650 1660 1670 1680

CCTCAGTTGG GATTACCCCG CAAACCGCAC AAATGTGGCC GCTACAACCT GGTTATCCTG

1690 1700 1710 1720 1730 1740

GCTATATCAG TGGATATCCC CTACCCTTTT CGGCCACTCC CTACCGAACG GCGAGAAGGA

1750 1760 1770 1780 1790 1800

TATCCTATGC CCGGGCATCG ATGCTCAACA GCCATCGAGA GTGGTGGAAA CTAACTCCAC

1810 1820 1830 1840 1850 1860

TCGCAAGGAG TTCTATTACT TCGACGGCGA CGAGGCCTTT GCCCGTTCTG CTGTGGACGA

1870 1880 1890 1900 1910 1920

TGAGGATGAG GATAAGGCTC ACCTGGGCGT CACAACTCAG CCACAGTCTT CGTCCCAATC

1930 1940 1950 1960 1970 1980

CATAGTTAAG CCAGAGTCAC CTCTGGTTAC GAAGGCCTTT GAGCAAAGCA GTTGCTCCTT

1990 2000 2010 2020 2030 2040

TGATTACATG AGGTACTTAA ACTTTCAGTC GACACTCTCT GGTTAACTAA ACCCTTGTGC

2050 2060 2070 2080 2090 2100

TTTTCGCCGC TAAGAAGTTG GTTCCCAAAC ATTTTTGGCT GAAGGTATAT TACAAGCTGG

GCAA

An open reading frame of 1962 bp exists, beginning with an AUG at nucleotide 132 and continuing through an AAG stop codon at nucleotide 2094 (putative start and stop codons are indicated in green and red, respectively, in Figure 10). A BLAST (Altschul et al. 1990) comparison of the available DNA databases has revealed no remarkable homology, outside of the CAG repeat, to any previously reported sequences.

Discussion

In total, 96.3 kb of genomic DNA from the 28A-28D region has been isolated by various means, possibly representing 20% of the total euchromatic DNA content in this location. That DNA has been mapped by standard restriction enzyme analysis. To reconcile the restriction and cytological maps, high resolution *in situ* hybridization of probes derived from the restriction map could be carried out. Well stretched polytene chromosomes, when carefully prepared, can be used to differentiate fluorescently labeled probes that hybridize as close as 10 kb apart, obtaining distinct signals which are visible with sophisticated computer-aided optical microscope data collection (Rykowski et al. 1988).

An interesting aspect of the molecular characterization in the 28A-28D region was investigating the Opa trinucleotide repeat. Opa repeats were first identified during sequencing of the *Drosophila melanogaster Notch* locus and have been subsequently identified in other genes in several organisms (Wharton et al. 1985). The repeat consists of $(CAG)_n$, presumably coding for strings of glutamine, that vary between and within species. The biological significance of Opa and other trinucleotide repeats was initially enigmatic. Large size differences between gene analogues in closely related species prompted arguments that they were devoid of function, or might serve as nonspecific hinge regions between protein domains (Beachy et al. 1985). The recent discovery that triplet repeat amplification is a mutagenic mechanism in about 10 human

diseases characterized by the phenomenon of genetic anticipation, including Fragile X Syndrome and Huntington's Chorea, has sparked wide interest in the sequences (Huntington's Collaborative Group 1993, Caskey et al. 1992, Oberlé et al. 1991, Kremer et al. 1990). For these diseases, a trinucleotide repeat exists within small copy number limits in unaffected individuals, with expansion of the repeat beyond these parameters leading to a premutation or disease allele. Trinucleotide repeat diseases fall into two mechanistic categories -- neurodegenerative diseases like Huntington's Chorea that contain CAG repeats within the translated portion of the gene, coding for polyglutamine tracts, and some non-neurodegenerative diseases, including Fragile X and myotonic dystrophy, with CGG or GTG repeats that are located in non-coding regulatory regions of the gene (Caskey et al. 1992).

The gene responsible for Fragile X syndrome, FMR-1, was the first human trinucleotide repeat amplified disease allele discovered (Oberlé et al. 1991, Kremer et al. 1991). Cloning and sequence analysis of FRM-1 suggests that the normal protein product, containing two KH domains known to be RNA binding sites *in vitro*, is involved in RNA metabolism (Pieretti et al. 1991, Verkerk et al. 1991, Siomi et al. 1993, Siomi et al. 1994, Musco et al. 1996). The untranslated regulatory region upstream of FRM-1 contains a $(CGG)_n$ repeat that can exhibit expansions of several thousand copies in the mutated state (Oberlé et al. 1991, Kremer et al. 1991, Caskey et al. 1992). Affected individuals show reduced or absent steady state FRM-1 RNA levels, and a lack of FRM-1 protein (Pieretti et

al. 1991), suggesting that the disease phenotype results from disruption of normal protein expression, perhaps caused by interference of the expanded gene form with transcription and translation processes. Discovery of several affected individuals lacking an expansion, but having a deletion or splicing mutation in the FRM-1 coding region is consistent with this theory (Luyebeel et al. 1995, De Boulle et al. 1993, Siomi et al. 1994). However, *Ubx* is an example of a gene with promoter region trinucleotide repeats that function as a silencer Beachey et al. 1985, Beachey et al. 1988). A distinct function for the $(CGG)_n$ sequence in the Fragile X regulatory region has not been ruled out. Myotonic dystrophy is another documented example of a disease associated with triplet repeat $(GTG)_n$ expansion in a regulatory region, but the molecular analyses of this disease are too premature to propose that a similar disease mechanism is responsible (Brook et al. 1992, Fu et al. 1992).

For neurodegenerative diseases, such as Huntington's, (Huntington's Collaborative Group 1993) that contain triplet repeats within their protein coding regions it appears that expansion may be the direct mechanism of disease. Small repeats within these genes code for polyglutamine tracts that appear to expand by moderate amounts, typically doubling the number of repeats from 11-35 copies in the unaffected allele to 40 to 70 in mutant alleles, with an inverse correlation between size of expansion and both age of onset and disease severity (Orr et al. 1995, Duyao at al. 1993, Kawaguchi et al. 1996). The gene responsible for Huntington's Chorea has been cloned and sequenced.

Biochemical analyses of the corresponding Huntingtin protein have shown that the glutamine stretches bind a key glycolysis enzyme, glyceraldehyde-3-dehydrogenase (GAPDH) *in vitro*, leading to speculation that Huntington's normal cellular role may possibly be involved in energy production (Trottier et al. 1995). Larger polyglutamine tracts that can bind more GAPDH, resulting in a gain-of-function mutation, would be consistent with the autosomal dominant inheritance pattern of Huntington's Disease.

Discovery of expandable trinucleotide repeats has provided insight into the peculiar inheritance pattern of Fragile X and other diseases that present with the phenomenon of genetic anticipation -- the so-called Sherman's Paradox. Genetic anticipation is an increase in the incidence and severity of a disease with subsequent generations. In Fragile X, for instance, which demonstrates an X-linked recessive mode of inheritance, pedigrees often include males with severely affected grandsons, who clearly should be carrying the disease allele, but are themselves unaffected or only mildly affected. Reduced penetrance could be, and has been, invoked as an explanation for this phenomenon, however the pattern of a more severely affected grandfather with a mildly affected grandson is rarely seen. Ascertainment bias was the leading theory used to explain what was widely presumed to be artifactual data (Howeler 1989). However, insight into the molecular make-up of the genes suggests that unaffected or mildly affected males have simply inherited a moderately expanded pre-mutation allele. The allele is passed to the daughter and

undergoes expansion in her germline to the fully mutated state, to be inherited by her affected sons. Another curious aspect of the genetics of Fragile X is that expansion appears to occur exclusively in the female germline (Barnes et al. 1989). It has been postulated that this sex difference is somehow related to X-inactivation or genomic imprinting, but no investigation of the phenomenon has occurred (Laird 1987).

The molecular mechanism of expansion has not been determined. *In vitro* mechanistic studies of DNA polymerase fidelity have shown that polymerases can be prompted to pause when provided with GC rich segments, and also when encountering repeats (Goodman 1987). This effect is partially controlled by conformational aspects of the DNA, likely hairpin formation during local unwinding (Kaguni 1982). The fact that trinucleotide repeats are all GC rich has prompted the suggestion that DNA pausing, or "stuttering", culminating with incompletely replicated ends switching template strands during replication, is a possible mechanism for trinucleotide expansion (Gacy et al. 1995). However, *in vitro* studies, done under limiting nucleotide conditions that are presumably never encountered *in vivo*, provide little insight into biologically significant roles that polymerases may play in expansion of trinucleotide repeats. Until a rigorous *in vivo* test is designed to explore the role of polymerase pausing during replication, such suggestions are purely speculative.

Analysis of the sequence of my OPA gene with a BLAST program, using the nonrepetitive portion of the gene, has revealed no similarity to a previously

identified sequence. Uncovering a *Drosophila* $(CAG)_n$ containing gene with no known human homologue raises the intriguing possibility that *Drosophila OPA* corresponds to an unidentified human neurodegenerative disease gene. Bipolar disorder is an example of a neurodegenerative disease that has not been linked to a single genetic locus. Probing a human brain cDNA library with a nonrepetitive portion of the OPA gene could potentially uncover a gene of medical interest. The strategy of using trinucleotide repeats to target gene identification has been successfully employed (Petronis et al. 1996).

Restriction mapping and sequencing of an identified novel $(CAG)_n$ containing gene near urate oxidase, and restriction mapping of 58.3 kb of newly isolated DNA comprised the molecular investigation of the 28A-28D cytological region. The scope of the analysis has been to follow the initial observation that clustered genes reside in interband and faint band areas, by attempting to amass more positive evidence for this phenomenon. Although the amount of DNA that can be isolated and characterized within a practical amount of time is not sufficient to allow the formation of a generalized conclusion about euchromatic DNA organization, the current success of the genome mapping project, with its attendant emphasis on uncovering the coding portions of the genome, will illuminate the organizational motifs.

References

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and D. J. Lipman, 1990 Basic local alignment search tool. J. Mol. Biol. **215**:403-410.

Antequera, F. and A. Bird, 1993 Number of CpG islands and genes in human and mouse. Proc. Natl. Acad. Sci. USA **90**:11995-11999.

Barnes, D. M., 1989 Fragile X Syndrome and its puzzling genetics. Science **243**: 171.

Beachy, P. A., Helfand, S. L. and D. S. Hogness, 1985 Segmental distribution of bithorax complex proteins during *Drosophila* development. Nature **313**: 545-551.

Beachy, P. A., Krasnow, M. A., Gavis, E. R. and D. S. Hogness, 1988 An Ultrabithorax protein binds sequences near its own and the Antennapedia P1 promoter. Cell **55**: 1069-1081.

Bender, W., Spierer, P. and D. S. Hogness, 1983 Chromosome walking and jumping to isolate DNA from Ace and rosy loci and the Bithorax Complex in *Drosophila melanogaster*. J. Mol. Biol. **168**:17-33

Briggs, M. R., Kadonaya, J. T. and S. P. Bell, 1986 Purification and biochemical characterization of the promoter-specific transcription factor, SP1. Science **234**: 47-52.

Brook, J. D., McCurrach, M. E., Harley, H. G., Buckler, A. J., Church, D., Aburatani, H., Hunter, K., Stanton, V. P., Thirion, J.-P., Hudson, T., Sohn, R., Zemelman, B. Snell, R. G., Rundle, S. A. , Crow, S., Davies, J., Shelbourne, P., Buxton, J. Jones, C., Juvonen, V., Johnson, K., Hayen, P. S., Shaw, D. J. and D. E. Housman, 1992 Molecular basis of myotonic dystrophy: Expansion of a trinucleotide (CTG) repeat at the 3' end of a transcript encoding a proline kinase family member. Cell **68**: 799-808.

Buckler, A. J., Chang, D. D., Graw, S. L., Brook, J. D., Haber, D. A., Sharp, P. A. and D. E. Housman, 1991 Exon amplification: A strategy to isolate mammalian genes based on RNA splicing. Proc. Natl. Acad. Sci. USA **88**: 4005-4009.

Burke, D.T., Carle, G. F. and M. V. Olson, 1987 Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors. Science **236**: 806-812

Cai, H. and M. Levine, 1995 Modulation of enhancer-promoter interactions by insulators in the *Drosophila* embryo. Nature **376**: 533-536.

Caskey, C. T., Pizzuti, A., Fu, Y-H., Fenwick, R. G. and D. L. Nelson, 1992 Triplet repeat mutations in human disease. Science **256**: 784-788.

Cross, S. H., Charlton, J. A., Nun, X. and A. P. Bird, 1994 Purification of CpG islands using a methylated DNA binding column. Nature Genet. **6:** 236-244.

De Boulle, K., Verkerk, A. J. M. H., Reenters, E., Its, L., Hendrickx, J., VanRoy, B., Van den Bos, F., de Graaf, E., Oostra, B. A. and P. J. A. Willems, 1993 Point mutation in the *FMR-1* gene associated with fragile X mental retardation. Nature Genet. **3**: 31-35.

Duyao M., 1993 Trinucleotide repeat length instability and age of onset in Huntington's disease. Nature Genet. **4**: 387-392.

Duyk, G. M., Kim, S. W., Myers, R. M. and D. R. Cox 1990 Exon trapping: A genetic screen to identify candidate transcribed sequences in cloned mammalian genomic DNA. Proc. Natl. Acad. Sci. USA. **87**: 8995-8999.

Elvin, P., Slynn, G., Black, D., Graham, A., Butler, R., Filey, J., Anand, R. and A. F. Markham, 1990 Isolation of cDNA clones using yeast artificial chromosome probes. Nucleic Acids Res. **18**: 3913-3917.

Friedman, T. B., Owens, K. N., Burnett, J. B., Saura, A. O. and L. L. Wallrath, 1991 The faint band-interband region 28C2 to 28C4-5(-) of the *Drosophila melanogaster* salivary gland polytene chromosome is rich in transcripts. Mol. Gen. Genet. **226**: 81-87.

Fu, Y.-H., Pizzuti, A., Fenwick, R. G., Jr., King, J., Rajnarayan, S., Dunne, P. W., Dubie, J., Nasser, G. A., Ashizawa, T., DeJong, P., Wieringa, B., Korneluk, R.,m Perryman, M. B., Epstein, H. F., and C. T. Caskey, 1992 An unstable triplet repeat in a gene related to myotonic muscular dystrophy. Science **255**: 1256-1258.

Gacy, A. M., Goellner, G., Juranic, N., Macura, S. and C. T. McCurray, 1995 Trinucleotide repeats that expand in human disease form hairpin structures *in vitro*. Cell **81**: 533-540.

Gal, A., Nahon, J.-L. and J. M. Sala-Trepat, 1983 Detection of rare mRNA species in a complex RNA population by blot hybridization techniques: A comparative survey. Anal. Biochem. **132**: 190-194.

Gardiner-Garden, M. and M. Frommer, 1987 CpG islands in vertebrate genomes. J. Mol Biol. **196**: 261-282

Gausz, J., Hall, L. M. C., Spierer, A and P. Spierer, 1986 Molecular genetics of the *rosy-Ace* region of *Drosophila melanogaster*. Genetics **112**: 43-64.

Goodman, M. F., 1987 DNA polymerase insertion fidelity. J. Biol. Chem. **30**: 14689-14701.

Hawley, R. S., 1980 Chromosome sites necessary for normal levels of meiotic recombination. I. Existence and mapping of the sites. Genetics **94**: 625-646.

Hochman, B., 1973 Analysis of a whole chromosome in *Drosophila*. Cold Spring Harbor Symp. Quant. Biol. **38**: 581-589.

Howeler, C. J., Busch, H. F., Geraedts, J. P., Niermeijer, M. F. and A. Staal, 1989 Anticipation in myotonic dystrophy: Fact or fiction? Brain **112(Part 3)**: 779-797.

The Huntington 's Disease Collaborative Research Group, 1993 A novel gene containing a trinucleotide repeat that is expanded and unstable in Huntington's Disease chromosomes. Cell **72**: 971-983

Judd, B. H., Shen, M. W. and T. C. Kaufman, 1972 The anatomy and function of a segment of the X chromosome in *Drosophila melanogaster*. Genetics **71**: 139-156.

Kaguni, L. S., 1982 Template directed pausing in *in vitro* DNA synthesis by DNA polymerase α from *Drosophila melanogaster* embryos. Proc. Natl. Acad. Sci. USA. **79**: 983-987.

Kawaguchi, Y., Okamoto, T., Taniwaki, M., Aizawa, M., Inoue, M., Katayama, S., Hawazkamiu, H., Nakamura, S., Nishimura, M. Akigucki, I., Kimura, J., Narumiya, S. and A. Kakizuka, 1996 CAG expansions in a novel gene for Machado-Joseph disease at Chromosome 14q32.1. Nature Genet. **8**: 221-228.

Kellum, R. and P. Shedl, 1991 A position-effect assay for boundaries of higher order chromosomal domains. Cell **64**: 941-950.

Kellum, R. and P. Shedl, 1992 A groups of SCS elements function as domain boundaries in an enhancer-blocking assay. Molec. Cell. Biol. **12**: 2424-2431.

Kimmerly, W. J., Stultz, K., Lewis, S., Lewis, K., Lustre, V., Romero, R., Benke, J., Sun, D., Shirley, G., Martin, C. and M. J. Palazzolo, 1996 A P1-based physical map of the *Drosophila* euchromatic genome. Gen. Res. **6**: 414-430.

Kremer, E. J., Pritchard, M., Lynch, M., Yu, S., Holman, K., Baker, E., Warren, S. T., Schlessinger, D., Sutherland, G. R. and R. I. Richards, 1991 Mapping of DNA instability at the Fragile X to a trinucleotide repeat sequence $p(CCG)_n$. Science **252**: 1711-1714.

Laird, C., 1987 Fragile sites in human chromosomes as regions of late-replicating DNA. Trends Genet. **3**: 274

Larsen, F., Gundesen, G., Lopez, R. and H. Prydz, 1992 CpG islands as gene markers for the human genome. Genomics **13**: 1095-1107.

Lefevre, G., Jr., 1981 The distribution of randomly recovered X-ray-induced sex-linked genetic effects in *Drosophila melanogaster*. Genetics **99**: 461-480.

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

Lewis, J. D., Meehan, R. R., Henzel, W. J., Maurer-Fogy, I., Jeppesen, P., Klein, F. and A. Bird, 1992 Purification, sequence and cellular localization of a novel chromosome protein that binds to methylated DNA. Cell **69**: 905-915.

Lim, J. K. and L. A. Snyder, 1974 Cytogenetic and complementation analyses of recessive lethal mutations induced in the X chromosome of *Drosophila* by three alkylating agents. Genet. Res. **24:** 1-10.

Liu, C. P. and J. K. Lim, 1975 Complementation analysis of methyl methanesulfonate-induced recessive lethal mutations in the *zeste-white* region of the X chromosome of *Drosophila melanogaster*. Genetics **79**: 601-611.

Lovett, M., Kere, J. and L. Hinton, 1991 Direct selection: A method for the isolation of cDNAs encoded by large genomic regions. Proc. Natl. Acad. Sci. USA **88**: 9628-9632.

Luyebeel, K. A., Peier, A. M., Carson, N. L., Chuydley, A. E. and D. L. Nelson, 1995 Intragenic loss of function mutations demonstrate the primary role of *FMR-1* in fragile X syndrome. Nature Genet. **10**: 483-485.

Maniatis, T., Fritsch, E. F. and J. Sambrook, 1982 Molecular cloning: A laboratory manual. Cold Spring Harbor Press, Cold Spring Harbor, New York.

Maniatis, T., Hardison, R. L., Lacy, E., Lauer, J., O'Connell, C., Quon, D., Sim, G. K. and A. Efstratiadis, 1978 The isolation of structural genes from libraries of eukaryotic DNA. Cell **15**: 687-705.

Monaco, A. P., Neve, R. L., Colletti-Feener, C., Bertelson, C. J., Kurnit, D. M. and L. M. Kunkel, 1986 Isolation of candidate cDNAs for portions of the Duchenne muscular dystrophy gene. Nature **323**: 646-650.

Mortlock, D. P., Nelson, M. R. and J. W. Innis, 1996 An efficient method for isolating putative promoters and 5'-transcribed sequences from large genomic clones. Genome Research **6**: 327-335

Muller, H. J., 1928 The gene as the basis of life. Proc. Fourth Int. Congr. Plant Sci. **1:** 897-921.

Musco, G., Gunter, S. Joseph, C. Morelli, M. A. C., Nilges, M., Gibson, T. and A. Pastore, 1996 Three-dimensional structure and stability of the KH domain: Molecular insights into the fragile X syndrome. Cell **85**: 237-245.

Oberlé, I., Rousseau, F., Heitz, D., Dretz, C., Devys, D., Hanauer, A., Boué, J., Bertheas, M. F. and J. L. Mandel, 1991 Instability of a 550-base pair DNA segment and abnormal methylation in Fragile X syndrome. Science **252**: 1097-1102.

O'brien, S. J., 1973 On estimating functional gene numbers in eukaryotes. Nature (New Biol.) **242**: 52-54.

Orr, H. T., 1993 Expansion of an unstable trinucleotide CAG repeat in spinocerebellar ataxia type 1. Nature Genet. **4**: 221-226.

Parimoo, S., Patanjali, S. R., Shukla, H, Chaplin, D. D. and S. M. Weissman 1991 cDNA selection: Efficient PCR approach for the selection of cDNAs encoded in large chromosomal DNA fragments. Proc. Natl. Acad. Sci. USA **88**: 9623-9627.

Petronis, A., Bassett, A. S., Honer, W. G., Vincent, J. B., Tatuch, Y., Sasaki, T., Ying, D. J., Klempan, T. A. and J. L. Kennedy, 1996 Search for unstable DNA in schizophrenia families with evidence for genetic anticipation. Amer. J. Hum. Genet. **59**: 905-911.

Pieretti, M., Zhang, F., Fu, Y.-H., Warren, S. T., Oostra, B. A., Caskey, C. T. and D. L. Nelson, 1991 Absence of expression of the *FMR-1* gene in fragile X syndrome. Cell **66**: 817-822.

Rasch, E. M., Barr, H. J. and R. W. Rasch, 1971 The DNA content of sperm of *Drosophila melanogaster*. Chromosoma **33**:1-18

Rommens, J. M., Iannuzzi, M. C., Kerem, B-S., Drumm, M. L., Melmer, G., Dean, M., Rozmahel, R., Cole, J. L., Kennedy, D., Hidaka, N., Zsiga, M., Buchwalk, M., Riordan, J. R., Tsui, L-C. and F. S. Collins, 1989 Identification of the cystic fibrosis gene: Chromosome walking and jumping. Science **245**: 1059-1065.

Rykowski, M. C., Parmelee, S. J., Agard, D. A. and J. W. Sedat, 1988 Precise determination of the molecular limits of a polytene chromosome band: Regulatory sequences for the *Notch* gene are in the interband. Cell **54**: 461-472

Sanger, R., Nichlen, S. and A. R. Coulson, 1977 DNA sequencing with chain inhibitors. Proc. Natl. Acad. Sci. USA. **74**: 5463-5467.

Shiraishi, M., Lerman, L. S. and T. Sekiya, 1995 Preferential isolation of DNA fragments associated with CpG islands. Proc. Natl. Acad. Sci. USA **92**: 4229-4233.

Siomi, H., Siomi, M. C., Nussbaum, R. L. and G. Dreyfuss, 1993 The protein product of the fragile X gene, *FMR-1*, has characteristics of an RNA-binding protein. Cell **74**: 291-298.

Siomi, H., Choi, M. C., Siomi, M. C., Nussbaum, R. L. and G. Dreyfuss, 1994 Essential role for KH domains in RNA binding: Impaired RNA binding by a mutation in the KH domain of *FMR-1* that causes fragile X syndrome. Cell **77**: 33-39.

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

Tagle, D., Swaroup, M., Lovett, M. and F. Collins, 1993 Magnetic bead capture of expressed sequences encoded within large genomic segments. Nature **361**: 751-753.

Tamkun, J. W., Deuring, R., Scott, M. P., Kissinge, M., Pattatuccci, A. M., Kaufman, T. C. and J. A. Kennison, 1992 *brahma*: A regulator of *Drosophila* homeotic genes structurally related to the yeast transcriptional activator SNF2/SW12. Cell **68**: 561-572

Trottier, Y., Lutz, Y. Stevanin, G., Imbert, G., Devys, D., Cancel, G., Saudou, F., Weber, C., David, G., Tora, L., Agid, Y., Brice, A. and J.-L. Mandel, 1995 Polyglutamine expansion as a pathological epitope in Huntington's disease and four dominant cerebellar ataxias. Nature **378**: 403-406.

Valdes, J. M., Tagle, D. A. and F. S. Collins, 1994 Island rescue PCR: A rapid and efficient method for isolating transcribed sequences from yeast artificial chromosomes and cosmids. Proc. Natl. Acad. Sci. USA. **91**: 5377-5381.

Verkerk, A. J. M. H., Pieretti, M., Sutcliffe, J. S., Fu, Y.-H., Kuhl, D. P. A., Pizzuti, A., Reiner, O., Richerds, S., Victoria, M. F., Zhang, R., Eussen, B. E., van Ommen, F.-J. B., Blonden, L. A. J., Riggins, G. J., Chastain, J. L., Kunst, C. B., Galjaard, H., Caskey, C. T., Nelson, D. L., Oostra, B. A. and S. T. Warren, 1991 Identification of a gene *(FMR-1)* containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell **65**: 905-914.

Voelker, R. A., Wisely, G. B., Huang, S-M. and H. Gyurkovics, 1985 Genetic and molecular variation in the RpII215 region of *Drosophila melanogaster*. Mol. Gen. Genet. **201**: 437-445.

Vollrath, D. and R. W. Davis, 1987 Resolution of DNA molecules greater than 5 megabases by contour-clamped homogeneous electric fields. Nucleic Acids Res. **15**: 7865-7876.

Wallace, M. R., Marchuk, D. A., Anderson, L. B., Letcher, R., Odek, H. M., Saulino, A. M., Fountain, J. W., Brereton, A., Nicholson, J., Mitchell, A. L., Brownstein, B. and F. S. Collins, 1990 Type I Neurofibromatosis gene: Identification of a large transcript disrupted in 3 NFI patients. Science **249**: 181-186.

Wharton, K.A., Yedvobnick, B., Finnerty, V. G. and S. Artavanis-Tsakonas, 1985 opa: A novel family of transcribed repeats shared by the *Notch* locus and other developmentally regulated loci in *Drosophila melanogaster*. Cell **40**: 55-62.

Woodruff, R. C. and M. Ashburner, 1979 The genetics of a small autosomal region of *Drosophila melanogaster* containing the structural gene for alcohol dehydrogenase. II. Lethal mutations in the region. Genetics **92:** 133-149.

Young, M. and B. H. Judd, 1978 Nonessential sequences, genes, and the polytene chromosome bands of *Drosophila melanogaster*. Genetics **88:** 723-742.

Appendix B

Table 16 lists the four overlapping deficiencies that define the A, B, and C cytological regions. Absence and presence of the l(2) / deficiency Cy^+ progeny were scored as previously described, where "+" = viable and "-" = lethal.

Table 16 Viability of Mutant/Deficiency Heterozygotes

Lethal	Df(2L)spdx4	Df(2L)A2	Df(2L)SS1	Df(2L)TE62x1	Group
1	-	-	-	-	A
3	-	-	-	-	A
4	-	-	-	-	A
5	-	-	-	-	A
6	-	-	-	-	A
7	-	-	-	-	A
8	+	+	+	+	C
9	-	-	-	-	A
10	-	-	-	-	A
11	-	-	-	-	A
12	-	-	-	-	A
13	+	-	+	+	B
14	-	-	-	-	A
15	-	-	-	-	A
16	+	-	+	+	B
17	-	-	-	-	A
18	+	+	+	+	C
19	-	-	-	-	A
20	-	-	-	-	A
21	+	+	+	+	C
22	-	-	-	-	A

23	+	+	+	+	C
24	+	+	+	+	C
25	+	+	+	+	C
26	+	+	+	+	C
27	-	-	-	-	A
28	-	-	-	-	A
29	-	-	-	-	A
30	+	+	+	+	C
31	-	-	-	-	A
32	-	-	-	-	C
33	+	+	+	+	A
34	-	-	-	-	C
35	+	+	+	+	A
36	+	+	+	+	A
37	+	+	+	+	A
38	+	+	+	+	A
39	-	-	-	-	C
40	+	+	+	+	A
41	-	-	+	+	
42	-	-	-	-	A
43	-	-	-	-	A
44	-	-	-	-	A
45	+	+	+	+	C
46	+	+	+	+	C
47	+	+	+	+	C
48	+	+	+	+	C
49	+	+	+	+	C
50	-	-	-	-	A
51	+	+	+	+	C
52	-	-	-	-	A
53	-	-	-	-	A
54	-	-	-	-	A
55	-	-	-	-	A
56	+	+	+	+	C

57	+	+	+	+	C
58	-	-	-	-	A
59	-	-	-	-	A
60	-	-	-	-	A
61	+	-	+	+	B
62	-	-	-	-	A
64	-	-	-	-	C
65	+	+	+	+	A
66	-	-	-	-	A
67	-	-	-	-	A
68	-	-	-	-	B
69	+	-	+	+	A
70	-	-	-	-	A
71	-	-	-	-	C
73	+	+	+	+	C
74	-	-	-	-	A
75	-	-	-	-	A
76	-	-	-	-	A
77	-	-	-	-	A
78	-	-	-	-	A
79	+	+	+	+	C
80	+	+	+	+	C
81	-	-	-	-	A
82	+	+	+	+	C
83	+	-	+	+	B
84	+	+	+	+	A
85	-	-	-	-	C
86	-	-	-	-	C
87	+	+	+	+	C
88	+	+	+	+	C
89	+	+	+	+	C
90	-	-	-	-	A
91	-	-	-	-	A
92	+	+	+	+	C

93	+	+	+	+	C
94	-	-	-	-	A
95	+	+	+	+	C
96	-	-	-	-	A
97	-	-	-	-	A
98	+	+	+	+	C
99	-	-	-	-	A

Table 17 Genetic Characterization of Deficiencies

	Df(2L) ade327	Df(2L) SS1	Df(2L) spdA2	Df(2L) spdx4	Df(2L) Az	Df(2L) J1-H52	Df(2L) TE6x1	Df(2L) TE6x2	Df(2L) 128x11	Df(2L) 12x14
Df(2)ade327	-									
Df(2L)SS1	+	-								
Df(2L)spdA2	-	-	-							
Df(2L)spdx4	-	-	-	-						
Df(2L)Az	-	-	-	-	-					
Df(2L)J1-H5	-	-	-	-	-	-				
Df(2L)TE6x1	-	N.D.	N.D.	N.D.	N.D.	N.D.	-			
Df(2L)TE6x2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	-		
spd-flag	+	spd	spd	spd	spd	spd	spd	spd	+	+
cui	+	+	+	+	+	+	+	+	cui	cui
wg-1	+	wg	wg	wg	wg	wg	+	N.D.	+	N.D.
wg-cx4	wg	-	-	-	-	-	-	-	N.D.	N.D.
Sp	Sp	-	-	-	-	-	-	-	Sp	Sp