Fly Pushing

The Theory and Practice of *Drosophila* Genetics

SECOND EDITION

RALPH J. GREENSPAN

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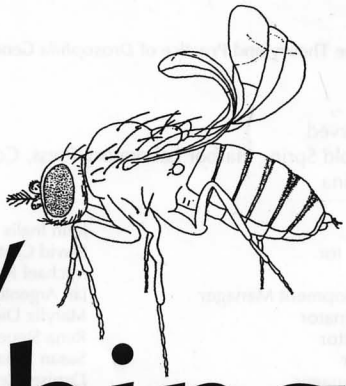

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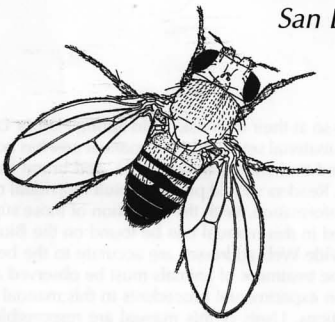

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Cover artwork: Commonly used marker mutant phenotypes. *Front cover:* (*top*) *white-apricot* (w^a/w^a); *Curly* (*Cy*/+); (*bottom*) *Dichaete* (*D*/+). *Back cover:* (*Top row, left*) heterozygous *Bar* (*B*/+) female; (*center*) *B/B* female or *B/Y* male; (*right*) *Stubble* (*Sb*/+). (*Second row, left*) *Sco* (*Sco*/+); (*center*) normal haltere; (*right*) *Ultrabithorax* (*Ubx*/+). (*Third row, left*) *Serrate* (*Ser*/+); (*right*) *forked* (*f*/*f*). (*Fourth row, left*) *singed* (*sn*/*sn*); (*right*) *eyeless-Dominant* (*eyD*/+). Illustrations on front and back covers and those on page xiii are from Lindsley and Zimm 1992, used and/or modified with permission of the publisher, Academic Press.

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O Genotype, O Phenotype,
This kiss had better last her;
He's off to see his other love,
Drosophila melanogaster.

from *Love on the Fly*
by Dani S. Grady

Contents

Preface

MOST OF WHAT MATTERS IN FLY PUSHING does not change with time, and we are grateful for that fact. As Jeff Hall has said, "How many fields are there in which you can read papers from 1916 (referring to Calvin Bridges' papers on nondisjunction) and find them as relevant today as they were then?" Since this book consists primarily of classical fly genetic techniques, it therefore does not go out of date.

The justification for a second edition is that a sufficient number of new techniques, or new variants of old techniques, have been developed to warrant their inclusion. Mostly, these involve the incorporation of a new molecular tool into a traditional genetic strategy. In addition, there has been a watershed in the evolution of fly genetics since publication of the original edition: the complete sequencing of the fly genome. This has made many aspects of gene identification in the fly much easier.

Some may feel that certain topics are obsolete, but part of the genius of fly genetics as it has developed over the years is its reuse and recombination of approaches from the past to create new innovations. For this reason, most of the approaches discussed in the first edition are revisited in this one.

This book has benefited from the comments and criticisms of many people, to whom I am enormously grateful: Michael Ashburner, Eric Wieschaus, Barry Ganetzky, Steve Small, Angus Wilson, Tim Karr, Nanci Kane, and Sue Broughton for the first edition, and Herman Dierick, Bambos Kyriacou, and Marla Sokolowski for the current edition. I thank Michael Regulski, Maryliz Dickerson, Rena Steuer, Susan Schaefer, and Denise

Weiss for their editorial assistance and input, and David Crotty for his patience as the revision was brought to fruition. Above all, I thank Jeff Hall, who first introduced me to the fly and taught me everything important that I know about its genetics.

Fly Pushing

The Theory and Practice of *Drosophila* Genetics

SECOND EDITION

FREQUENTLY USED MARKERS

heterozygous ♀ hemizygous ♂

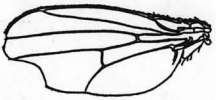
Bar (*B*) *X*
eye narrower than usual, oval shape

forked (*f*) *X*
bristles short with split or bent ends

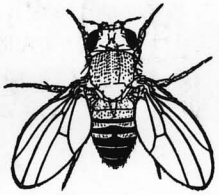
singed (*sn*) *X*
bristles short, gnarled, and wavy

Curly (*Cy*) *2nd*
wings curled upward instead of flat

Scutoid (*Sco*) *2nd*
missing bristles, especially from posterior thorax

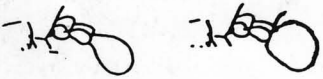


Serrate (*Ser*) *3rd*
(also called *Beaded-Serrate*, Bd^S)
wings notched



Dichaete (*D*) *3rd*
wings extended like jet plane instead of straight back

Stubble (*Sb*) *3rd*
bristles short and stubby



+/+ *Ubx*/+

Ultrabithorax (*Ubx*) *3rd*
haltere larger and rounder than normal

eyeless-Dominant (ey^D) *4th*
very small eyes

Introduction

WITH EACH PASSING YEAR, MORE AND MORE SCIENTISTS are attracted to work with *Drosophila*, lured by the potential for combining genetic and molecular approaches to questions of gene expression, cell biology, development, and neurobiology. They are aware of a large folklore of classical genetic tools lurking somewhere in the fly field—they have seen the results in some of the dazzling findings that have been made—but access to these tools somehow seems to be limited. At times, it may even appear that the wielders of "hard-core" fly genetics preside over a coven with secret rites of initiation. The situation has led many to bemoan the lack of a simple and rational way to gain access to Drosophilid mysteries.

Publication of a compendium of facts and commentaries on *Drosophila* genetics and biology by Michael Ashburner (originally in 1989, with a new edition, Ashburner et al. 2004) has provided fly workers with a comprehensive reference source for all of the folklore (and much more). Still lacking, however, is a bridge to that folklore for the uninitiated.

This book attempts to provide that bridge. It is designed for those graduate students, postdocs, and even laboratory heads wishing to use fly genetics in their work. An elementary knowledge of genetics (e.g., undergraduate level) is assumed, but not much more. The approach used in this book has been worked out over the years in lectures given as part of intensive short-term courses at the Cold Spring Harbor Laboratory, at the University of California at San Francisco, and in my own laboratory. This approach owes a major debt to the pedagogical tradition of the late Larry Sandler as modified and transmitted by his student (my mentor), Jeff Hall.

Although the book does not attempt to be comprehensive, it does refer to more complete treatments of particular topics elsewhere (such as the aforementioned Talmudic commentaries of Ashburner).

Each subject is addressed from a practical standpoint, with a bit of the theoretical foundations (such as they are) to make it intelligible. Illustrations of each technique are taken from the literature and practice problems are provided. Although the working out of problems is helpful in learning the principles, it must be borne in mind that there is no substitute for actually doing genetic experiments and crosses—"fly pushing," as it is affectionately known.

CHAPTER 1

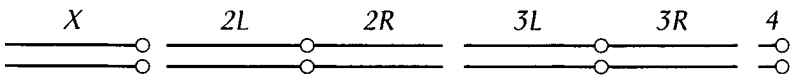
The Basics of Doing a Cross

OVER THE PAST CENTURY, FLY GENETICS HAS BEEN developed to a high art. Some of this is the result of time and accumulated information but much of it is due to the construction of several unique genetic "tools," plus a few intrinsic features of fly biology. The net effect is to make mating schemes more reliable and unambiguous by controlling the randomizing and shuffling effect of recombination in meiosis, and to make the physical location of genes on chromosomes easier to determine. The following sections present some of the basics of fly genetics and looking after fly cultures, as well as a beginner's Rosetta stone of fly nomenclature.

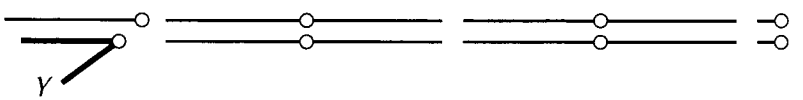
FLY CHROMOSOMES

Flies have four pairs of chromosomes, usually represented as lines and circles for arms and centromeres:

Female



Male



L refers to the left arm and *R* to the right. The *X* and fourth chromosomes

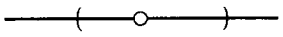
have major left arms and tiny right arms (standardly drawn with no right arm). The size of the *X, 2L, 2R, 3L,* and *3R* are roughly comparable, whereas chromosome *4* is only about one-fifth as large.

Sex determination in *Drosophila* is based on the ratio of *X* chromosomes to autosomal sets. In males, one *X* with two autosomal sets gives a ratio of 0.5, whereas females have a ratio of 1.0. The *Y* chromosome contains few genes and is not required for most aspects of male development, only for proper sperm motility.

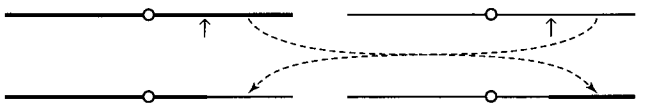
An important feature of fly genetics is the total absence of recombination in males. Whereas recombination is usually lower in the heterogametic sex of a species (the sex with two different sex chromosomes, usually the males), in *Drosophila melanogaster* it is effectively zero. Recombination in females, on the other hand, is alive and well. Its control is achieved by the use of the fly pusher's most distinctive tool: balancer chromosomes. These are chromosomes whose normal gross sequence is so scrambled (the result of multiple breaks and rejoinings induced by radiation) that they are no longer capable of pairing or recombining with their normal homolog during meiotic prophase. In addition, their presence in a fly is easily recognizable by a dominant marker mutation and they contain recessive markers as well. Consequently, their transmission to progeny can be tracked unambiguously. Since they effectively block any recombination with their homologs, the transmission of the homolog to progeny can also be tracked unambiguously. This holds true even if the homolog has no dominant marker, because homologs segregate reliably. Thus, if the progeny did not get the balancer, it must have gotten the homolog. This is the single most important principle in fly mating schemes.

Balancers, which are discussed in more detail later in this chapter, are a special case of the more common kind of chromosome rearrangement known as inversions. Other rearrangements that appear in the course of our discussions are translocations, compound chromosomes, deficiencies, and duplications. The major categories of rearrangement with their representations are listed below.

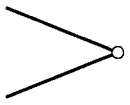
- Inversions, in which two breakage and repair events have occurred in the same chromosome, resulting in an inverted segment (the breakpoints are symbolized by parentheses):



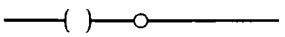
- Translocations, in which breakage events have occurred in two different chromosomes and repaired so that the pieces are swapped:



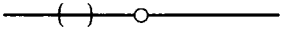
- Compound chromosomes, in which two left or two right arms have become attached to the same centromere (e.g., attached-*X*, attached-*2L*, attached-*4*):



- Deficiencies (also called deletions), in which two breaks have occurred in the same chromosome and the repair event has excluded the excised piece:

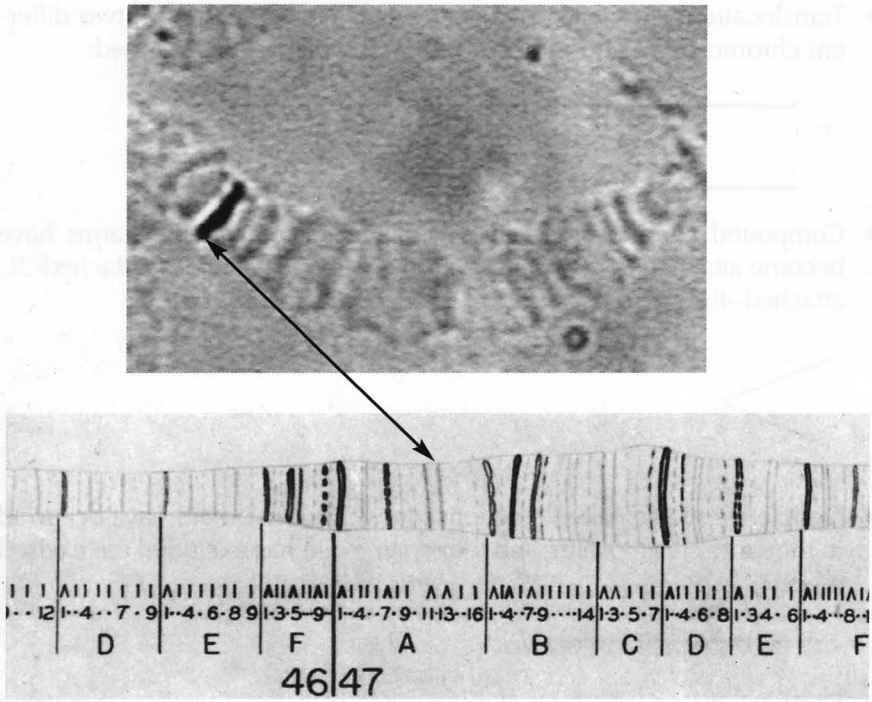


- Duplications, in which an excised piece is inserted into another chromosome:



Another important intrinsic feature of the fly is the presence of polytene chromosomes in the salivary glands. These have distinctive, high-resolution banding patterns. Early on, they made it possible to correlate map positions of genes with physical features of the chromosome and determine the breakpoint locations of chromosome rearrangements. In the molecular era, they have facilitated the mapping of cloned DNA sequences to physical locations.

Each of the major chromosome arms is divided into 20 numbered segments: 1–20 for *X*, 21–40 for *2L*, 41–60 for *2R*, 61–80 for *3L*, and 81–100 for *3R*. Chromosome *4* is divided into regions 101–104. Each numbered region is then divided into lettered regions (A,B,C,D,E) and each lettered region into numbered bands. (The number of lettered regions and numbered bands for each numbered region is not constant along the chromosome, but depends on local topography.) Centromeric regions are 20 for the *X*, 40–41 for the second, 80–81 for the third, and 104 for the fourth



In situ hybridization to larval polytene chromosome. (*Top*) Enzymatically stained DNA hybridized to band 47A11-14 region (courtesy of P. Tolias). (*Bottom*) Bridges' original drawing of the same chromosome region (Bridges 1916).

chromosome. The corresponding telomeric regions are 1 (*X*), 21 (*2L*), 60 (*2R*), 61 (*3L*), 100 (*3R*), and 101 (*4*).

RECOGNIZING MARKERS

Marker mutations are the key to deciphering genotypes. Sometimes they are used to mark the chromosomes that you are specifically trying to follow, but more often they mark the chromosome that you are trying to lose. In any event, a vast array of mutations affecting eye color, eye shape, wing shape, wing vein morphology, bristle color, bristle shape, and cuticle pigmentation—to name the major categories—serve to tag the various chromosome arms.

Descriptions of mutant phenotypes can be found in Lindsley and Zimm (1992), for those preferring the printed page, and on FlyBase for the cybernetically inclined (FlyBase Consortium 2003; see the Appendix). One simply becomes accustomed to recognizing these descriptions. The important points to bear in mind about markers concern their consistency of expression and their interactions with each other.

Consistency of expression is reflected in the likelihood that a fly of mutant genotype will show a mutant phenotype (penetrance) and if so, the extent of the range of those phenotypes (expressivity). The "rank" assigned to mutations in Lindsley and Zimm embodies these parameters, with the highest (RK1) showing the greatest consistency. When you select positively for a given marker, you can tolerate a certain amount of inconsistency. The worst that will happen is that you will miss a few flies. Much more dangerous, however, is selecting against a particular marker (i.e., saving flies based on the absence of the marker). In these cases, it is crucial to be able to rely on the marker's consistency of expression—or at least to be aware of any possible inconsistency. The RK rating helps you to discern the ones that are problematic until you get a feel for it.

Interactions between markers become important as soon as you find yourself in the situation of using two that affect the same trait. For instance, if you are using two different mutations affecting bristle shape, it is crucial to be familiar with the double mutant to determine whether it is distinguishable from each single mutant. Rarely can this information be found in Lindsley and Zimm or on the FlyBase (except for certain eye-color mutations). Instead, one often has to proceed empirically.

NOMENCLATURE

Fly nomenclature is a paper tiger.

From the uncollected sayings of Mao Zedong.

The shorthand of *Drosophila* genetics can be reduced to a few simple rules, illustrated in the examples that follow (see also FlyBase Consortium 2003).

1. $f;\ cn\ bw;\ \frac{TM2}{tra}$

This example demonstrates several points:

- The genotype of a chromosome is indicated only if there is a mutation or some other kind of variant on it, and the chromosomes are always listed in the order *X/Y, 2, 3, and 4*. In this example, *f; cn bw; TM2/tra* refer to the *X, 2,* and *3* chromosomes, respectively. If the pertinent mutations are on the *X* and *3* only, then you would skip any designation for chromosome *2*, e.g., *f; TM2/tra*.
- Fly genotypes (e.g., *f*) are always italicized, as are mutant and gene names. This book follows the traditional convention.
- Mutant names are abbreviated with three or fewer letters (although this rule has been abandoned in recent years because of the amount of mutants to name [e.g., *norpA* and *disco*]; *f* denotes *forked,* affecting bristle morphology, *cn* is for *cinnabar* and *bw* for *brown* (both affecting eye color such that together they produce a white eye), *TM2* indicates the balancer chromosome "third multiple 2," and *tra* denotes *transformer,* a gene required for sex determination.
- Lowercase abbreviations indicate recessive phenotypes and uppercase indicate dominants, with locus names taken from enzyme or protein names (e.g., *Adh* for the structural gene for *Alcohol dehydrogenase*) or, as in this case, a particular chromosome rearrangement (the balancer chromosome *TM2*).
- Semicolons separate the genotype symbols for different chromosomes. In the example above, genotypes of the *X, 2,* and *3* chromosomes are indicated.
- Commas follow the name of a rearrangement and indicate mutations on that chromosome (e.g., the full name of *TM2* is *TM2, Ubx^{130}* because it carries a mutant allele of *Ultrabithorax* known as number 130).
- A chromosomal genotype written on a single line indicates that the stock is homozygous for that genotype; heterozygosity is denoted by a two-line genotype (as in *TM2/tra* above), each line corresponding to one of the homologs present (in publications, the genotype would be denoted as *TM2/tra,* all on one line).
- Anything that is not shown is presumed to be wild type. Thus, *f* means that the *X* chromosome carries a mutant allele of *forked;* all other *X*-chromosome loci are presumed to be wild type. Similar-

ly, when heterozygosity is indicated, only the mutant loci (or rearrangements) are shown for each chromosome.

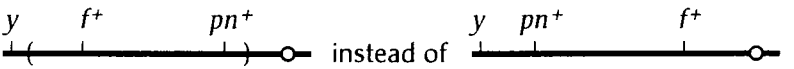
2. $\frac{C(1)RM,\ y^2}{Y}$; $\frac{In(2LR)O,\ Cy}{Sco}$; $\frac{ci^D}{ey^D}$

- *C(1)RM* denotes a compound chromosome. *C(1)* shows that it is a compound of the first chromosome, and *RM* refers to the fact that it is reversed metacentric, i.e., the centromere is in the middle (metacentric) and in the linear order of the chromosome. One arm is reversed relative to the other (i.e., both are attached at the same end); a common shorthand for *C(1)* is $\widehat{XX}$, attached-*X*.
- This particular attached-*X* is homozygous for the y^2 allele (the second one found) for the gene affecting cuticle color yellow; it has black bristles and a yellow cuticle, which differs from (the first) yellow by the fact that its bristles are yellow as is its cuticle.
- Since the attached-*X* contains both homologs of the *X* on one centromere, they do not segregate from each other. Usually these stocks are kept such that both males and females carry a *Y*; this is to ensure that all of the males will be fertile. (The presence of a *Y* chromosome has nothing to do with sex determination and has no effect on females with two *X*s, but it is essential for sperm motility. Since the *Y* segregates from the *X* in males, the only way their sons can receive a *Y* is if they obtain it from their mothers, a result of its segregation from the attached-*X*. This occurs reliably by having a *Y* present in all flies of the stock.)
- The genotype of the second chromosome in this stock is heterozygosity for two different chromosomes: One is a balancer known as *In(2LR)O,Cy* (sometimes referred to colloquially and variously as "*Curly-O*," *CyO*, "*Curly-Oster*," or "*Curly of Oster*," named after Irwin Oster, who produced it), which carries the dominant mutation *Cy* causing wing curling, and the other is a chromosome with the dominant mutation *Scutoid* (*Sco*, subsequently renamed noc^{Sco} as an allele of *no ocelli*), which eliminates certain thoracic bristles; the *In(2LR)* refers to the multiple inversions (*In*) involving the left (*L*) and right (*R*) arms of the second (*2*) chromosome.
- The final chromosome genotype refers to the fourth chromosome, heterozygous for two dominant alleles of genes for which most other alleles are recessive—*eyeless* and *cubitus interruptus*. This

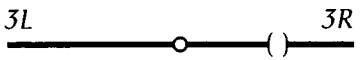
represents an exception to the convention of uppercase letters for dominant mutations, instead using a superscript *D* to indicate dominance of these alleles. Since both of these alleles are recessive lethal, all flies will be heterozygous for both (this apparent exception to the naming convention follows the rule that loci originally defined by recessive mutations, such as *ey*, will continue to be designated by lowercase letters even after dominant alleles are found).

- Chromosome rearrangements are designated by an abbreviated symbol followed by the chromosome affected (*1, 2, 3,* or *4*) and the name of the rearrangement. For example,

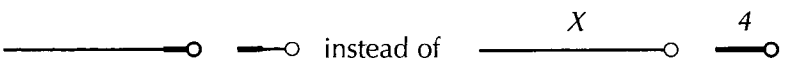
In(1)sc^{4} inversion on the *X* chromosome called "*scute 4*," producing a mutant phenotype because of a break in the *scute* locus (breakpoints are symbolized by parentheses):



Df(3R)P14 a deletion (deficiency) of part of the right arm of the third chromosome (*3R*), whose name, *P14*, stands for Pasadena 14:

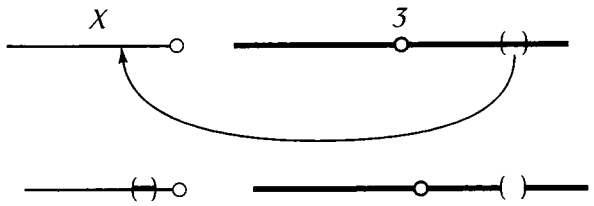


T(1;4)B^{S} translocation between the first and fourth chromosomes, in which there is a break in each and a reciprocal rejoining, producing a severe *Bar* eye phenotype called *Bar of Stone* (*B*^{S}):

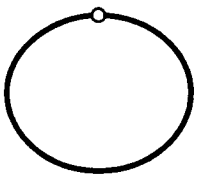


Tp(3;1)ry^{35} transposition (*Tp*) of a piece of the third chromosome into the *X*, one of whose breakpoints produces a mutant phenotype in the eye-color gene *rosy*; sometimes designated as translocation *T(3;1)ry*^{35}; transpositions can also refer to the movement of a segment from one place to anoth-

er in the same chromosome. When this *X* is present in a strain whose third chromosomes are both normal, it becomes a "duplication," designated by *Dp(3;1)ry*^{35}:

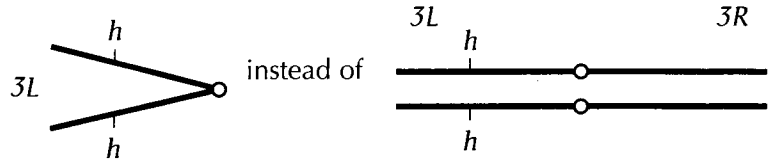


R(1)w^{vC} ring *X*, an *X* chromosome with no free ends. This particular one has abnormal expression (variegation, hence the "*v*") of the *white* gene affecting eye color (the "*C*" refers to Catcheside, its originator):



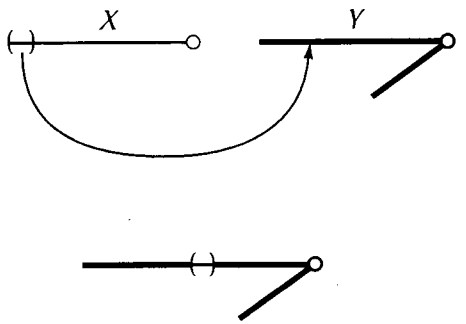
C(3L)RM, h compound chromosome consisting of two left arms of the third chromosome, also known as "attached-*3L*," homozygous for the original allele of the mutation *hairy* (*h*), producing extra hairs on the thorax and head. "*RM*" stands for reversed metacentric, jargon meaning that the two left arms of chromosome *3* are attached to the same centromere instead of each one being attached to a separate centromere with a right arm. "Metacentric" refers to the fact that the centromere is in the middle and "reversed" refers to the order of genes from one *3L* to the next. This reverses when you pass through the centromere, such that the normal *3L*-chromosome tips are still at the tips. This contrasts with a "tandem metacentric," in which the gene order would repeat identically

when passing through the centromere, and where the tip of one arm is now at the centromere:



F(2L) "free" left arm of the second chromosome, in which only a single left arm (*2L*) is on one centromere:

y^{+}*Y* duplication on the *Y* chromosome of the chromosomal segment carrying a wild-type allele of *yellow*, also designated as *Tp(1;Y) y*$^{+}$:



Note: On above figures, *Y* signifies the chromosome, *y* the *yellow* locus.

A new kind of gene name has emerged after sequencing the fly genome: "*CG*" (see FlyBase Consortium 2003). These genes, not previously identified, are those predicted from the genome sequence. Each one is designated by a number preceded by the letters CG (referring to Celera Genome), e.g., *CG5170*. CG names are temporary and will eventually give way to more descriptive gene names as functions are assigned to this vast set of many thousands of genes.

EVOLUTION OF GENE NAMES

Since genes are generally pleiotropic, independently isolated mutations with different phenotypes and different names often eventually turn out to be alleles of the same locus. When this occurs, the name that prevails for the locus is identified first, and the other name becomes a superscripted allele designation. For example, the olfactory mutation *smellblind* (*sbl*) turned out to be an allele of the sodium channel gene *paralytic* (*para*). The final name thus becomes $para^{sbl}$. Several of the dominant markers used routinely in fly crosses, and whose names permeate the fly literature, have suffered the same fate. In this book, the traditional names are used, but are noted if recently subsumed into another locus name.

WHAT MAKES FLIES SO GREAT? BALANCER CHROMOSOMES

Balancer chromosomes are what set fly genetics apart from genetics in all other organisms. Most recessive mutations are invisible in heterozygous condition. The ability to carry out crosses such that invisible genotypes can be scored in progeny with virtually 100% reliability has given fly genetics a degree of ease and power unmatched in other diploids.

H.J. Muller invented the idea of balancers, as he did much of the rest of what has become hard-core genetic analysis in the fly (Alfred Sturtevant was responsible for most of the rest), when he first identified the chromosome *C1B* as a suppressor of exchange on the *X* and used it to isolate new *X*-linked lethal mutations (Muller 1918). Since then, the principle that multiply inverted chromosomes are highly unlikely to undergo exchange with their normal homologs has been elaborated. When these chromosomes are also carriers of marker mutations, they become powerful tools in segregation analysis and the predictable synthesis of defined genotypes. Since the markers are often recessive lethals themselves, the chromosomes provide a means for constructing true-breeding stocks for defined lethal mutations—"balanced lethal" stocks in which only those adults doubly heterozygous for the balancer and for the lethal-bearing homolog survive.

Many balancers exist for the *X*, *2*, and *3* chromosomes. They are not necessary for chromosome *4* because there is no exchange on that chromosome (although balancing of recessive lethals with a dominant marker that is also a recessive lethal is still needed). The most effective balancers are those that suppress exchange all along the chromosome. Those that fail to do so usually have a large enough portion in normal order to per-

mit occasional synapsis with a homolog and consequent double crossovers within the short intervals that succeed in pairing. This can result in "breakdown" of the balancer—replacement of portions by a normal sequence, with transfer of some markers to the normal homolog. These are clearly situations to be avoided because they confound the usefulness of balancers.

Since the *X* chromosome must exist in hemizygous condition in males, most *X*-chromosome balancers do not contain recessive lethals. Instead, some *X*-chromosome balancers carry recessive female sterile mutations to prevent them from "taking over" the stock (i.e., becoming the only *X* chromosomes present, which will occur if the other chromosome carries mutations that are unhealthier than those on the balancer).

Balancers are usually named with a letter for their chromosome (*F* for first, which is the *X*; *S* for second; and *T* for third), with an *M* for multiply inverted and with a number and sometimes a lowercase letter to identify its place in a series. The name is sometimes followed by the genetic symbol for the principal markers carried by that balancer. The most efficient balancers are as follows:

X chromosome

- *FM7a* (real name *In(1)FM7, y^{31d} sc^{8}*), which carries the dominant marker *Bar* (*B*), as well as recessive alleles of *yellow* (y^{31d}), *scute* (sc^{8}), *white-apricot* (w^{a}), and *vermilion* (v^{Of}).
- *FM7b*, which carries y^{31d}, sc^{8}, w^{a}, and a recessive female-sterile allele of *lozenge* (lz^{sp}).
- *FM7c*, which carries y^{31d}, sc^{8}, w^{a}, and a recessive female-sterile allele of *singed* (sn^{X2}) in addition to alleles of *vermilion* (v^{Of}) and *garnet* (g^{4}).

Second chromosome

- *SM6* (real name *In(2LR)SM6, al^{2} Cy dp^{lv1} cn^{2} sp^{2}*), which carries the dominant marker *Curly* (*Cy*) as well as various recessives *dumpy* (*dp*), *cinnabar* (*cn*), and *speck* (*sp*).
- *In(2LR)O, Cy dp^{lv1} pr cn^{2}* (sometimes referred to as *CyO* or "*Curly of Oster*"), and it carries *Curly* (*Cy*) and the recessives *dumpy* (*dp*), *purple* (*pr*), and *cinnabar* (*cn*).

Third chromosome

- *TM3* (real name *In(3LR)TM3, y^{+} ri p^{p} sep bx^{34e} e*), which carries the wild-type allele of *yellow* (y^{+}) and the recessives *radius incompletus* (*ri*), *pink peach* (p^{p}), *sepia* (*sep*), *bithorax* (bx^{34e}), and *ebony* (*e*). More useful are those versions carrying the dominant marker *Serrate* (*Ser*) sometimes accompanied by *Stubble* (*Sb*).

- *TM6* (real name *In(3LR)TM6, Hn^{P} ss^{P88} bx^{34e} Ubx^{P15} e*), which carries the dominants *Henna* (*Hn^{P}*, not very easy to score) and the more reliable *Ultrabithorax* (*Ubx^{P15}*) plus the recessives *spineless* (*ss^{P88}*), *bithorax* (*bx^{34e}*), and *ebony* (*e*).
- *TM6B* (real name *In(3LR)TM6, Hu e*), which carries a dominant allele of *Antennapedia* (*Hu*), often with the additional dominants *Dichaete* (*D^{3}*) or *Tubby* (*Tb*, a good marker for larval and pupal stages as well), and the recessive ebony (*e*).
- *TM8* (real name *In(3LR)TM8, l(3R)DTS th st Sb e*), which carries a dominant temperature-sensitive lethal (*l(3R)DTS*), the dominant *Stubble* (*Sb*), and the recessives *thread* (*th*), *scarlet* (*st*), and *ebony* (*e*). *TM9* is a further derivative of this.
- *T(2;3) CyO; TM9*, which is a double balancer for chromosomes *2* and *3*, the result of a radiation-induced reciprocal translocation between *In(2LR)O,Cy* and *TM9*. Since it is a reciprocal translocation, in which part of *In(2LR)O,Cy* is now linked to part of *TM9* and the remainders are also linked together, the only progeny that you will recover are those that have all the pieces present in the same gamete for it to be euploid (i.e., to have a complete haploid genome). This helps to preselect progeny if you only want those that have inherited balancers for both chromosomes.

A great many variants of these balancers have been derived that have useful transgenes inserted into them. A few of these are discussed in this book; all are listed at http://flystocks.bio.indiana.edu (the Bloomington Stock Center's list).

DECIPHERING MATING SCHEMES

After nomenclature, the next most obfuscated realm of fly lore is the mating scheme. Mating schemes are shorthand for the genotypes that you need to collect and mate to get the progeny you want. What is confusing is that the schemes do not show all of the possible progeny from a cross, only the genotype of the unique desired class. These flies will be unique in phenotype as well as genotype if the author of the scheme has planned correctly. The unwritten assumptions are that homologous chromosomes pair and segregate from each other in the first meiotic division, all possible combinations of haploid segregants will be produced with equal frequency in the male's sperm and female's eggs, and likewise all possible combinations of diploid genotypes will be produced with equal frequency when eggs are fertilized. Whether they all survive is a separate matter.

Our assumption is that they will be produced initially (with the caveat that some abnormal chromosomes deviate from normal expectations). The shorthand indicates the genotypes of the relevant pairs of homologs for the cross at hand. A typical, simple scheme is shown below.

$$\text{♂♂}\quad \frac{nd}{Y} \times \frac{FM7a}{FM7a};\ \frac{In(2LR)O,Cy}{Sco} \quad \text{♀♀}$$

Males ↓ Virgin females

$$\frac{FM7a}{nd};\ \frac{In(2LR)O,Cy}{+}$$

This represents a cross between *notchoid* (*nd*) males (they must be males because they have one *X* and one *Y*, and the fact that more than one male is symbolized by ♂♂) and females (virgins, of course, symbolized by ♀♀) homozygous for the balancer chromosome *FM7a* on the *X* and heterozygous for the balancer *In(2LR)O,Cy* and the dominant marker *Scutoid* (*Sco*) on the second chromosome. Since the male's genotype does not show anything about his second or third chromosomes, he is assumed to be free of genetic variations and mutations (+). (In the Methods section of a paper, these balancers would be designated by their formal names: *In(1)FM7a* and *In(2LR)O,Cy*. In laboratory shorthand, they would usually be written as above. As mentioned, some forms of *FM7* carry a recessive, female-sterile mutation; *FM7a* does not and so would work in this cross.)

This cross produces many different classes of progeny. Only one of them is shown: females heterozygous on the *X* for *FM7a* and *nd*, and heterozygous on the second chromosome for *In(2LR)O,Cy*. Meanwhile, elsewhere in the bottle you will also find

$$\underset{\text{♂}}{\frac{FM7a}{Y};\ \frac{In(2LR)O,Cy}{+}} \text{ and } \underset{\text{♂}}{\frac{FM7a}{Y};\ \frac{Sco}{+}} \text{ and } \underset{\text{♀}}{\frac{FM7a}{nd};\ \frac{Sco}{+}}$$

Each is phenotypically, as well as genotypically, unique. The dominant marker on the *FM7a* chromosome, *Bar* (*B*) eye, makes any progeny carrying *FM7a* unambiguously recognizable. Similarly, on the second chromosome, the dominant marker on *In(2LR)O,Cy*, the *Curly* (*Cy*) wing mutation, and the dominant marker mutation, *Scutoid* (*Sco*), which eliminates

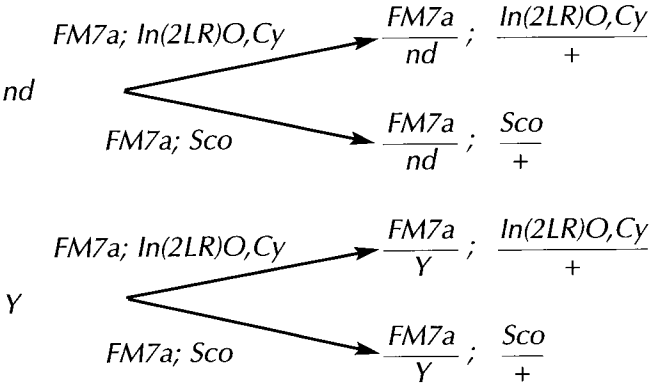
posterior thoracic bristles, make any fly carrying these mutations recognizable in any combination, since the mutations do not mask each other. The *nd* mutation is effectively invisible. It has no morphological effect when heterozygous (in females). (*Bar* is particularly well suited as a dominant marker for the *X* chromosome because it is viable in homozygous as well as heterozygous condition, and the two are distinguishable—*B/B* females are more severely affected than *B/+*. Males have no homozygosity for the *X*, only hemizygosity [*B/Y* in males is as severe as *B/B* in females], so *Bar*'s viability is very helpful in allowing survival of males carrying *FM7a*. In contrast, the dominant markers on autosomal balancers never need to survive in hemizygotes because hemizygosity for chromosome *2* or *3* is lethal.)

The unwritten assumption in these schemes is that meiosis causes each pair of homologs to segregate from each other so that only one of each pair is transmitted to any given progeny (Mendel's law of segregation). This occurs independently for each pair of homologs (Mendel's law of independent assortment). The parental males in the cross are hemizygous for their sex chromosomes, *nd/Y*, and consequently display the mutant *nd* phenotype. Since the *X* and *Y* pair and segregate at meiosis, they give rise to gametes of two possible genotypes: *nd* or *Y*. Even though they carry balancers and marker mutations for two different chromosomes, the parental females are only heterozygous on the second chromosome and thus give rise to only two possible gametes: *FM7a; In(2LR)O,Cy* or *FM7a; Sco*. These four gametic genotypes can combine in four possible combinations, as shown above. Heterozygosity on any other chromosomes increases the possible types of gametes and the corresponding classes of progeny. If in doubt about the classes of progeny that might arise from a cross, you can always set up a Punnett square (below) to ensure that you are imagining all possible combinations.

Female gametes

Male gametes	*FM7a ; In(2LR)O,Cy*	*FM7a ; Sco*
nd	$\frac{FM7a}{nd} ; \frac{In(2LR)O,Cy}{+}$	$\frac{FM7a}{nd} ; \frac{Sco}{+}$
Y	$\frac{FM7a}{Y} ; \frac{In(2LR)O,Cy}{+}$	$\frac{FM7a}{Y} ; \frac{Sco}{+}$

Even better than the Punnett square is the "algebraic" or branching approach, which is better suited to multiply mutant genotypes:



It is important to ensure that the genotypic class you want is phenotypically unique and thus recognizable among all the other progeny. This is the key to fly genetics.

BASIC FLY HUSBANDRY

Fortunately for many of us in the fly field, working with *Drosophila* does not take much technical skill—care, yes, but not manual skill. Much of the power of fly genetics comes from the ability to perform crosses in which each possible genotype in the progeny is recognized easily and unambiguously. To achieve this, be certain that the only progeny you obtain are from the intended cross, rather than from stray flies or unwanted pregnancies (i.e., nonvirgin females). Often, the class of progeny you are trying to generate will be a small fraction of the total and not particulary healthy. This means that it is equally important to start the cross with enough flies to recover enough progeny to continue the experiment.

The easiest way to grow flies is at room temperature. This also protects your stocks from incubator failures—by far the major cause of catastrophic loss of flies. The healthiest way to keep fly stocks, on the other hand, is at 25°C, 60% relative humidity. At this temperature, you get the fastest generation time (approximately 9–10 days from egg to emerged adult) and the best viability. (The generation time increases by roughly 2 days if the mold inhibitor Tegosept is present. See Ashburner et al. 2004.) Stocks can also be maintained routinely at temperatures as high as 29°C and as low as 18°C with correspondingly faster or slower generation times (see Ashburner et al. 2004) but with poorer yield.

For stocks that are true breeding (i.e., all adult progeny are the same genotype), the only time limitation on the life of the culture is that of health—older cultures produce less healthy flies and serve as a breeding ground for mold and mites, the scourge of all stock collections. A good rule of thumb for these cultures is to transfer them every 2 weeks and keep them a maximum of 18 days at 25°C. Cultures that are produced from a specific cross have an additional constraint: Eighteen days after the start of the culture, you will start to obtain second-generation progeny whose genotype will probably be a complete mystery, since you will not have known their parents.

True-breeding stocks can be transferred without anesthetization. This technique constitutes one of the only manual skills in fly pushing: Tap the flies down to the bottom of the old vial (gently, so they do not all get stuck), quickly remove the vial's plug (usually cotton, rayon, or foam rubber), and place an open fresh vial down on top of it, holding the two vial mouths together tightly. Flip them over and tap the flies down into the new vial (gently, so you do not transfer the old food as well). Then quickly plug the new vial. For the first few weeks, many stray flies will probably escape. To keep the number of stray flies in the laboratory to a minimum, use some kind of fly trap. Low-tech versions consist of a clean culture bottle with some vinegar in the bottom and a dash of detergent, topped with an open funnel to impede the flies' escape before they fall into the liquid. High-tech fly traps are the commercial "bug lights" familiar to suburban backyards. Screen doors have sometimes been used (S. Hawley, pers. comm.), but may violate fire regulations.

Since fly stocks can only be maintained by live culturing (they do not readily survive freezing) it is worthwhile to keep duplicates of each stock. This also ensures cultures available for virgin collection whenever you want. For stocks kept in vials, which is true for most of what you keep, this can be done either by keeping two copies on the same schedule or staggering by a half generation. Labeling stocks is equally important, most easily accomplished by using a movable ring tag (a cardboard disc attached to the vial by a rubber band) with the full genotype of the stock indicated on the vial. The date of initiation of each culture (when flies are first put on that food) should also be written on the vial or bottle so that you can easily tell their age.

The number of flies needed to start a culture varies with the viability of the genotype, and a golden mean exists for each. A little empiricism goes a long way when it comes to figuring out how many flies to put in a bottle. In general, the more mutants present (especially dominants), the

poorer the viability. Chromosome rearrangements (e.g., balancers, compound chromosomes, etc.) reduce the fertility of stocks: Too few flies cause the culture not to "take," or it becomes overwhelmed by mold or bacteria. Too many flies results in the culture becoming so soggy from the cumulative waste products that when you dump flies from it, the food will come out as well, leaving a mess on your anesthetizer and fly desk. In addition, soggy cultures cause the flies' wings to stick to their bodies, making it difficult or impossible to score the wing phenotype of many useful genetic markers. If a culture looks like it is becoming too soggy, it is possible to rescue it by stuffing a small piece of paper towel into the food. To prevent the paper from growing mold, it too may need to be treated with mold inhibitor.

Wild-type stocks or those with a single marker mutation may require only 10–15 females for small bottles (see below), 25–35 females for a large bottle, and 4–8 for a vial. Fewer males are required, since one male can make himself known to many females. Stocks with multiple markers, dominants, or rearrangements may require two or three times as many flies. The best way to compensate for not knowing exactly how many flies to start with is to watch the culture as it develops. When the food starts to look churned up (especially near the surface), enough larvae have emerged and it is time to dump out the parents (empty them from the bottle before the first progeny appear). This can take roughly 4 days, depending on the genotypes. When doing a cross, it is also important to dump the parents to eliminate the danger of mistaking progeny for their parents. This is another good reason for writing the date on the culture at the beginning.

CODDLING DIFFICULT STRAINS

Also known as cosseting (in the U.K.), this refers to the gentle art of keeping sickly stocks, rescuing those that have deteriorated to very few flies, or carrying out crosses with a single male and female pair. The basic principles of sound fly husbandry apply: Use a fresh food vial garnished with fresh yeast paste ("Baker's Dried Active Yeast" dried to the consistency of peanut butter), keep the culture in ideal conditions (25°C, 60% humidity), and say an occasional prayer.

If you managed to start out with both males and females in the vial (which does not always happen with a deteriorating stock) and you find that progeny are appearing, it is best to collect them as they emerge and place them in a fresh vial. (They will not live long if left in the original cul-

ture vial. In this fashion, you can start a new culture.) If you have obtained enough flies for the next culture to proceed more quickly (i.e., larval churning of the food after several days), you may transfer the parents to a fresh vial midway and thus increase the number of progeny you ultimately obtain.

COLLECTING FLIES FOR CROSSES

To carry out crosses cleanly, you must start with virgin females. At 25°C, female flies will not mate within the first 8 hours of emergence as adults. This means that virgins are most easily obtained by collecting flies twice a day—once in the morning and again 7–8 hours later. Morning is when the majority of flies emerge from the pupa case (if your incubator is on a light:dark cycle); this is one of their most predominant circadian rhythms (also the origin of the name *Drosophila*, as you may have learned in high school). Although you cannot assume that all of the females present in the culture in the morning are virgins, many of them will be newly emerged and thus recognizable by their pale pigmentation and a dark spot in their translucent abdomens. (This dark spot is the fly's version of meconium, leftovers in the intestine from their last meal as larvae.) If you have been careful about clearing all the adults from the bottle in the morning (including those that have stuck to the side or the food), then any females present 7–8 hours later will also be virgins, even if they no longer look newly emerged.

A more efficient method for maximizing the number of virgins present in the morning is to place the cultures at 18°C overnight, after your collection at the end of the day. Development is slowed down sufficiently at this temperature so that it is roughly 98% probable that newly emerged females will not mate for 16 hours. Thus, your morning collections can be assumed to contain all virgins (if the bottles have been properly cleared at the last collection) and you can simply alternate the cultures to between 25°C during the day and 18°C at night. The 2% error will not matter if nonvirginity is distinguishable among progeny of the cross (see below). If nonvirginity is indistinguishable, then the more conservative approach is better.

For many crosses, it is possible to control for nonvirginity by the use of a "virginity marker"—any recessive marker mutation that is homozygous in the stock from which the females come and which will not be homozygous in the intended cross. If progeny that carry this marker appear, you know that their parents are not virgins and they can be tossed.

When you are in the middle of a mating scheme in which you do not start from homozygous strains, it is still often possible to compensate for nonvirginity by anticipating the possible genotypes and marker combinations that you would see if there were some nonvirgins in the cross. If you can arrange the scheme so that these will be different from the markers that distinguish the progeny you want, you will be safe.

Males for a cross may be collected at almost any time; the only consideration is that they must be at least 3 days old at the time that they are placed with virgins, since they will not mate efficiently if they are too old (>10–15 days). This is because of the maturation process that males experience after eclosion, which makes them more likely to court and mate females. In this sense, females are more mature than males when they emerge as adults. (So what else is new?) Although the sex ratio is indeed 1:1, it is not necessarily so on each day's collection. Female flies develop faster than males, so there will be more of them in the earlier days of the culture than later.

It is often most convenient to store the flies you have collected before mating them so that you can start the cross all at once—perhaps because it takes several days to collect enough flies, the food has not yet been made, or you may prefer to start all of your crosses on Fridays. (The virtue of starting crosses on Fridays is that the first progeny emerge 10 days later, on Monday. Thus, you can collect progeny for 5 days, start a new cross the next Friday, and in this fashion free up the occasional weekend day. But do not tell your advisor where you heard this.) Fresh food vials (i.e., those that have not had flies in them) are the best place to store flies, 20–30 flies to a vial. If they are to be kept this way for more than a few days, you will need to change the vials to keep the flies healthy. In uncrowded, fresh vials flies can live for 40–60 days, but their fertility gradually decreases to near zero by this time.

During this storage period, it is also possible to ensure that you have virgins by making certain that no larvae begin to churn up the food. Toward the end of the storage period, you can also ensure that the culture will go quickly when you transfer the flies to bottles by "preincubating" males and females a day or so in advance in vials. In this way, many of the females will have mated by the time you put them in bottles. Like any second-order reaction, mating proceeds more quickly in the crowded conditions of a vial, in which collisions between males and females are more likely.

Nowadays, most people use CO_2 to anesthetize flies for virgin collection and for examining markers. Various gadgets have been devised for

delivering the CO_2 through diffusers on a flat surface so that a low level of gas can be maintained while you sort through the flies. (The porous polyethylene that is used to plug the bottoms of chromatography columns works well for this purpose.) Usually, a CO_2 hose is inserted into the bottle or vial to anesthetize the flies (while keeping the vial inverted to prevent the flies from sticking on the food) or else they are dumped into an anesthetizer—a porous vessel that exposes the flies to the gas.

The traditional way of anesthetizing flies is with ether. Ether anesthetizers consist of an enclosed container (often an empty fly bottle) with a cotton wad in the bottom into which ether is poured and a porous tube that holds the flies. Care is always needed to avoid overanesthetizing the flies—something that is much less likely with CO_2. Once the flies are anethestized, it is necessary to sort before they reawaken, which could happen quickly during summer heat.

The principal ill effects of such anesthetization are behavioral. Both CO_2 and ether impair neural activity for some time afterwards. For this reason, wait a minimum of 24 hours (sometimes longer) after anesthetization before conducting any behavioral test.

CHAPTER 2

Isolating New Variants

THE REASON THAT WE WORK WITH FLIES IS NOT BECAUSE they are cute, although Ed Lewis has said that you have to love your organism. We work with them primarily because of the potential for getting and analyzing mutations. Much of today's enormous edifice of molecular manipulation in *Drosophila* is predicated on this central fact.

Most forms of mutagenesis do not permit targeting the event to a predetermined gene—as in the so-called "knock-out" technique with mice (a name as descriptive of the effect on one's thinking as on the gene in question). Instead, they approximate a random process from which lesions in the desired gene are generally obtained by genetic testing of individual mutation-bearing flies for complementation or homozygosity. Few selection schemes of the sort that are used with microorganisms are applicable to flies.

Mutagenesis will never become obsolete, if only because random chance always produces more interesting lesions than those that we can think up. But the day is approaching when every gene in the fly will be mutated (i.e., "knocked out") as part of a genome-wide effort of the Berkeley Drosophila Genome Project and Exelixis (Spradling et al. 1995, 1999; http://www. fruitfly.org/p_disrupt/index.html; Thibault et al. 2004). (At that moment, we may hear little cries coming from our fly cultures that will sound something like, "We give up!")

Principles

Mutagens are not completely random in their action, but they approximate it closely enough for the purposes of designing mutant screens. Generally, you treat male flies with a mutagen, or perform a cross to produce

males with mobilized transposable elements, and then "clone" individual treated chromosomes in the F1 generation. This is necessary because after a mutagenesis treatment each sperm is unique. Thus, unless you are able to recognize and recover a newly induced mutation in the next generation, you will be out of luck. Many of the mutations you will want are either hard to recognize or recover. For instance, it is hard to get a lethal mutation out of a dead fly.

Males are most often used because (1) mature sperm are quite sensitive to mutagens, (2) the flies are still capable of performing their conjugal duties after the treatment, and (3) one male will mate with many females to propagate the treated chromosomes. Treatment of females creates problems because of the deleterious effects of mutagens on the oocyte itself and the tendency of the oocyte to soak up mutagen, decreasing its effective concentration in the nucleus. However, females may be the only choice because the mutagenic event requires the presence of both homologs and must therefore occur in a cell whose chromosomes have not yet completed the first meiotic segregation. The induction of compound chromosomes, whose homologous arms are attached to the same centromere, is an example.

Most individual F1 flies carry a mutagenized set of chromosomes in all of their germ cells. However, since chemical mutagens often act on a single strand of the DNA double helix, F1 progeny will potentially be mosaic for the lesion as a result of the semiconservative replication of that chromosome. That is, alteration of the sequence on one strand of the DNA by the mutagen will be transmitted to one of the two daughter cells at the first mitosis. As a result, a new mutation will be present in some cells of the animal. This could produce a mutant phenotype without being transmissible if it is present in the tissues crucial to the phenotype, but not in the germ cells. However, the chances are low that the germ cells in one F1 individual will be mosaic. This means that the screen should be designed so that F1 flies will be testcrossed in such a way as to reveal the presence of new mutations in one class of progeny, while allowing recovery of the mutation-bearing chromosome in sibling progeny (see below).

It is generally assumed that with sufficient ethylmethanesulfonate (EMS) mutagenesis, "saturation" can be obtained for any phenotype or chromosomal segment. Although this is true in principle, the claim must be qualified by the realization that mutagens all show some bias with respect to sequence or chromatin structure and in addition, saturation is only as good as your ability to recognize the phenotype of a mutated gene.

MUTAGENS

The kind of mutagen to use depends on the kind of mutation you want and the amount of time you want to spend looking for it. Chemical mutagens are most suitable for obtaining point mutations (or small, intragenic deletions) at a reasonable rate. Thus, they are best for obtaining an allelic series, conditional (e.g., temperature-sensitive) mutations, and screens aimed at obtaining predefined phenotypes (e.g., learning mutants). Radiation is most suitable for producing rearrangements, i.e., translocations, duplications, deletions, and inversions. Insertional mutagenesis gives the best leverage for rapid molecular cloning of the mutated gene. Its derivative, enhancer trapping, allows you to identify genes on the basis of enhancer-driven expression patterns. A detailed account of various mutagens, their properties, and uses can be found in the Ashburner et al. (2004) commentaries. The following account is abbreviated.

Ethylmethanesulfonate

EMS is the most commonly used chemical mutagen. It is an alkylating agent that produces a high proportion of point mutations, although it also produces small deletions and, occasionally, other rearrangements as well (Pastink et al. 1991). The dose required depends on the kind of screen: a lower dose when searching for a new mutation and a higher dose when isolating new alleles of existing mutations. This is because the chromosome will be made homozygous in the former case, whereas it will be tested in *trans* with another chromosome in the latter. The rationale is that when you are making treated chromosomes homozygous to look for a particular phenotype, you do not want to complicate the situation by having more than one homozygous mutation present—especially a recessive lethal mutation.

For a low dose, males are fed a solution of 0.025-mM EMS in 1% sucrose overnight (see Ashburner et al. 2004). This results in an average of one lethal hit per chromosome arm, or a rate of one hit per 1000 chromosome arms per locus. (That is, if you test progeny from a mutagenized male, you will find an average of one new lethal mutation on each individual's *X*. If you are screening for mutations at a particular locus, you will find it in approximately one out of 1000 chromosomes you screen. This corresponds to the fact that there are roughly 1000 lethally mutable genes on each of the major chromosome arms [*X, 2L, 2R, 3L,* and *3R*].) A twofold higher dose may be used for screens in which treated chro-

mosomes are scored when heterozygous (as in a screen for new mutations uncovered by a deletion).

The treated flies are mated to females for 4–5 days, after which time the males are removed. This is done to maximize the proportion of uniquely mutant chromosomes, since the mutagen can affect gonial stem cells as well as mature sperm. When gonial cells are affected, multiple sperm with the same mutation will be produced. Since the stem cells are less sensitive, the overall frequency of individual hits will be lower. To avoid this, the treated males are discarded before stem cell daughters have had time to become mature sperm, thus ensuring that mutations will be independently induced. The virtue of having independently induced mutations is that you do not waste your time analyzing the same allele more than once and, if you are attempting saturation mutagenesis, the only way to estimate when you have attained saturation is by the frequency of repeated, independent mutations of the same locus.

As an alkylating agent, EMS acts on one strand of the double helix. This means that F1 progeny will be mosaic, at least to some extent, for the new mutation. In practice, this only matters if you are trying to score a mutant phenotype in the F1 since then you are counting on the fact that the new mutation will be present in the germ cells as well as in the somatic tissues you have scored and that a somatic phenotype can be detected even when all cells might not be mutant.

It is best to treat males that are 3–5 days old, the age at which they mate most readily. But be careful since many will not survive the overnight exposure to mutagen, not all will be fertile after the treatment, and many of the F1 progeny males will be sterile. It is thus a good rule of thumb to mutagenize a large number of males—equal to half the number of F1 individuals that you plan to testcross (see below). Equally wise is to run a pilot to assess the mutagen sensitivity of the flies that you actually plan to use. Another virtue of a pilot run is that it tells you whether your crosses will produce the classes of progeny you expect. Many ideas look great on paper but fail utterly when it comes to real flies.

Ethylnitrosourea

We assume, for operational purposes, that chemical mutagens such as EMS are completely random in their site of action. This is not true, in fact, and nowhere has it been revealed more clearly than in the different spectrum of mutations obtained with another alkylating agent, ethylnitrosourea (ENU). With ENU, the mutability of genes is different, as are the kinds of

mutations obtained, although the overall frequency is comparable. The frequency of rearrangements also appears to be reduced, as does the frequency of mosaicism in the F1 progeny (Pastink et al. 1988). In all other respects, the same principles and practices apply as with EMS. A feeding dose of 7.5 mM produces lethals (standard jargon for "lethal mutations") on approximately 40% of the treated chromosome arms.

ENU is even more hazardous for mammals than EMS; it causes an extraordinarily high frequency of brain tumors in mice. Many fly workers do not consider its benefits worth the added risk and the extreme precautions it requires.

Radiation

Radiation in the form of X rays was the first mutagen ever to be used, and it remains an essential tool for geneticists. The most conveniently available sources of radiation are X-ray machines and cobalt or cesium sources (for gamma rays). All are capable of inducing chromosome breaks, which are then sometimes repaired to produce translocations, deletions, transpositions, and inversions (Pastink et al. 1987). The breakpoints that comprise these rearrangements also cause mutant phenotypes if they fall within a gene or if, by juxtaposing certain chromosome regions, they produce a phenomenon known as the "position effect" (see Ashburner et al. 2004). Radiation can also cause point mutations.

Mature sperm is the most susceptible target for irradiation. The possible range of rearrangements produced by irradiation of mature sperm is limited by the fact that each sperm has only a haploid set of chromosomes. Thus, events involving pairs of homologs (including the *X* and *Y*) cannot occur.

The frequency of these events is considerably lower than for chemical mutagenesis. A dose of 4000r to males yields a frequency of roughly 5% lethal hits (i.e., lethal mutations) per chromosome arm, as opposed to 60% with the standard dose of EMS. This dose is calibrated as the highest dose that does not cause excessive sterility. Since the breakage event is double stranded, there are no problems with mosaicism in the progency. Otherwise, the flies are handled similarly as with chemical mutagens.

Insertional Mutagenesis

Disruption of genes by the insertion of transposable elements is not the easiest form of mutagenesis, but the advantages conferred by having an

insertion to "tag" a gene or an enhancer trap to reveal an expression pattern often outweigh the inconveniences. The principle is that of mobilizing a mobile element to transpose and reintegrate at a new site in the genome; this is also known as hybrid dysgenesis. P elements are the most commonly used transposons, with *hobo* and *PiggyBac* as subsequent additions. (The above-mentioned inconveniences are steadily decreasing with the Berkeley Drosophila Genome Project and Exelixis' comprehensive production of insertions throughout the fly genome [Spradling et al. 1995, 1999; http://www.fruitfly.org/p_disrupt/index.html; Thibault et al. 2004]. In time, one will be able to order insertional lesions for any and all genes.)

P-element mutagenesis may be carried out with either intact transposons, starting with strains carrying many such elements in their genome, or with various kinds of inactive elements capable of being transposed, but which lack the activity itself. Inactive elements must be activated by the addition of the transposase activity separately, usually in the form of a chromosome carrying a stably integrated transposase gene.

The advantage of the inactive elements is that the induction of jumps can be controlled by simply adding and removing the transposase activity through appropriate crosses (see below). With this technique, the equivalent of a mutagenized male is the progeny from a cross that brings together the transposable element and the transposase activity in the same fly. Each sperm in these males is treated as if unique, just as if the fly had been fed a mutagen. In subsequent generations, there is no mosaicism to be resolved, just the separation of transposase activity from transposable elements to prevent further jumps. (P-element mutagenesis also works in females, but unlike chemical mutagenesis, it does not cause random damage to the egg.)

The frequency of jumps is a combination of (1) the frequency of excisions, which seems to depend in a somewhat unpredictable way on the size and sequences present in the transposon as well as the site of insertion, and (2) the frequency of insertions, which depends on the chromatin structure present at a given locus (Liao et al. 2000). Some loci are hot spots for insertions, such as the *singed* (*sn*) locus, at which insertions occur at a rate of roughly 1/100. Other loci seem to be completely refractory to insertions, such as *Alcohol dehydrogenase* (*Adh*). The overall average is roughly 1/2000. Part of the rationale for developing alternatives to P elements, the *hobo* and *PiggyBac* systems, was to exploit a different set of insertion biases (Smith et al. 1993; Horn et al. 2003; Thibault et al. 2004). These other systems work on the same principle: A disabled transposable element carries an exogenous gene and a source of transposase activity. The genetic crosses for manipulating them (outlined below) are correspondingly similar.

GENETIC SCHEMES FOR DETECTING MUTANTS

Tests for new mutations vary with the situation but usually fall into two categories: those that are concerned with a particular locus or chromosome region and those that are focused on a particular phenotype regardless of where the genes map. The first kind involves testing the treated chromosome in heterozygous condition—usually in *trans* with a known mutation or deletion-bearing chromosome. The second kind requires that the treated chromosomes be made homozygous.

New Alleles at an Autosomal Locus

A frequent source of insight into a gene's action comes from the analysis of an allelic series. The more complex the process in which a gene is involved, the more important such analyses become. The basic strategy is the classic complementation test, whereby the newly mutagenized chromosome is made heterozygous with a known allele of the locus in question. Crucial to this strategy is the ability to distinguish the chromosome bearing the new allele from the old. The following shows a simple scheme for isolating new alleles of a recessive lethal, third-chromosome mutation (*naked cuticle, nkd*).

Mutagenize wild-type males and mass mate to virgins carrying a balancer *TM6* marked with the dominant mutation *Ubx*, heterozygous with a different dominant marker *Sb*. Actually, one rarely uses wild-type males, instead using males homozygous for some gratuitous marker such as the cuticle color mutation *ebony* (*e*).

♂♂ $\frac{e}{e}$ × $\frac{TM6,\ Ubx}{Sb}$ Balancer dominant ♀♀

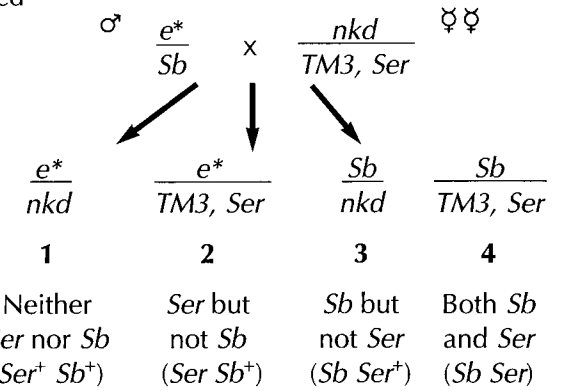
↓

produces

$\frac{e}{TM6,\ Ubx}$ and $\frac{e}{Sb}$ F1 progeny, both ♂♂ and ♀♀

Of the two types of F1 progeny produced, one is *Ubx* but not *Sb*

(i.e., *Sb*$^+$) and the other is *Sb* but not *Ubx* (i.e., *Ubx*$^+$). Select the males that are *Sb,Ubx*$^+$ and mate them individually to virgins carrying *nkd* balanced by *TM3, Ser.*



F2 progeny will be one of these four genotypes (both ♂♂ and ♀♀).

As always, the shorthand shows the genotypes of the relevant pairs of homologs, but the genotypes of all classes of progeny are uncharacteristically drawn out. This scheme illustrates the principle of "cloning" mutagenized chromosomes (symbolized with an asterisk). The first mating is done en masse, since every sperm from a mutagenized male is genetically unique. Each of their F1 progeny now carry a single set of mutagenized chromosomes and thus can be tested for the presence of the desired mutation by mating males singly to several virgin females. (Base the amount of males to mutagenize at the outset on the number of the individual F1 males you plan to testcross. That is, at the beginning, mutagenize twice as many males as you plan to testcross at this stage. Your decision on the number to testcross at this stage will depend on how much of your time you want to spend on this mutagenesis.) The "wild-type" males carry a gratuitous marker mutation (*e,* in this case) to aid in identifying the mutagenized chromosome in subsequent tests of homozygous survival or just to guard against the introduction of spurious chromosomes from stray flies that might contaminate a stock.

This scheme also illustrates the principle of making each genotype unambiguously identifiable. Each class of progeny can be easily recognized by the presence or absence of the dominant markers *Stubble* (*Sb*) and *Serrate* (*Ser*). (You can also use F1 males carrying *Ubx* instead of *Sb.*) Class 1 has none of the dominant markers. **If no class 1 progeny are found, then you have induced a new mutation that fails to complement**

the lethality of *nkd*. These are rare, thus the majority of your vial crosses will have 25% of the progeny as class 1, with no abnormalities. This class is also the one for which you will see new, viable alleles of *nkd*, if they are recognizable—that is, they may or may not differ phenotypically from the original *nkd* allele.

If one or more of your vials indicates the presence of a new mutation, then the scheme also provides a convenient way of recovering that chromosome without having to worry whether the original male is still alive. (In fact, he is almost certainly dead, since you undoubtedly dumped him and his harem into your morgue before the emergence of the F2 generation.) The flies carrying the new mutation are recognizable as class 2 progeny, who have inherited the dominant marker *Ser* (but not *Sb*) carried by their mothers on the balancer chromosome *TM3*.

The key feature shown here is the use of two different dominants in the second cross, such that they "trade" markers. That is, in the F1 generation, *Sb* marks the progeny carrying the mutagenized chromosome (*e**). Similarly, the females mate with the F1 males carry the test mutation (*nkd*) heterozygous with a different dominant (*Ser*). Since these dominant markers will segregate from their respective homologs carrying these mutations in the next generation, you can be assured that F2 progeny carrying *Ser* will also carry *e**, whereas those carrying *Sb* will also carry the original *nkd*. Thus, they have "traded" markers. This is one of the most useful techniques for identifying genotypes, especially since most of the mutations with which you will work are recessive and thus invisible in heterozygous condition. As described previously, the success of this technique depends on the absence of recombination in males and the suppression of recombination by balancer chromosomes in females.

A final point about this scheme is that it allows you to set up a true-breeding stock of heterozygotes for the new lethal mutation. By collecting males and virgin females of class 2 (*TM3*, *Ser*/*e**), a "balanced" stock can be started in which the only viable progeny will have the same genotype as their parents; homozygotes for either chromosome are nonviable.

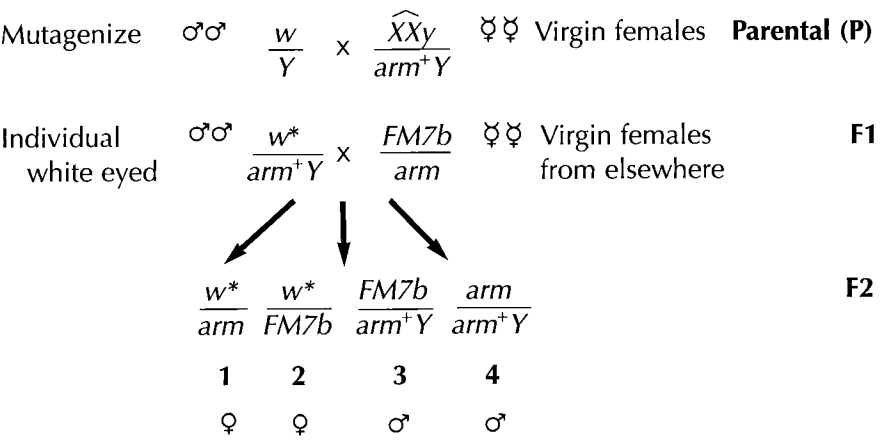
Note: The balancers *TM6* and *TM3* actually carry the recessive *e* mutation. If you take this fact into account, how does that change the recognizability of the various classes of progeny?

New Alleles on the *X*

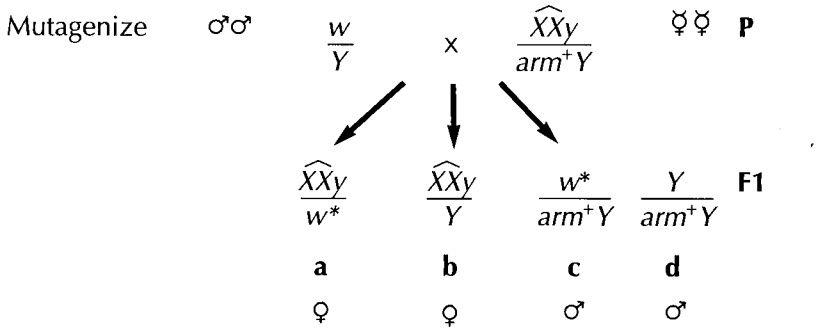
Picking up new alleles of a locus is straightforward on the autosomes but can be difficult on the *X* if the new alleles are lethal in males. This

derives from the unavoidable fact that males have only one *X*, which exposes any recessive mutations to full expression. A male carrying a lethal on its *X* is in big trouble when it comes to propagating.

The test of a newly induced mutation for allelism must involve complementation. To introduce the mutant allele from both parents, you must allow the survival of males carrying it. The easiest solution is to use a duplication of the normal chromosomal region containing the locus. This can be an insertion of the region into either the *Y* chromosome or an autosome, and such duplications do exist for many parts of the *X*. (They can also be made for unrepresented parts; see Chapter 4.) A representative scheme for isolating new alleles of the *X*-linked lethal *armadillo* (*arm*) is shown below. The "wild-type" males to be mutagenized start out with the gratuitous *X*-chromosome marker *white* (*w*).



This scheme illustrates several features that are peculiar to the *X*. Focusing on the parental (**P**) cross that produces the **F1** generation, the $\widehat{XXy}/arm^{+}Y$ females in the first cross carry an attached-*X* marked with *yellow* (*y*) and also a *Y* chromosome that has a duplication of the wild-type *armadillo* locus on it. Recall that the presence of a *Y* chromosome in females affects neither their femaleness nor their fertility. In this situation, the *Y* will segregate from the attached-*X* at meiosis so that progeny from the cross will inherit either the attached-*X* or the $arm^{+}Y$ from their mother. When fertilized by sperm carrying either of the father's sex chromosomes, the resulting progeny of the first cross above are the following:



Only two of these genotypes, **b** and **c**, are potentially viable. Lethal are **a** with three *X*s and **d** with no *X*. **b** is female, having inherited the mother's $\widehat{XX}$ and the father's *Y*, whereas **c** has the normal chromosome constitution of a male.

Problem 1 Show how this represents a reversal of the normal transmission of *X* and *Y* from parents to offspring by predicting the genotypes from a cross of a wild-type male with a female of the genotype *w/FM7b*.

For purposes of our mutagenesis, class **c** in the F1 generation contains males with a mutagenized *X* chromosome and a *Y* duplication that "covers" *armadillo* (i.e., it contains a wild-type copy that rescues the mutant phenotype). This means that if the *X* carries a newly induced mutation of *arm*, the male will be rescued by the arm^+Y. (If it carries a new lethal mutation outside of the region covered by the duplication, it will not survive, but you did not want it anyway.)

In the F2 generation, the class 1 progeny will constitute the complementation test for a new allele of *armadillo*: *w*/arm*. By the same token, this new chromosome is recoverable in the sibling females of class 2, balanced by the *FM7b* chromosome. A stock that is effectively true-breeding can then be established by simply mating the females of class 2 (as virgins) to *FM7b/Y* males. (The balancer *FM7b* carries the lz^{sp} allele of *lozenge*, which confers female sterility when homozygous, so that homozygous *FM7b* females produce no progeny. Thus, the only productive matings will be between *FM7b/Y* males and *w*/FM7b* females.)

Problem 2 How could you carry out a similar screen without resorting to an attached-*X*? Also, how could you do this using an insertion of arm^+ into an autosome (*Dp(1;2)* arm^+) instead of an arm^+Y?

Identifying New Genes by Mutagenesis

Much of our contemporary picture of the genetic control of pattern formation in the fly embryo is derived from the panel of mutants isolated by Nüsslein-Volhard and Wieschaus in the late 1970s (Nüsslein-Volhard and Wieschaus 1980). Their strategy is a classic illustration of using a mutant screen to identify new genes based on phenotype. They assumed only that mutable genes alter the cuticle pattern of mature embryos. One of the principal strengths of their approach is its lack of bias—any molecular component that contributes to this process is fair game—and this has been borne out by the identification of genes as diverse as transcription factors, receptor tyrosine kinases, and tight junction molecules.

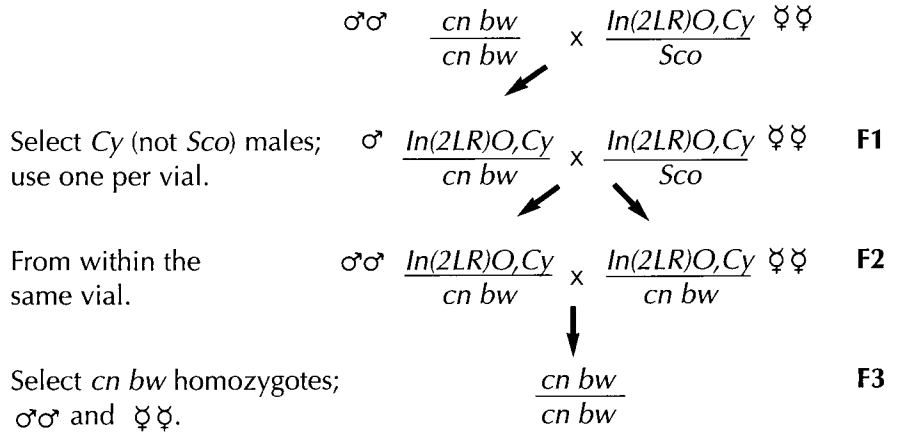
The screens were designed to reveal loci anywhere on each chromosome affecting cuticular phenotype. To do this, they needed to generate many lines containing homozygous, mutagenized chromosomes and a simple and rapid method of screening for pattern alterations in them. The basic strategy behind these screens, widely applicable to many situations, is outlined below.

Isogenizing the Starter Stock

Since the scheme relies on screening for lethal mutations in homozygous lines, it is very important that the strain to be mutagenized does not start out with lethal mutations. This may seem a trivial concern at first glance, since it is obvious that if you start with live flies from a homozygous stock, there must not be any lethals present. But the genetics gods are not very kind and although the spontaneous mutation rate is low enough to be ignored most of the time, it is not zero. Spontaneous mutations will occur in any culture and can become predominant as a result of genetic drift in small culture populations, such as those in a vial. Some of these will be recessive lethals, and some of the live flies will carry them in heterozygous condition. If as few as 1% of the fliés carry these, when this stock is used as the source of wild-type chromosomes for the mutagenesis, the vast majority of supposedly new mutations you isolate will preexist in the stock. Although this may be a fine project for a recalcitrant undergraduate or for the son of the Dean whom you "volunteered" to take into the laboratory for the summer, it is not exactly a worthwhile way to spend time.

The preventive steps are easy. First, isogenize the starter stock. That is, start a new stock from a single chromosome, then use it promptly before the genetics gods can do their mischief (i.e., within a month or so). A sim-

ple scheme for isogenizing a second chromosome is shown below. (The chromosome carries eye-color markers *cinnabar* [*cn*] and *brown* [*bw*], which together produce a white-eyed fly. This facilitates the scoring of lethals later in the mutagenesis screen for chromosome *2*.)



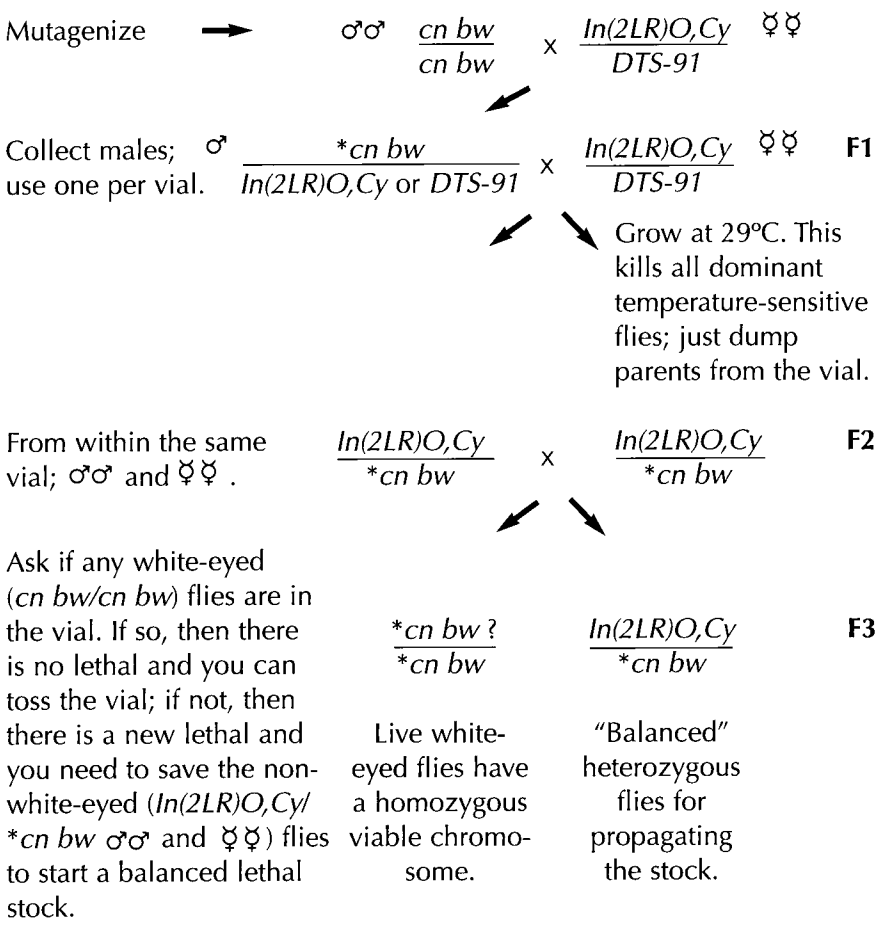
In the F1 stage of the scheme, a single *cn bw* chromosome is propagated. All the progeny in the F2 generation carry the same *cn bw* chromosome, which cannot recombine with its homolog, the balancer *In(2LR)O,Cy*. By the F3 generation, the stock is isogenic. It is wise to set up more than one vial at the F1 stage, since all of your work will be for naught if there is a lethal lurking on the one *cn bw* chromosome you choose; you can rest assured that if you only set up one male, he will be carrying a lethal. In addition to ensuring that your *cn bw* chromosome is viable, monitor its health by counting the proportion of *cn bw* progeny in each vial compared to the *In(2LR)O,Cy* progeny. If healthy, the ratio of *cn bw:In(2LR)O,Cy* should be 1:2.

Problem 3 Why is it unnecessary to carry out these steps before an *X*-chromosome screen?

The Mutagenesis Screen

This little detour has only set you back by 8 weeks, which gave you enough time to expand the other stocks to the level needed for a large-scale screen. It should have also provided time to test all other stocks that you plan to use to ensure that they have the right chromosomes and

behave properly (more later). Now, back to the mutagenesis. The scheme is illustrated below.



In principle, this scheme is very similar to the one just used to isogenize the *cn bw* chromosome. Mutagenize males that carry marked chromosomes (*cn bw*), cross them to females with a balancer chromosome and another dominantly marked chromosome, derive unique mutagenized chromosomes in the F1 males that are heterozygous with either the balancer (*In[2LR]O,Cy*) or the dominant-bearing chromosome (*DTS-91*), cross these F1 males to females from the same balancer/dominant stock, and then make the mutagenized chromosomes homozygous.

What distinguishes this scheme from the one described earlier is the use of a dominant temperature-sensitive (DTS) lethal. As the name implies,

this chromosome carries a mutation that kills all progeny that carry it at the nonpermissive temperature of 29°C. It acts during development, so to be effective, the bottles must be shifted to the higher temperature after eggs are laid. The value of a DTS in this scheme is that it obviates the need to collect virgins at the F2 generation, which is quite difficult for 1000 vials, let alone 10,000. Instead, the DTS kills all but the **cn bw/In(2LR)O,Cy* progeny. By dumping parents from the vial, those progeny will constitute the crucial cross for which the **cn bw* chromosomes become homozygous. This leaves the F1 cross as the only stage requiring a large amount of effort at virgin collection. (Balancer chromosomes containing DTSs have also been made. They are particularly useful for generating homozygotes automatically, as in screens for maternal effect mutations in which homozygous mutant females must be generated to be tested.) DTS techniques interfere with efforts to find temperature-sensitive mutations.

When the F3 progeny appear, simply look in the vial for any white-eyed progeny. If some are present, then the **cn bw* chromosome does not carry a new lethal, and the vial can be thrown out. If none are present, then you may have an already balanced new lethal. On the other hand, if you are looking for viable mutations, this indicates that you need to save the vial.

This scheme contains some pitfalls, but a little caution will keep you from developing difficulties. First, the reason for indicating only that you *may* have a new lethal is that not enough progeny in the vial were present to be certain that there were no **cn bw/*cn bw* progeny. Expanding the stock in the next generation will remedy this. Second, it is crucial to dump parents before the emergence of progeny at each generation, so that you only perform the crosses intended. Finally, DTS stocks must be tested before you start the scheme, to ensure that they work and that they are not leaky. (This principle actually applies to all stocks used in any fly mutagenesis. A little paranoia never hurts.)

An alternative to DTS-bearing chromosomes makes use of the cell death gene, *hid* (*head involution defective*, also known as the *Wrinkled* locus), driven by a heat-shock promoter, *P{hs-hid}*, which acts as a dominant lethal when induced (Grether et al. 1995). A brief heat shock delivered on 2 consecutive days at the end of 5 days of egg laying will eliminate any progeny carrying this on a chromosome, and it has been hopped onto both *CyO* and *TM3, Sb* (van Doren 1996).

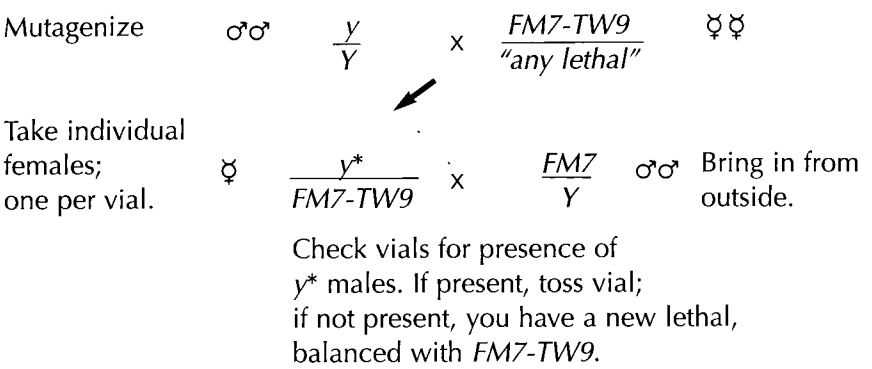
The virtues of mutagenizing a marked chromosome such as *cn bw* are several. The ease of lethal detection has already been mentioned. The two markers also make it easy to carry out a preliminary mapping of the new

mutation (see Chapter 3). Finally, they help guard against "reisolating" a mutation with which someone else in the laboratory is working. Flies do escape and contaminate other crosses occasionally. If your mutagenized chromosomes are distinctly marked, then you will not accidentally pick up your benchmate's mutant line by cross contamination (unless that stock has the same markers or this is the only way you can get your benchmate to give you her mutant line). Although this may sound unlikely, it has actually occurred enough to be taken seriously. Sometimes, the error was not discovered until the "new alleles" were sequenced and found to be identical to the original.

Lethals are not the only possible products of such a screen. The homozygous **cn bw/*cn bw* progeny of the F3 may carry all sorts of viable but nonetheless interesting mutations—death is not the only interesting phenotype to study in flies.

Screening the X for New Genes

Given the special problems of handling lethals on the *X*, it should come as no surprise that Nüsslein-Volhard and Wieschaus needed to devise the following efficient scheme for screening the *X*:



This is simple, but eloquent. The genotype of virgins in the first cross, *FM7-TW9/"any lethal,"* is the key to this scheme. *FM7-TW9* is a variant of the *X*-chromosome balancer *FM7* on which Ted Wright induced a recessive lethal (hence the "*TW*"). The other chromosome can carry any other lethal, so that these females are alive but cannot produce viable male progeny. This has therefore become a "virginizing" cross because the only viable progeny will be female and therefore virgin. This means that you can collect **y/FM7-TW9* virgins from these vials at your leisure and mate them individually to

FM7 males. If a new lethal has been induced on the **y* chromosome, this cross will produce no viable *y* males, and it will be balanced.

This scheme carries the usual caveat that you must have enough progeny (≥ 25 for a first pass, then retest with more the next generation) to believe that there are no yellow males. Since the cross starts with only one female, this confirmation in the next generation becomes all the more important. Similarly, not as many of the vial crosses will go with only one female starting them. This is not fatal to the scheme, only to the particular flies, and can be anticipated by starting more vials than usual.

Insertional Mutagenesis and Enhancer Trapping

Not long after the inception of P-element transformation in *Drosophila*, the potential use of transposable elements for insertional or "tagged" mutagenesis became apparent to many in the fly world. It started out as a cottage industry, using simple transformation vectors carrying either the wild-type allele of the eye-color gene *rosy* (ry^+) or the bacterial *neomycin* gene for resistance to the drug G418 (neo^+) (Cooley et al. 1988). With the development of "enhancer-trap" P elements (O'Kane and Gehring 1987; Bellen et al. 1989; Bier et al. 1989) expressing β-galactosidase (*lacZ*) in a tissue-specific manner depending on the site of insertion, the cottage industry grew into a multinational conglomerate. More recently, green fluorescent protein (GFP) and its various derivatives, the most common of which is the brighter fluorescing *EGFP* (Brand 1999; Morin et al. 2001), have replaced *lacZ* and made the screening process even easier. Now there is hardly a fly laboratory that has not generated at least one enhancer-trap line of its own. With the Berkeley Drosophila Genome Project's and Exelixis'comprehensive program of inserting a transposable element into every locus (predicted or otherwise) in the fly genome (Spradling et al. 1995, 1999; http://www.fruitfly.org/p_disrupt/index.html; Thibault et al. 2004), such variants will soon be available for any gene.

The initial appeal of insertional mutants was the ease of cloning the disrupted gene, an advantage compensated for by the considerably lower frequency of mutagenesis (see earlier in this chapter). In other words, the technique was simply another way of mutagenizing. The incorporation of easily recognizable eye-color markers into P elements confers an advantage in that recessive mutations become dominantly recognizable. With the advent of enhancer trapping, an altogether new strategy for identifying genes has emerged, based on pattern of expression rather than mutant phenotype.

Enhancer trapping is based on the observation that an inserted gene, containing its own transcriptional start site, is expressed under the control of those endogenous enhancers that are present in the neighborhood of the insertion site. New insertion lines can be generated in large numbers and screened very easily, since the position-dependent expression of *lacZ* or *EGFP* does not require homozygosity of the insert. Lines that show "interesting" patterns of expression can then be saved and made homozygous to see if the insertion has disrupted a gene capable of producing an obvious phenotype. These features, along with the fact that insertions into different parts of a gene have different effects, make it likely that fly workers will continue to generate new transpositions even after the comprehensive genome-wide projects are complete.

Schemes for mobilizing P elements and recovering new insertion lines resemble the standard mutagenesis schemes in many respects. However, a new wrinkle is added by genetically introducing the elements that do the mobilizing and are mobilized, instead of administering a physical or chemical mutagen. Two separate genetic elements are used: a transposable element and a "transposase" activity to catalyze the excision and insertion events. (Both of these elements were originally part of the same primordial P element, which catalyzed its own movements. For convenience they have been separated.) The *hobo* transposon system is also capable of producing deletions (see Chapter 3). *PiggyBac*, in contrast, has not yet shown a comparable ability (Horn et al. 2003).

Enhancer-trap Screens

The transposable elements used in enhancer trapping consist of P-element flanking sequences, capable of being acted on by the transposase, within which are included *lacZ*, a wild-type eye-color gene for detecting the element (either *rosy*, ry^+, or *white*, w^+) and plasmid rescue sequences (usually ampicillin resistance and an *E. coli* origin of replication). The w^+ used in these vectors is a "minigene," whose expression and subsequent eye color is also affected by the site of insertion so that it is often possible to distinguish homozygotes from heterozygotes, as well as differentiate between various lines by eye color. To make life even easier, the transposable element can be excised from a balancer chromosome, such as *In(2LR)O,Cy, P[ArB]A4.1M2*, a variant of *In(2LR)O,Cy* containing a *lacZ*, ry^+ enhancer-trap vector (also written as *P[lacZ*, ry^+*]*). In this way, all of the chromosomes that contain the original, unmobilized element can be effectively screened out in the next generation by selecting Cy^+ progeny. The source of transposase is a defective P element that is stably integrated

into the third chromosome near the right tip, *P[ry⁺Δ2-3]* (known less formally as *Δ2-3*), because of its deletion of the intron between open reading frames 2 and 3 (ORF2 and ORF3) (Robertson et al. 1988). Usually, this is introduced on a chromosome also carrying the dominant bristle mutation *Stubble* (*Sb*) for easy detection of its presence.

The approach relies on the ability of the *P[ry⁺Δ2-3]* element to mobilize the transposable element when both are present in the male germ cells. A "mutagenic" male is produced by crossing flies carrying each element separately. By introducing them on different chromosomes, it is possible to limit the conjunction of the transposase and the transposable element to a single generation, so that one does not confound the isolation of single new insertions by the continual generation of a heterogeneous population of new transpositions within each line. Absence of the transposase is monitored by scoring for *Sb⁺*, but this only works if the chromosome carrying *P[ry⁺Δ2-3]* and *Sb* is not permitted to recombine with its homolog, an event that might separate the marker *Sb* from the transposase. It is accomplished by ensuring that it is balanced when present in a female (i.e., heterozygous with a balancer chromosome such as *TM6*) or kept in a male in which recombination does not occur.

Presence of the transposable element is monitored by scoring for the wild-type eye-color marker contained in the element (either *ry⁺* or *w⁺*). When this element has been jumped out of a balancer such as *In(2LR)O,Cy*, it becomes possible to select only those progeny containing a new insertion site simply by picking flies carrying the wild-type eye-color marker and lacking *Cy*. Of course, this necessitates that the normal loci for *ry* or *w* are mutant. A representative "jump-starter" scheme is shown below.

♂♂ $\frac{In(2LR)O,Cy,\ P[lacZ,\ ry^+]}{+};\ \frac{ry^{506}}{ry^{506}}$ × $\frac{Sb\ P[ry^+\Delta 2\text{-}3]}{TM6,\ Ubx}$ ♀♀ **P**

These males are *Cy*. These virgins are *Sb* and *Ubx*.

Select ♂♂ *Cy Sb Ubx⁺* males that are dysgenic and mate to *rosy* virgins. $\frac{In(2LR)O,Cy,\ P[lacZ,\ ry^+]}{+};\ \frac{Sb\ P[ry^+\Delta 2\text{-}3]}{ry^{506}}$ × $\frac{ry^{506}}{ry^{506}}$ ♀♀ **F1**

Select single *ry⁺ Cy⁺ Sb⁺* males or females; they will have possible insertions (*). $\frac{+^*}{Y};\ \frac{+^*}{+};\ \frac{ry^{506*}}{ry^{506}}$ or $\frac{+^*}{+};\ \frac{+^*}{+};\ \frac{ry^{506*}}{ry^{506}}$ **F2**

♂ ♀

This scheme starts with males heterozygous for a second-chromosome *In(2LR)O,Cy* balancer containing the P element with *lacZ* and the wild-type allele of *rosy* (ry^+) in it, and homozygous on the third chromosome for the *rosy* allele ry^{506}. These are mated to virgins heterozygous on the third chromosome for *Sb P[ry⁺Δ2-3]*, the source of transposase activity, and the balancer *TM6*. The purpose of this cross is to obtain F1 males carrying the *In(2LR)O,Cy, P[lacZ, ry⁺]* and *Sb P[ry⁺Δ2-3]* chromosomes because it is in these males that hybrid dysgenesis will occur, resulting in the transposition of the P element to new sites on any chromosome. To detect and recover these transposition events, mate these F1 males to homozygous ry^{506} virgins. It is essential to maintain homozygosity for ry^{506} on the third chromosome so that the ry^+ P element will be detectable based on its ability to provide ry^+ activity.

In the F2 generation, select for flies that have a new insertion of the P element and discontinued transposase activity that could otherwise continue to destabilize the P elements. These flies do not have *Sb* (and consequently no *P[ry⁺Δ2-3]*) nor *Cy*, which eliminates the original sources of the ry^+ P element, the *In(2LR)O,Cy, P[lacZ, ry⁺]* chromosome. The absence of the original *In(2LR)O,Cy, P[lacZ, ry⁺]* chromosome permits you to detect the presence of the P element on a new chromosome because the new insert will be the only source of ry^+ activity. This is why it is essential to maintain homozygosity for ry^{506} at the third-chromosome *ry* locus. The + and * symbols represent the *X* and second chromosomes, and ry^{506}* represents the third chromosome derived from the F1 dysgenic males. These are the chromosomes that could contain new insertions of *P[lacZ, ry⁺]*.

Any new insertion can be propagated simply by continuing to select ry^+ progeny and mating them to ry^{506} homozygotes. For lines that you wish to keep or test for viability by making them homozygous, it is essential to determine the chromosomal linkage of the insert and balance it. For this purpose, it is useful to construct homozygous ry^{506} stocks that carry *FM7* or *In(2LR)O,Cy*. For the third chromosome, the balancers *TM3,* ry^{RK} *Sb e* and *TM2, ry Ubx* contain a *rosy* mutation. Simply follow this with a linkage test (see Chapter 3) by mating the insert-bearing flies to one of the balancer stocks, picking up progeny carrying both the insert and the balancer, and determine if they always segregate from each other in the next generation. If they do, the insert is linked to the homolog of the balancer. (ry^{506} is the allele traditionally used in P-element schemes because it is healthy and not spontaneously revertible, the result of a small internal deletion of the *rosy* gene.)

Problem 4 Design the crosses for determining linkage and testing homozygous viability of a newly mobilized w^+ insert. Decide on the stocks you will need for the tests and work out the expected results for inserts on each of the four chromosomes.

New Insertional Alleles of Known Loci

If your goal is to produce a new insertional allele of an existing mutation, your scheme needs to be modified to maximize the number of jumps in each germ cell and to set up a complementation test for putative new insertions at the locus. Given the low frequency of inserts at most loci, it is important to start out with as many transposable elements as possible to optimize the chances of getting a hit in your favored gene. This was initially accomplished with a second chromosome (*Birm2*) containing 17 P elements capable of being transposed, but lacking in wild-type transposase activity themselves (Bingham et al. 1982). Since this approach suffered from the lack of a visible marker tag on each P element, an attached-X ($\widehat{XX}$) was constructed with eight $P[w^+]$ inserts (Bier et al. 1989). The disadvantage is that there are fewer elements to jump and they do not jump with as high a frequency as those in *Birm2*.

These problems are fast becoming moot as the P-element saturation of the genome progresses, rendering such schemes increasingly less necessary. For the cases in which it is still desirable to generate a new insertion, it is probably easiest to start with a nearby insertion, since the most common transpositions are to nearby sites.

Mutating Genes For Which There Are No Allelic Differences

The majority of the fly genome (Adams et al. 2000; FlyBase Consortium 2003) consists of genes whose function is presently unknown and for which no mutant allele exists. Different approaches are required depending on whether there is a simple way of knowing a priori how to recognize when the locus is mutant.

Screening Over a Deletion of the Locus

If you have some idea of the type of phenotype to expect, the simplest way to isolate a mutation is to obtain a deletion that uncovers the locus and then to simply blast away at it with mutagens. A standard screen for fail-

ure to survive over the deletion will turn up all the lethals, useful if you have reason to believe that your gene can mutate to lethality. If you do not expect lethality, but some tissue-specific phenotype (such as roughening of the eye, for which there appears a virtually unlimited number of genes in the fly), then you can focus on that tissue and its morphology. Here, the mutagen used becomes important, since it is crucial to mutate the gene in a way that will be easily detectable at the DNA level. Radiation (see above) is preferable for this purpose.

Targeted Insertional Mutagenesis

Gene targeting is the only way to obtain mutations in a gene that makes no assumptions whatsoever about the type of phenotype the mutant will exhibit. An early approach to obtaining mutations targeted to a specific sequence was a PCR-based technique for detecting P-element insertions into a defined site (Ballinger and Benzer 1989; Kaiser and Goodwin 1990; Dalby et al. 1995). The genetic aspects of this scheme are very similar to those for generating new enhancer-trap lines and do not require any other genetic tools for analyzing the locus, such as deletions or breakpoints. Putative new insertion chromosomes are then assayed by PCR, looking for the presence of a replicated segment using one primer from the P-element flanking sequences and the other from the target genomic region. These approaches generally work at very low frequency and efficiency, and they are laborious.

Homologous Recombination

Some genes are refractory to chemical or insertional mutagenesis. This may be a result of size (the target size is too small), sequence (transposons do not like the neighborhood), or just plain bad luck. For other genes, mutability may not be a problem, but recognizing that the gene has been mutated may be difficult especially if you do not know what phenotype to expect. The large number of predicted genes with unknown function that has emerged from the Drosophila Genome Project makes this problem even more compelling. Also, for very large genes, the ability to assay and manipulate regulatory sequences in the appropriate combinations and with the appropriate spacing may run up against the impossibility of introducing a transgene that incorporates far-flung enhancer sequences.

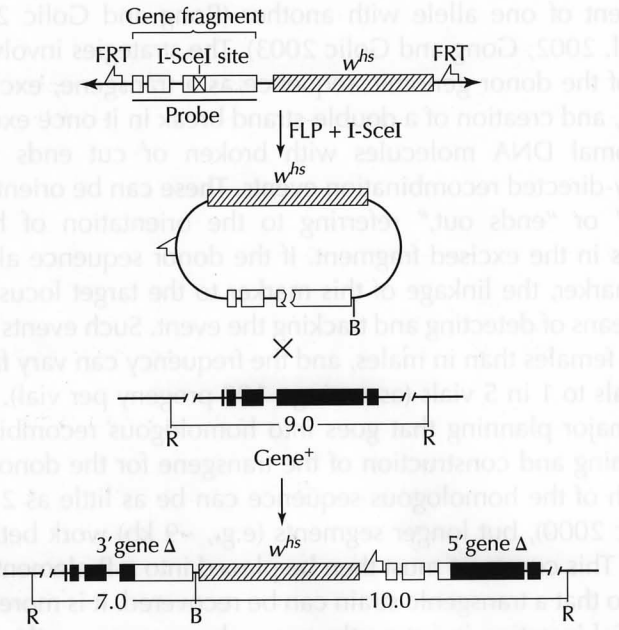
To deal with these issues, Golic and colleagues have developed strategies for homologous recombination either by disruption of the gene or by

replacement of one allele with another (Rong and Golic 2000, 2001; Rong et al. 2002; Gong and Golic 2003). The strategies involve the introduction of the donor genomic sequence as a transgene, excision of this sequence, and creation of a double-strand break in it once excised. Extrachromosomal DNA molecules with broken or cut ends can initiate homology-directed recombination events. These can be oriented as either "ends in" or "ends out," referring to the orientation of homologous sequences in the excised fragment. If the donor sequence also carries a genetic marker, the linkage of this marker to the target locus becomes a simple means of detecting and tracking the event. Such events occur more readily in females than in males, and the frequency can vary from 1 event in 225 vials to 1 in 5 vials (assuming ~100 progeny per vial).

The major planning that goes into homologous recombination is in the designing and construction of the transgene for the donor sequence. The length of the homologous sequence can be as little as 2.5 kb (Rong and Golic 2000), but longer segments (e.g., ~9 kb) work better (Rong et al. 2002). This construct must then be placed into a P-element vector and injected so that a transgenic strain can be recovered. It is more convenient if this initial insertion is not on the same chromosome as the locus being targeted. Experience with this approach is still relatively limited, although independent confirmations have been obtained (e.g., Elmore et al. 2003).

Excision of the donor sequence is brought about by adapting a yeast system of site-specific recombination known as "FLP/FRT," consisting of a sequence-specific recombinase (FLP) and its flip recombinase target (FRT) sequence (Golic and Lindquist 1989). When two FRTs flank a sequence and are oriented in the same direction, the excision event produces a closed circle. A double-strand break is introduced into this closed circle by means of another unique enzyme and target sequence: the yeast endonuclease I-SceI and its 18-nucleotide target sequence. By placing the I-SceI site inside the homologous donor sequence, the resulting broken ends are appropriately placed to initiate a recombination event with the homologous target sequence in the host genome. The "ends-in" strategy is illustrated below for a generic gene. The "ends-out" strategy (Gong and Golic 2003) produces a disruption without duplicating the locus.

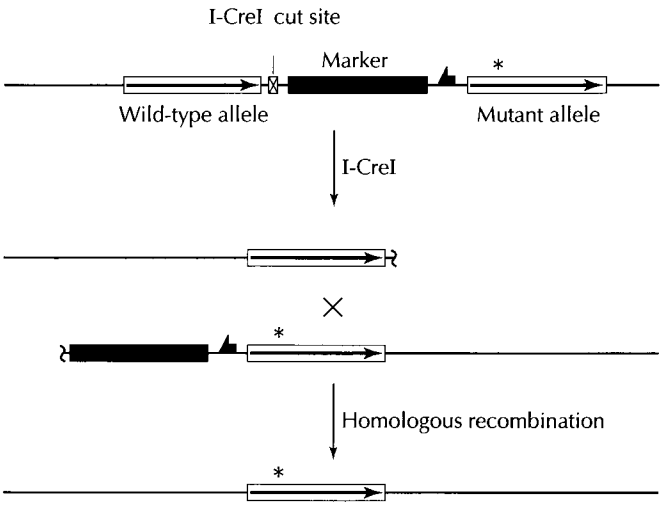
The w^{hs} gene in the construct carries a wild-type allele of the *white* gene to serve as a marker for following the incorporation and linkage of the newly inserted donor sequence. The FLP and I-SceI enzymes are induced from separate transgenes using heat-shock (*hsp70*) promoters. These heat-shock enzyme transgenes are available on different chromosomes with visible markers other than $white^{+}$ (see FlyBase Consortium



"Ends-in" homologous recombination. The donor construct is diagrammed at the top. FLP excision and I-SceI cutting produce the extrachromosomal targeting molecule, which then recombines with the endogenous locus to produce a tandem duplication. Arrows at the donor site represent P-element ends. (Modified, with permission, from Rong and Golic 2001. © 2001 The Genetics Society of America.)

2003). hs-FLP and hs-I-SceI are available together on either the second or third chromosome, and marked with *rosy*$^+$ (FLP) and *vermilion*$^+$ (I-Sce). One can thus choose the appropriate stock for carrying these transgenes on a chromosome, other than the one carrying the target locus, and allow the donor construct to be distinguishable by its w^+ marker from the enzyme transgenes as long as the endogenous *white* and *vermilion* (or *rosy*) loci are mutant. This way, the wild-type markers on the transgenes can be recognized.

Two wrinkles on this basic strategy are the abilities to replace an allele and eliminate any extraneous sequences from the insertion site (Rong et al. 2002; Gong and Golic 2003). The scheme illustrated above produces allelic substition (two copies worth) if instead of a gene fragment, the entire gene is used in the construct. In this instance, a mutation introduced at any desired site is inserted in two copies. Elimination of extraneous



Single copy substitution. A duplication produced by "ends-in" targeting can be reduced to a single copy by I-CreI-induced recombination between repeated sequences. (Redrawn, with permission, from Y.S. Rong et al. 2002. © 2002 Cold Spring Harbor Laboratory Press.)

sequences requires one additional engineered site and its corresponding enzyme: the I-CreI enzyme and its 22-bp recognition site.

As with the other enzymes, the I-CreI coding sequence, driven by the *hsp70* promoter in a *vermilion*$^+$ vector, is available in stocks with either second- or third-chromosome insertion sites (see FlyBase Consortium 2003). Since the final step in this scheme can only occur after the recovery of a homologous recombination event, the crosses to introduce the I-CreI enzyme can be carried out after the other enzyme transgenes have been crossed out of the stock.

The genetic schemes required for homologous recombination are principally concerned with synthesizing the genotypes that bring the donor construct, at its initial P-element (nonhomologous) insertion site, together with the heat-shock-inducible *FLP* and *I-SceI* transgenes. This is an example of manipulating multiple chromosomes, a topic dealt with in Chapter 4, Synthesizing Specific Genotypes. Confirming that homologous recombination has initially occurred requires linkage analysis to see if the marker gene in the donor sequence has become associated with the normal, genomic location of the target locus. This involves standard linkage mapping techniques described in Chapter 3, Mapping.

RNA Interference

An alternative method for silencing genes of known sequence is RNA interference (RNAi), first developed in the worm and subsequently shown to work in the fly (Carthew 2001). The technique makes use of double-stranded RNA (dsRNA) that causes degradation of endogenous mRNA when the dsRNA's sequence is derived from the coding sequence of the gene to be silenced. The dsRNA can either be made in vitro and injected (Kennerdell and Carthew 1998), or transcribed off of a transgene in vivo as a hairpin (Kennerdell and Carthew 2000), two separate cDNAs (Kalidas and Smith 2002), or an intron-containing sequence spliced into two complementary RNAs (Lee and Carthew 2003). Injections have been shown to work in embryos (Kennerdell and Carthew 1998) and in adults (Dzitoyeva et al. 2001, 2003).

Like homologous recombination, this approach is well suited to genes whose functions and expected mutant phenotypes are unknown. However, RNAi does have limitations. It does not work well on all genes and it is not necessarily capable of eliminating every last bit of expression. Thus, if tiny amounts of residual gene product are sufficient to produce a normal phenotype, the technique may fail. Genetically, no crosses need to be done other than those normally associated with transgenes (in the case of in vivo expression of dsRNA). Detailed methods for all aspects of RNAi can be found at http://www.biochem.northwestern.edu/carthew/manual/RNAi_Protocol.html/.

Searching for Gain of Function

The majority of mutations decrease the activity of a gene product by eliminating it, reducing its amount, or diminishing its biological activity. All of these effects fall into the category of "loss-of-function" mutations (see Chapter 5, Analysis of Mutations: I. Characteristics of the Allele, for a full discussion). The converse, "gain-of-function" mutations, involves some increase in gene product activity, such as increased amount, greater activity, or expression in a new time or place. A variant form of insertional mutagenesis has been designed specifically for the purpose of creating gain-of-function mutations. The strategy is to incorporate an enhancer and a promoter into the transposable element (dubbed "EP" for enhancer and promoter) to drive transcription of sequences flanking the insertion site (Rørth et al. 1998). The system uses the GAL4-UAS expression system imported from yeast (Brand and Perrimon 1993), consisting of a transcrip-

tion factor (GAL4) and its target sequence (UAS). The GAL4 binding sites in the UAS sequence constitute the "enhancer" portion of the EP transposon. Originally developed as a means of directing transgene expression to specific tissues or stages (see Chapter 6), the GAL4-UAS system lends itself easily to the gain-of-function screen. The EP vector is randomly jumped around the genome and gain-of-function phenotypes can be assayed by crossing to a strain that expresses GAL4 in the tissue or stage of choice to direct transcription to a particular tissue or developmental stage. A second version of this strategy created a double-headed enhancer promoter so that transcription is driven on both sides of the insertion (Toba et al. 1999). These screening systems thus permit a degree of control over the targeting of a defect that is not possible with most other forms of mutagenesis.

Generating Mutants: One Size Does Not Fit All

As the foregoing discussion indicates, there are many ways to generate mutants, each with advantages and disadvantages. Above all, no single method is suitable for all cases. The varieties of gene sequence and chromosomal environment ensure that some genes will be refractory to any particular method. But the very existence of so many different approaches makes it likely that at least one of them will work for any given gene.

CHAPTER 3

Mapping

ONCE YOU HAVE FOUND A NEW MUTATION, YOU WILL NEED TO KNOW where it maps. If it has been induced in a scheme that is not directed at a particular region of the genome, you must resort to the tried and true techniques of linkage and recombinational analyses.

Linkage analysis relies on Mendel's principle of segregation of alleles, that the two homologs of a chromosome segregate reliably and inexorably from each other during the first meiotic division. Therefore, if your mutation is on the opposite homolog from a dominant marker and if there is no recombination between the two homologs, they will segregate from each other in every meiosis and thus in all the progeny. This segregation cannot be confounded by recombination in males because they have none. In females, you can prevent ambiguities resulting from recombination by using balancer chromosomes.

Chromosome segregation, like all things biological, is not foolproof. Improper segregation does occur, but generally at a rate so low that it is of no consequence for our purposes. It can become significant and troublesome, however, when rearranged chromosomes such as translocations or multiple inversions are involved. Under certain circumstances, these will not pair and segregate normally. The resulting gametes do not contain a normal genetic complement and may have too much or too little genetic material. This condition is called **aneuploidy** (the proper genetic complement is called **euploidy**). Since at the level of whole chromosomes (with the exception of chromosomes *4, XXY,* and *XO*) aneuploidy is lethal to embryos, occasional failures to segregate will not even be noticeable in the adult progeny that you score in your analysis.

Recombinational analysis relies on the fact that the frequency of chromosomal exchange between two loci is related to the distance between them. The recombination occurs in females who are heterozygous for your mutation and for other markers on the same chromosome. The basic analysis has not changed since 1913 when Sturtevant first used it to establish the order of genes on the *X* chromosome of the fly. This technique allows you to establish an approximate position for your gene before zeroing in more accurately with deletions, duplications, and breakpoints.

Mapping strategies can also be used to identify genes if there is a convenient assay for the product or for a particular phenotype. Such strategies rely on the use of chromosomal rearrangements to systematically create deletions or duplications of regions of the genome and can serve as an efficient precursor to mutagenesis. That is, you can often identify the general region containing your favored gene and then screen for mutations in that region.

SEGREGATION ANALYSIS

Tracking a mutation by its segregation from a dominant marker is fundamental to all mating schemes in the fly. As shown previously in the discussion of mutagenesis schemes, tracking allows you to unambiguously identify those flies carrying recessive mutations. It requires that you have dominant markers and balancers for the second and third chromosomes. Linkage to the *X* is easier to see, since recessive mutations will be hemizygous in males and, if introduced from a male, only transmitted to female progeny. The fourth chromosome is usually left out of these schemes. If a mutation does not map to the *X*, second, or third chromosome, you can confirm linkage to chromosome *4*. Dominant markers for chromosome *4* include the dominant alleles of *eyeless* (ey^D) and *cubitus interruptus* (ci^D) but no balancers, since this chromosome does not undergo exchange even in females (except in triploids).

Dominant mutations are always the easiest to map, simply because they can be directly scored in the next generation. You mate the mutant flies to a stock carrying dominant markers on one or more of the other chromosomes, select male progeny heterozygous for all of the dominants, and mate them to a wild-type strain to score segregation in the next generation. Using males allows you to avoid the risk that exchange will cause your mutation to become linked to one of the markers. This could become a problem if your mutation maps far from the marker for its chromosome, because loci that are distant from each other on the same chromosome

will appear to assort independently, as if they were unlinked, when exchange is active. The following shows an example of a cross to test segregation of a dominant (*Dom*):

$$\frac{Dom}{+} \times \frac{In(2LR)O,Cy}{Sco} ; \frac{TM6}{Sb}$$

$$\downarrow$$

$$\frac{Sco}{Dom?} ; \frac{Sb}{} \times \frac{+}{+}$$

Next, score whether the new dominant segregates from *Sco* or *Sb*.

P-element insertions, either new transformants or enhancer-trap jumps, can be mapped by the same strategy since these lines are usually marked with the wild-type alleles of *rosy* (ry^+) or *white* (w^+) and will act as dominant on a mutant background of *ry* or *w* (see p. 20).

$$\frac{w}{Y} ; \frac{P[w^+]}{+} \times \frac{w}{w} ; \frac{In(2LR)O,Cy}{Sco} \text{ or } \frac{w}{w} ; \frac{TM3, Ser}{Sb}$$

$$\downarrow$$

$$\frac{w}{Y} ; \frac{In(2LR)O,Cy;}{P[w^+]?} \frac{+}{} \text{ or } \frac{w}{Y} ; \frac{+; \quad TM3, Ser}{P[w^+]\ ?} \times \frac{w}{w} ; \frac{+}{+}$$

Next, score whether w^+ segregates from *Cy*, *Ser*, or neither.

Recessive mutations are not much more difficult to map, requiring only the generation of males and females heterozygous both for the mutation and the markers. When testing for segregation of a recessive mutation, mate heterozygotes together to reveal the presence of the mutation. This requires that balancer chromosomes be used as the source of dominant markers in the heterozygous females to prevent exchange.

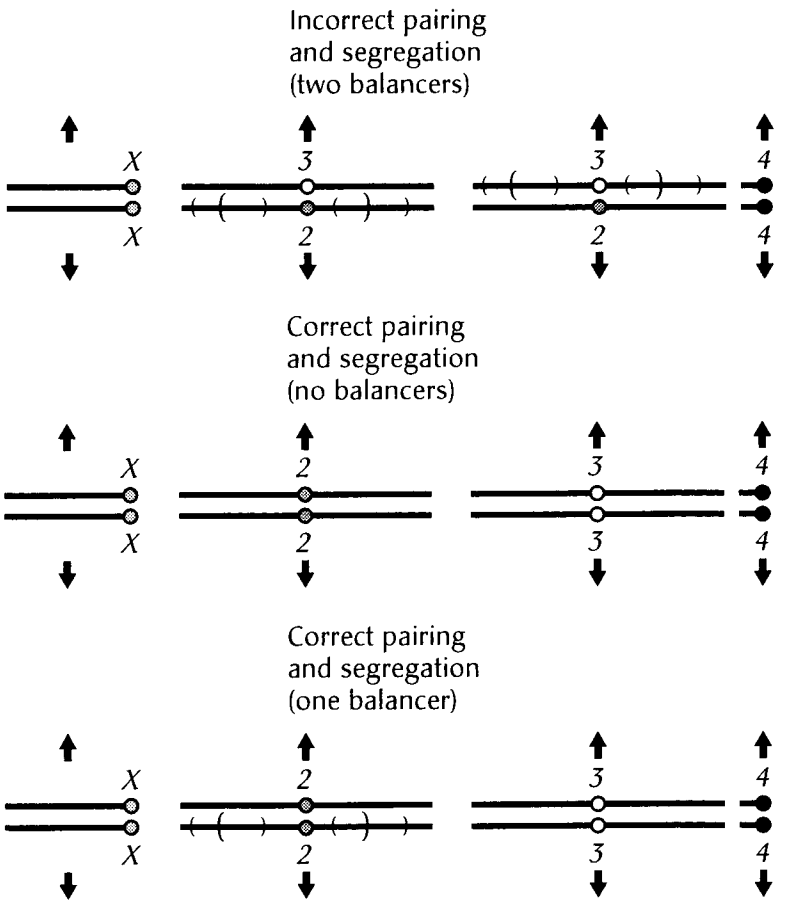
Problems with Balancers

It would be too good to be true if balancers did not also present some problems. Although these are not all pertinent to the use of balancers for segregation analysis and meiotic mapping, and will matter more in Chapter 4 (Synthesizing Specific Genotypes), there is enough reason to mention these problems here.

One kind of problem, already alluded to, is the probability (albeit low)

of a recombination event causing balancer breakdown. For this reason, when keeping and using balanced stocks it is wise to check routinely that all flies have the markers that they are supposed to have. This simple verification can save a great deal of time and trouble by allowing you to detect such an event before it takes over your stock. The best ways to deal with the discovery of an inappropriate fly are to discard that particular bottle or vial if other copies show correct markers or to start several new lines from single pairs of males and virgin females carrying the appropriate markers.

A more common problem occurs when a cross requires that you generate flies carrying balancers for two different chromosomes. Females het-



erozygous for two different balancers produce far fewer viable offspring than females carrying only one. This is because of the dependence of chromosome segregation at meiosis on exchange between homologs. When no rearrangements are present, the vast majority of *X*-chromosome and all of the second- and third-chromosome homologs recombine during female meiosis. When one copy of an inversion chromosome is present, it too will still segregate correctly from its homolog. But when two different chromosomes have heterozygous inversions, segregation runs amok. Exchange is prevented and they pair inappropriately. The result is that the wrong chromosomes segregate from each other (e.g., a second chromosome can segregate from a third chromosome or an *X* from a second chromosome) to produce aneuploid gametes and dead embryos. An attached-*X* in the same stock with a second- or third-chromosome balancer will have the same problems of incorrect pairing and segregation. In contrast, males do not have such problems. Even in the presence of multiple balancer chromosomes, segregation occurs normally.

A secondary difficulty that surfaces when multiple, heterozygous inversions are present in the same stock is that the likelihood of balancer breakdown increases. This is the result of another anomaly, the interchromosomal effect, in which the heterozygous rearrangements suppress exchange in part of the genome, increasing the likelihood of recombination occurring elsewhere. Thus, the presence of one balancer increases the probability of a rare breakdown event in another balancer. Although this is not nearly as common a problem as the incorrect segregation described above, it can still be an annoyance. It becomes more than an annoyance when mapping a new mutation meiotically, since it distorts the map distances and increases the chances of otherwise rare exchange events.

The remedy for these problems is to anticipate that crosses involving females with two balancers will go poorly and to try to circumvent them in the design of your mating scheme. The easiest way to do this is to transmit the two balancers through males, since they segregate chromosomes perfectly well regardless of the presence of inversions. If you have no alternative, use many more flies than you would normally and keep a sharp eye out for balancer breakdowns in the progeny.

MEIOTIC MAPPING

Once the linkage of a new mutation is known, in the absence of cloned pieces or sequence information, the most efficient way to find its location on the chromosome is to map it by recombinational analysis. This gener-

ally involves a multiply marked chromosome, whose mutations lie at intervals along the chromosome, and a similar chromosome that also carries a dominant marker. The basic strategy is to generate females heterozygous for both the chromosome bearing the new mutation and the marker chromosome. These will recombine during meiosis and the various classes of recombinant chromosomes, produced in proportion to the distance between markers and mutation, will go into her eggs.

The task is then to measure the proportion of different recombinant chromosomes and determine the position of the new mutation relative to the markers. To obtain a preliminary map interval, it is easiest to cross the heterozygous female to another version of the marker chromosome—one that contains a dominant marker as well. Then, males carrying recombinant chromosomes can be recognized and picked up as heterozygotes for the dominant marker. Whether a male has a particular recombinant interval can easily be scored by the amount of recessive markers that are now homozygous. These males can then be mated individually to the original stock carrying the lethal mutation to assess the presence of the lethal on the recombinant chromosome.

A simple mapping scheme follows for a new, recessive lethal (ℓ) on the third chromosome. The scheme uses a multiply marked chromosome known affectionately as "*rucuca*," which carries *roughoid* (*ru*) 3-0.0, *hairy* (*h*) 3-26.5, *thread* (*th*) 3-43.2, *scarlet* (*st*) 3-44.0, *curled* (*cu*) 3-50.0, *striped* (*sr*) 3-62.0, *ebony* (*e*) 3-70.7, and *claret* (*ca*) 3-100.7, and its cognate chromosome, "*ruPrica*," which also has the dominant marker *Prickly* (*Pr*) 3-90.0.

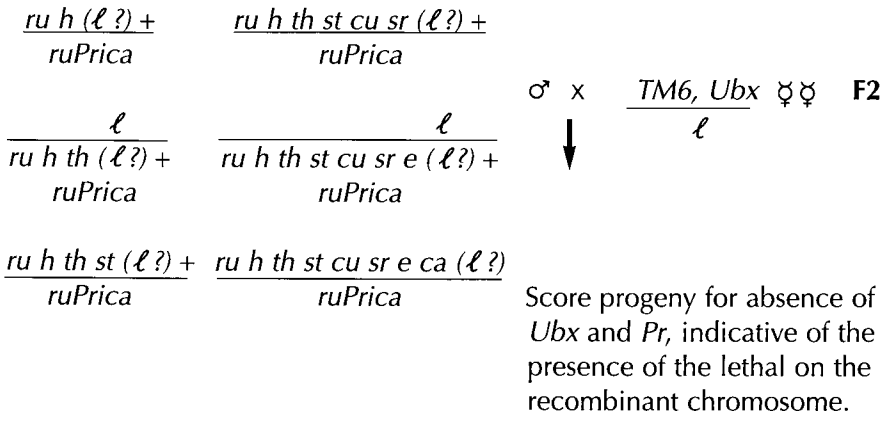
$$\frac{TM6,\ Ubx}{\ell} \times \frac{rucuca}{rucuca} \quad \textbf{P}$$

It is essential to collect females ♀♀ heterozygous for ℓ and markers. Select males heterozygous for *ruPrica* that have crossovers in each interval, recognizable by homozygosity of appropriate markers. Cross individually to the original lethal-bearing chromosome.

$$♀♀\ \frac{rucuca}{\ell} \times \frac{ruPrica}{+} \quad ♂♂ \quad \textbf{F1}$$

$$\downarrow$$

$$\frac{ru(\ell\ ?)+}{ruPrica} \qquad \frac{ru\ h\ th\ st\ cu\ (\ell\ ?)+}{ruPrica}$$



(These hypothetical recombinant chromosomes are drawn to reflect single crossover events. In reality, many would have double crossovers [e.g., *ru h + + + sr e ca*], but could still serve your purposes.)

This analysis defines the chromosomal interval in which the new mutation lies and can be accomplished with a few flies of each genotype. Finer-grain localization can then be carried out either by deletion mapping with available deficiencies of the region or by collecting many recombinants between the two markers in an interval and scoring the actual percentage of crossovers separating the *lethal* from one versus the other.

As reported by FlyBase or Lindsley and Zimm (1992), map positions are denoted by the chromosome followed by a normalized value for the meiotic map position, e.g., 2-36.8. The normalization is an attempt to order the genes on the chromosome in an approximate fashion by adding map values from the left tip to the right tip of the chromosome. Thus, it is possible for a locus to have a map position of greater than 50 map units, even though a value greater than 50 has not yet been measured in a single mapping experiment. These numbers and the gene order derived from them must be taken and used with caution. Direct mapping between specific loci is the only way to obtain reliable results. Meiotic map positions are standardly measured using heterozygous females that have been raised at 25°C. This is because recombination frequency is sensitive to temperature (Ashburner et al. 2004).

Some of the value of traditional meiotic map positions has diminished as the molecular mapping of the fly genome has progressed with its correlation of loci with sequences. The persisting value of meiotic mapping lies in the narrowing down of a new mutation's position from that of the

whole chromosome to a rough interval and in the demonstration that a phenotype results from a single gene. This can be readily accomplished by using a few markers and obtaining a relative map. Once the interval is known, you can obtain a finer localization with deletions or marked P-element insertions mapped to the region (see below).

It is important to remember that your markers should not interfere with the phenotype of your new mutation. If the new mutation causes an embryonic pattern defect that can be scored in an individual offspring, there will be little danger of interference from markers. If, on the other hand, scoring requires a population of flies of like genotype (e.g., for a behavior mutant), significant trouble can occur as a result of marker phenotypes that blur the distinction between behavioral mutant and wild type. In such a case, you might set up crosses that test for the presence of two benign markers (*straw* [*stw*] 2-55.1 for bristle color and *brown* [*bw*] 2-104.5 for eye color) and generate the population of flies to be tested for the new mutation (*n*) in parallel as follows:

$$\frac{stw;\ bw}{stw;\ bw} \times \frac{In(2LR)\ O,\ Cy}{n}$$

↓

Virgin females, ♀♀ in which crossing-over can occur.

$$\frac{stw;\ bw}{n} \times \frac{In(2LR)\ O,\ Cy}{Sco}$$

↓

Collect males heterozygous for either *In(2LR)O,Cy* or *Sco*, who will carry potentially recombinant chromosomes, and mate them individually in vials to a mixture of two types of females.

$$\frac{stw;\ bw}{stw;\ bw} \times \frac{In(2LR)\ O,\ Cy}{n\ ?} \text{ or } \frac{Sco}{n\ ?} \times \frac{In(2LR)O,Cy}{n}$$

♀♀ Single ♂ ♀♀

After a suitable mating period (5–7 days), place the two types of females into separate vials and score progeny.

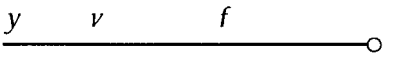
↓ ↓

What markers are present? Is *n* present?

The relative proportion of lines that have *stw* with *n*, *bw* with *n*, both with *n*, and neither with *n* will tell you the marker to which *n* is closer and whether it is between them or outside of them. (The closer *n* is to a marker locus, the less often will recombination occur between them and the more often will *n* segregate with the allele [marker +, in this case] to which it was originally linked.)

With the proliferation of P-element insertions in the fly genome, dominantly marked by *white*$^+$ or *rosy*$^+$ eye-color genes, it is possible to use a series of such P elements, distributed at a density of 7–10 per chromosome arm, to obtain a meiotic map position. In this case, the flies must also be mutant at the endogenous locus to be able to score the eye-color marker. The experiment is done by setting up a series of crosses in parallel between the mutation-bearing strain and each of the P-element strains. Females heterozygous for the mutation and the P element are then backcrossed to the mutation-bearing strain and their progeny are scored for presence or absence of the P element and the mutation (Zhai et al. 2003).

Problem 5 Assume that you have just isolated a new, viable learning mutation on an *X* chromosome already carrying *vermilion* (*v*) at 1-33.0. Design a scheme for mapping the new mutation relative to *v* and *yellow* (*y*) at 1-0.0 and *forked* (*f*) at 1-56.7, also *X*-chromosome recessives.



Relative positions of *y*, *v*, and *f*.

DELETION MAPPING

Once the approximate location of a mutation is known from meiotic mapping, a more accurate placement can often be assigned by testing various chromosome deletions for their ability to uncover a recessive mutant phenotype. When a mutation is caused by an insertion element, you can test concordance of the mutant phenotype with the site of insertion most rapidly by deletion mapping—the smaller the deletion, the more accurate the localization down to the limiting case of a chromosomal breakpoint.

This kind of mapping is indistinguishable from a complementation test in which you test for failure of complementation between a deletion and a mutation (see Chapter 2). After testing a variety of deletions for a given region, the gene can be localized to the shortest interval between the available breakpoints.

Most deletions are induced by radiation. Some result from chemical

mutagenesis (e.g., EMS) and an increasing number are being created by imprecise P-element excisions. These latter excision-generated deletions are often (but by no means always) small, generally not affecting more than one locus or its immediate neighbor. They are listed by Lindsley and Zimm (1992) and in FlyBase (FlyBase Consortium 2003; see the Appendix).

For those regions of the genome that cannot be conveniently tested with existing deletions, it is possible to synthesize a deletion using chromosome rearrangements specifically designed for that purpose (see the section below, Synthesis of Duplications and Deletions). Alternatively, it is also possible to generate new deletions by imprecise P-element excision (see the section Induction of Deletions, Chapter 4).

DUPLICATION MAPPING

Precise localization of a locus can make use of any kind of chromosome aberration to bracket the gene between known breakpoints. Duplications serve this purpose when a recessive phenotype can be covered (i.e., rescued) by the presence of a duplication carrying the wild-type locus. Duplications are often the reciprocal products of the radiation events that produce deletions, i.e., a chunk of chromosome is chopped out and reinserted elsewhere.

Since a test of rescue by a duplication requires introducing three components (the two mutant alleles and the duplication), it is desirable to use duplications in which the insertion has gone into a different chromosome. An illustration of such a test is shown below for a second-chromosome lethal (ℓ) mutation with a duplication of the segment containing the wild-type engrailed locus inserted into the third chromosome.

$$\male\male\ \frac{In(2LR)O,Cy}{\ell}\ ;\ \frac{Dp(2;3)\ en^{+}}{Sb} \quad \times \quad \frac{In(2LR)O,Cy}{\ell} \quad \female\female$$

$$\downarrow$$

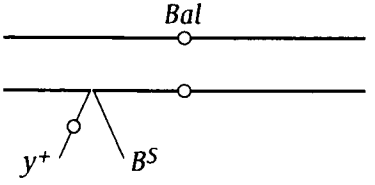
$$\frac{\ell}{\ell}\ ;\ \frac{Dp(2;3)\ en^{+}}{+} \qquad ??$$

Check for Cy^{+}, Sb^{+} flies in the progeny. If they are present, this indicates that $Dp(2;3)\ en^{+}$ covers the lethality of ℓ. This localizes the mutation inside the breakpoints of the duplication. The approach is not foolproof, however, if a second lethal has by chance been induced on the same chromosome. A duplication capable of rescuing one lethal would not rescue the other, producing a false negative. To circumvent this problem, you

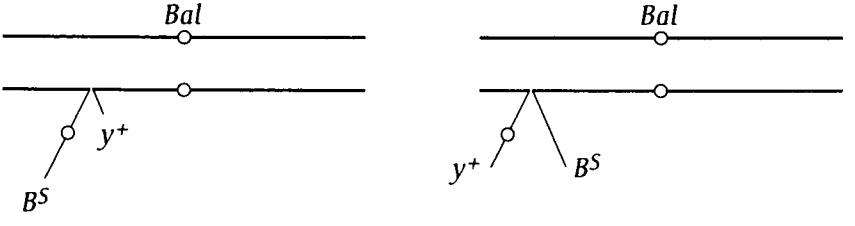
can use a different, independently isolated allele of the gene if it exists, since it would never have the same two random lethals induced. (See Chapter 4 for techniques to synthesize strains.)

SYNTHESIZING DELETIONS AND DUPLICATIONS

Not all regions of the genome are represented by existing deletions and duplications, although more are being covered all the time (Parks et al. 2004). Those that are can be obtained in a kit from the Drosophila Stock Center at Bloomington, Indiana (see the Appendix). If you are stuck with solving the problem yourself, strains constructed by Lindsley, Sandler et al. (1972) permit synthesis of duplications (almost at will) and, to a lesser extent, deletions. These strains are translocations between either the second or third chromosome and a *Y* chromosome marked with y^+ (the wild-type allele of *yellow* body color) and B^s (an allele of the dominant eye mutation, *Bar of Stone*) known as *T(Y;A)*s. Each translocation has the following general structure:

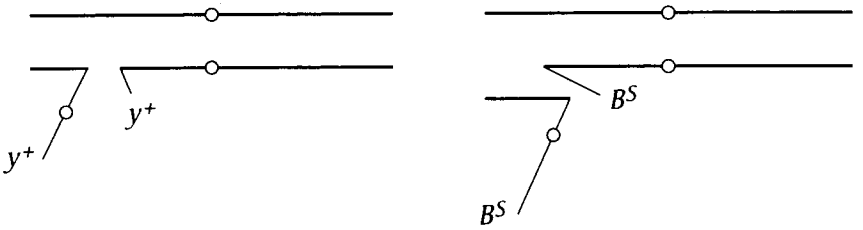


Here, a reciprocal translocation can be found between marked *Y* and the autosome such that each piece of the broken autosome has a different *Y*-linked marker and a centromere. (Chromosome fragments have no future if they have no centromeres.) The entire autosome is present, but it is now broken into two pieces. These are balanced by an appropriate autosomal balancer chromosome (*Bal*). By choosing two *T(Y;A)*s with relatively nearby breakpoints and with opposite configurations of y^+ and B^s,



one class of progeny produced gets the short end of the translocations (producing a deletion), another class the long ends (producing a duplica-

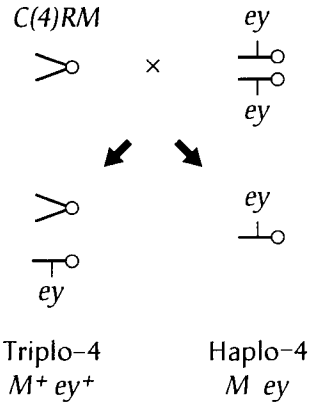
tion), and all the others are euploid (with a normal amount of genetic material).



The deletion is uniquely marked with two doses of y^+ but not B^s, whereas the duplication is unique with two doses of B^s and none of y^+. The aneuploid (deleted or duplicated) progeny are a small fraction of the total progeny at best, and reduced viability associated with aneuploidy can decrease the recovery even further. An analogous set of translocations between the *X* and the same marked *Y*, generated by Stewart and Merriam (1973), are known as *T(X;Y)*s.

The initial study for which these rearrangements were made surveyed the "gross structure" of the *Drosophila* genome (Lindsley, Sandler et al. 1972) and defined several haplolethal loci (lethal when present as a heterozygous deletion), one of which was also triplolethal (lethal when duplicated). In general, they found that heterozygous deletions were lethal when larger than one numbered region and when duplications were larger than four or five numbered regions. These are only averages, however, and some examples of very large duplications (e.g., the entire left arm of chromosome *2*) and deletions (e.g., 37B-40B) are viable.

In the paleolithic age before cloning, these stocks were successfully used to identify structural loci for several enzymes of neurobiological interest by "dosage effects." The strategy relied on the fact that duplications and deletions of these loci were not dosage compensated. That is, flies with a duplicated enzyme locus would have roughly 1.5 times as much enzyme as a normal, euploid fly. Those that were heterozygous for a deletion would have roughly 0.5 as much as euploids. (In reality, the values are quite variable and average 1.3 for duplications and 0.6 for deletions.) Crosses of 30 *T(Y;A)*s produce a series of contiguous duplications for chromosomes *2* and *3*. Crosses of two appropriately chosen *T(X;Y)*s are sufficient to produce duplications for each half of the *X*. The fourth chromosome can be tested in one cross between a stock carrying an attached-*4*, *C(4)RM*, and a stock homozygous for a fourth-chromosome marker (e.g., *eyeless*, *ey*), producing progeny that are either triplo-*4* or haplo-*4*.

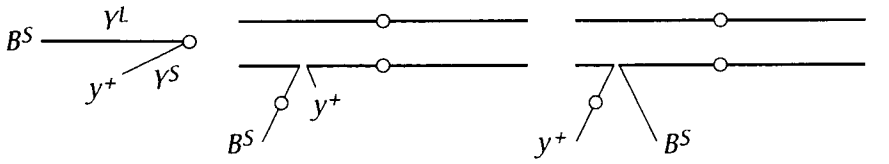


Half of the progeny will have three copies of chromosome *4* and half will have one copy. Those with three copies will look wild type and those with one copy will look eyeless, a result of hemizygosity for the mutation. *Minute* (*M*) is a phenotype characterized by thin bristiles and retarded development, a result of hemizygosity for the *Minute* locus on chromosome *4* (see Chapter 4 for a discussion of *Minutes*).

In this fashion, loci for *Acetylcholine esterase* (Hall and Kankel 1976), *Dopa decarboxylase* (Hodgetts 1975), *cAMP Phosphodiesterase* (Kiger and Golanty 1977), and *Choline acetyltransferase* (Greenspan 1980) were initially found. It was because of this cytological localization of *cAMP Phosphodiesterase* that the molecular identity of the learning mutant *dunce* was solved, based on the initial realization that it mapped to the same place (Byers et al. 1981; story recounted in Greenspan 1990).

If you want to use these stocks, it is best to carry out a series of pilot crosses (using one or two vials) to ensure that they are behaving as they should. This is much easier than making chromosome squashes from each; *Y*-autosomal breakpoints are difficult to score. The markers indicating duplication and deletion progeny are one indicator. If the two strains in the cross have breakpoints that are nowhere near each other, no duplication or deletion progeny will be produced. If they are several numbered regions apart, duplications should survive but not deletions, and so on. A more detailed characterization involves scoring the proportion of duplication progeny that are male versus female (Lindsley, Sandler et al. 1972; Ashburner et al. 2004).

To determine the markers that indicate duplication and deletion, consult the list of these stocks to see the arm of the *Y* chromosome that becomes broken in the translocation—either the short arm (Y^S) or the long arm (Y^L). In the original *Y* chromosome, B^s was on Y^L and y^+ on Y^S:



Original *Y* | *T*(*Y*;*A*) with Y^S breakpoint | *T*(*Y*;*A*) with Y^L breakpoint

Over the years, the attrition of these stocks has been significant, but many still remain. Some have lost the B^s marker, but this is not always a problem if one of the two strains retains it.

Problem 6 Determine the classes of progeny that you could distinguish if you mated two *T(Y;A)*s, both of which have breakpoints in Y^S. Determine the classes of progeny that you could distinguish if you mated two *T(Y;A)*s, one of which has a breakpoint in Y^S and one a breakpoint in Y^L, but with its B^s marker lost.

The strength of this technique for adult flies is obvious. For embryos and larvae, on the other hand, B^s is completely unrecognizable and y^+ scorable only in third-instar larval mouth parts and setae (tiny hairs). It is thus impossible to know which progeny are duplicated or deleted for the segment at these preadult stages. This precludes most kinds of analyses except those in which you need only a distinctive phenotype in some fraction of the progeny, such as in the identification of new loci.

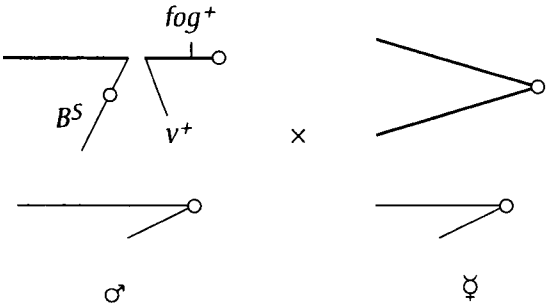
Identifying New Embryonic Genes by Deletion Mapping

Most deletions are homozygous lethal. This severely limits their utility for identifying new loci based on phenotype unless the phenotype can be scored at the lethal stage. For embryonic development, this is quite feasible and a variety of new loci has been identified starting from a morphological defect in 25% of the progeny of a strain heterozygous for a deletion, or in the aneuploid progeny of a *T(X;Y)*. Even when multiple genes are deleted, as is virtually always the case, the lethal phenotype is predominantly caused by a single, early-acting gene. This is ultimately sorted out by isolating individual mutations in the deleted region (see Chapter 2) and seeing if any recapitulate the phenotype of the homozygous deletion. You are limited only by the ability to screen for morphological aberrations.

The *single-minded* (*sim*) locus was identified in this fashion, starting from a screen of embryos from heterozygous stocks of autosomal deletions. Dead embryos were stained and examined in whole mounts. Aberrations of the ventral nerve cord were found in homozyotes for a 14-band deletion *Df(3R) ry*^{3} (Thomas et al. 1988). EMS mutagenesis and a screen for lethal mutations uncovered by this deficiency confirmed that this phenotype was the result of a single gene defect.

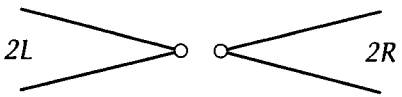
More dramatically, one of the earliest acting zygotic genes was identified through deletion mapping as being predominantly responsible for

the phenotype produced in an embryo having no *X* chromosome (*nullo X*). *Nullo-X* embryos have long been known to arrest development very early with no cellularization at the blastoderm stage (Poulson 1940). This was always assumed to be a "syndrome" due to the loss of one fifth of the genome. Wieschaus and Sweeton (1988) created large deletions of the *X* to define the region responsible for this phenotype. They mated *T(X;Y)* males to attached-*X* females (each with its own intact *Y*) and cleverly scored the portion of the *X* an embryo received by the presence or absence of the *folded-gastrulation* (*fog*) phenotype. fog^- embryos fail to form a posterior midgut and so are easily recognized.



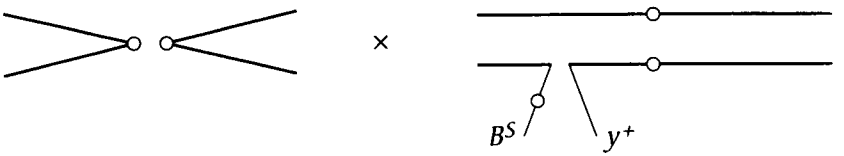
Those embryos receiving the *Y* from their mothers and a fragment of the *T(X;Y)* from their fathers will have large deletions of the *X*, distinguishable by the presence or absence of the posterior invagination induced by fog^+ activity. After obtaining a rough localization by this method, the authors zeroed in on the locus by means of careful deletion mapping of the embryonic phenotype. This revealed, contrary to previous expectation, that a single, two-band region was responsible for the *nullo-X* phenotype of failed cellularization. The *nullo* locus was ultimately identified as the culprit (Rose and Wieschaus 1992).

Taking this approach to the autosomes, Merrill et al. (1988) utilized a different set of chromosome rearrangements to produce complete or partial deletions of autosomal arms. They used compound autosome stocks in which the two left arms are attached to a common centromere and the two right arms to a common centromere. In most stocks of this sort, each set of compound arms is also homozygous for a recessive marker to easily determine if the stock is uncontaminated (see p. 12).

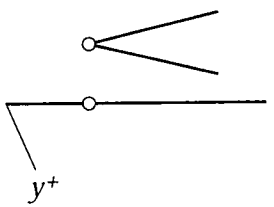


When males and females of this stock are mated together, some will lack *2L* and others *2R*. (Segregation of these compound autosomes in males occurs randomly, whereas in females *2L* will segregate regularly from *2R*. Thus, males will produce gametes containing random mixtures of the various chromosome rearrangements, whereas females will produce gametes that contain one or the other.) A similar stock exists for the third chromosome. To monitor the frequency of the various classes of segregants in these stocks, they were crossed to embryonic cuticle pattern mutants located on each arm.

From this analysis, Merrill et al. (1988) determined the chromosome arms that carried genes acting before gastulation, similar to the *nullo-X* locus previously identified. By combining various *T(X;Y)*s with the *C(2L;2R)* stock, they were able to create smaller deletions of the appropriate arms.

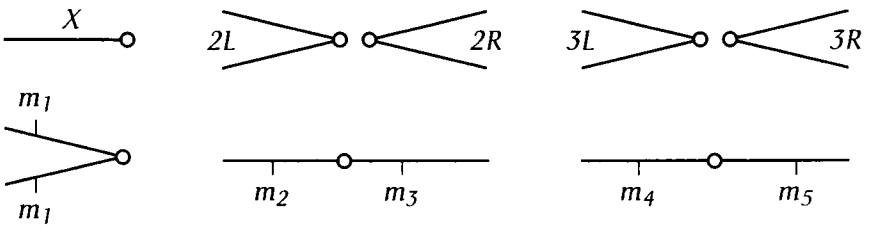


A small fraction of the progeny embryos from this cross will be totally deleted for the distal left arm:



The upshot of this analysis was the identification of seven new loci with developmental defects preceding gastrulation, two of which affecting cellularization.

Compound chromosomes of this sort are produced by radiation. To introduce recessive markers onto their arms, essential for knowing what you have, one produces triploid females in which the one normal set of chromosomes carries the desired recessive mutations m_1, m_2, m_3, m_4, and m_5 (e.g., see Hardy 1975).



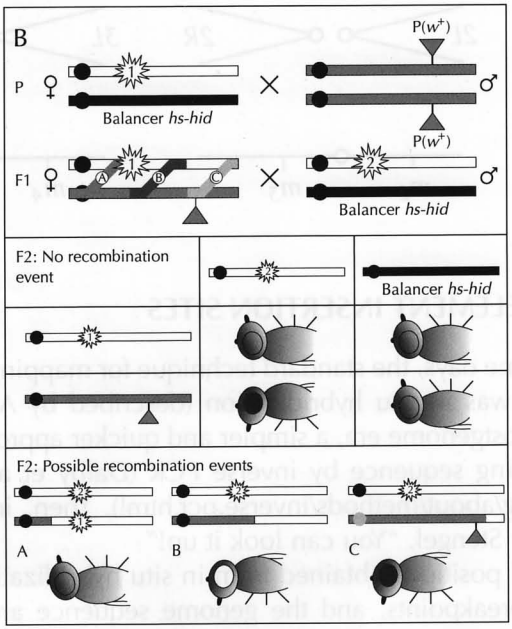
MAPPING P-ELEMENT INSERTION SITES

In the pregenome days, the standard technique for mapping insertion sites of P elements was in situ hybridization (described by Ashburner et al. 2004). In the postgenome era, a simpler and quicker approach is to determine the flanking sequence by inverse PCR (Dalby et al. 1995; http://www.fruitfly.org/about/methods/inverse.pcr.html). Then, in the immortal words of Casey Stengel, "You can look it up!"

Cytological positions obtained from in situ hybridization, analysis of chromosome breakpoints, and the genome sequence are expressed as "band numbers" with regard to the chromosome bands visible in preparations of larval salivary gland chromosomes (see p. 6). The correspondence between meiotic map positions and cytological band positions is not a simple one, varying with position along the chromosome. Specific correlations can be found (or guessed at) in FlyBase (FlyBase Consortium 2003; http://flybase.bio.indiana.edu/maps/fbgrmap.html). Remember, however, that a mutant phenotype must be independently mapped to the site of a new insertion, either by excision, deletion, or recombination analysis (if only to avoid embarassment).

FINE-GRAIN TRADITIONAL MEIOTIC MAPPING WITH P-ELEMENT MARKERS

With a sufficient density of P-element insertions in the vicinity of a mutation, traditional meiotic mapping can also be used to localize its position in the genome sequence (Zhai et al. 2003). Choose a set of four or five P elements per cM spanning the locale in which the mutant has been meiotically mapped and perform a series of crosses to place the mutation heterozygous with each of the P elements. Mate such heterozygous females to test for complementation to males carrying the same mutation, another allele of the gene, or a deficiency of the gene, over a balancer.



Scheme for P insertion mapping. Mutant chromosomes are indicated by open bars, with P-containing chromosomes in gray and balancer chromosomes in black. Mutant alleles are denoted by stars and are numbered to be distinguished from one another. All flies are on a w^- background so that w^+ P elements are detectable. The top box shows P and F1 crosses, the middle box shows nonrecombinant F2 progeny, and the bottom box shows three classes (A, B, and C) of recombinant F2 progeny. (Redrawn, with permission, from Zhai R.G. et al. 2003. © 2003 National Academy of Sciences, U.S.A.)

Provided that the endogenous locus for the eye-color marker (*white* or *rosy*) is also kept mutant, any of their progeny that have undergone a recombination event (which will necessarily be a single recombination in that small of an interval) between the mutation and the P element will be recognizable. By scoring 10,000 progeny for each P insertion, you can obtain a map resolution of 0.1 cM. Since the flanking sequence of each P element is known, the mutation can be placed into an interval between two adjacent elements and its distance from each roughly estimated.

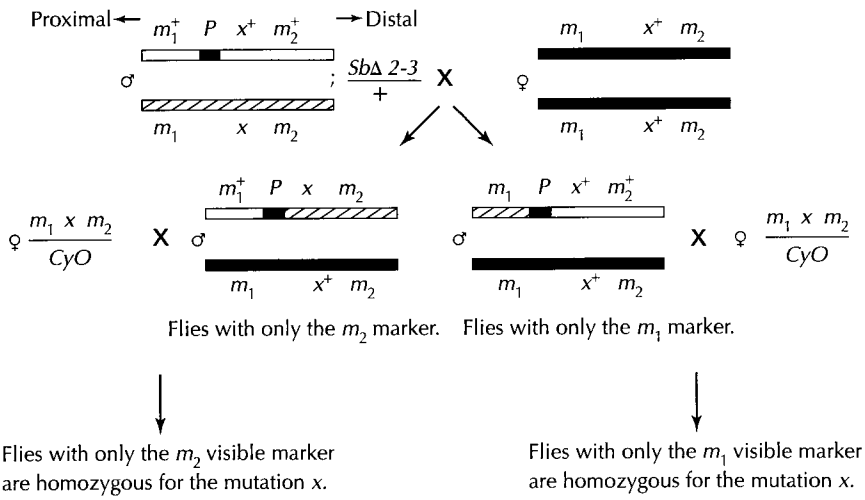
This approach has been used to estimate the correspondence between recombinational distance and molecular distance, which can vary from ~100 kb/cM in the middle of an arm to ~1500 kb near a telomere (Zhai

et al. 2003), in line with very early estimates of relative physical distance versus recombination frequency obtained from comparing chromosome rearrangements and meiotic map distances (Painter and Muller 1929). The results also show that recombinational distances can vary significantly even within small chromosomal segments, introducing some uncertainty in the accurate prediction of molecular position based on map position alone.

MAPPING BY P-ELEMENT-INDUCED MALE RECOMBINATION

The absence of recombination in *Drosophila* males has been one of the great serendipities in genetics. In the early days, it gave the little fly its major advantage for producing the first genetic maps because of the ability to transmit intact chromosomes through males and score recombination cleanly in females (Sturtevant 1965; Kohler 1994). In the more recent past, it was the anomalous occurrence of male recombination that provided one of the first observations that eventually led to the identification of P elements (Kidwell et al. 1977). Twenty years later, the anomaly of P-element-induced male recombination reappeared as a technique for fine-grained mapping of mutations in the fly, once their general location was known. The approach capitalizes on the fact that many P-element insertions exist, their exact location in the genomic sequence is known, and the vast majority of male recombination events occur very close to the site of an inserted P element as the result of a double-strand break made at one site in the element by its transposase (Preston and Engels 1996).

The strategy (Chen et al. 1998) makes use of flanking markers to detect the recombination event. These are chosen to be easily scorable and can be located virtually anywhere on a chromosome, since the recombination event occurs in the vicinity of the P element. As diagrammed below (from Chen et al. 1998), m_1 and m_2 represent the flanking markers, *x* represents the mutation being mapped, and *P* represents an inserted transposon. The recombination event is induced by the stable transposase insert on the third chromosome, *Δ2-3*, and visualized by separation of the two flanking markers. All progeny will have both flanking markers (m_1 and m_2) except those few that have undergone a recombination event; they will have one or the other. You may then score the recombinant flies for the presence or absence of the mutation (*x*), which tells you whether the P element is proximal or distal to the mutation. In the



Mapping by P-element-induced male recombination. m_1 and m_2 are proximal and distal flanking markers that flank the P element. Recombination events are induced by transposase (*Δ2-3*) at the ends of the P element in the male flies. Recombinants, identified by the presence of only the m_1 or m_2 visible marker, are crossed to *x*-bearing females to test for the mutation's presence on the recombinant chromosomes. If *x* is distal to the P element, the recombinant chromosomes m_1^+ m_2 do not complement *x*, and m_1 m_2^+ do complement *x* (shown here). If *x* is proximal to the P element, m_1 + m_2 recombinants complement *x*, whereas m_1 m_2^+ do not (not shown). (Redrawn, with permission, from Chen B. et al. 1998. © 1998 The Genetics Society of America.)

example above, the m_2-containing recombinants should retain the mutation, whereas the m_1-containing recombinants should not.

By carrying out the same procedure sequentially on a series of P-element inserts that are clustered in the region containing the mutation, a clear transition can be obtained between P elements mapping proximal and those mapping distal on the chromosome relative to the mutation. The two closest P elements giving opposite mapping results (proximal versus distal) define the interval containing the mutation. Depending on the availability of inserts, this can be as small as 40 kb (Chen et al. 1998; Ostrowski et al. 2002), an interval that is likely to contain a small number of candidate genes. The frequency of such recombination events depends on the particular P element. In these studies, some never yielded recom-

binants, but most (30/37) did and varied from 1 to 10%. A safe level of progeny to score is 10,000 for each P element, but this is not so difficult if your markers are chosen to be easily scorable at a glance for the presence of both versus the presence of either one alone (e.g., *cn* and *bw* for the second chromosome, which are white in the double mutant, or *st* and *ca* for the third, which are orange in the double mutant).

FINE-GRAIN MAPPING BY TRANSPOSON-INDUCED DELETIONS

The same phenomenon that produces male recombination sometimes produces small deletions adjacent to the P element (Preston et al. 1996). These range in size from a few base pairs to 100 kb and because they retain a portion of the P element, they extend unidirectionally from the same site. The cross to produce them is the same as the one diagrammed above and as many as one third of the recombinants harbor deletions. The presence of a new deletion is detected by PCR of flanking sequences.

By now, you may be wondering about the fate all of the deletions that must have been produced in the previous section when this scheme was used to map a mutation as being either proximal or distal to a P element. The answer is that they do not affect the analysis one way or another, provided that the P elements used are far enough apart that an adjacent deletion would not take out the wild-type locus from the P-element-bearing chromosome. Occasional inconsistencies in the results will arise, however, and these are likely to be due to the occurrence of an interfering deletion or duplication from the male recombination event. However, if one scores a series of 3–5 recombinants from each P element, the overall trend will be clear.

As discussed earlier, an alternate transposon was developed using the *hobo* element as a two-component system in which the transposase can be introduced separately from the transposon (Smith et al. 1993). Elaborating further on this system, Huet et al. (2002) produced another strategy for producing deletions adjacent to an insertion site, analogous to the system just described. In the presence of *hobo* transposase, *P{hsH/T-2}*, the *hobo* element replicates and jumps into a nearby site, and then a recombination event deletes the intervening sequence. To accomplish this, they introduced a *hobo* element into the middle of a P-element vector, *P{wHy, w^+ y^+}*, such that the *hobo* element was flanked by *white*$^+$ on one side and *yellow*$^+$ on the other. Knowing the orientation of the P-element insertion

in the genome allowed deletions to be selected for directionality simply by scoring the loss of one marker versus the other.

Deletion events have been confirmed to extend beyond the limit of the P element by PCR for terminal sequences of the transposon. Roughly one third of the marker loss events were internal to the P element. Using inverse PCR, a second test determines the sequence that now flanks the transposon and confirms that it is in the correct chromosomal region, on the correct side of the transposon, in the correct orientation, and also that the *hobo* element is only present in one copy. This was true for over 80% of the events that were not internal to the P element. This yielded a deletion frequency of 28% of all jumps. Taken together with a frequency of 19–26% for initial jumps, this yielded an overall frequency of 5–7%. Most of the deletions were <60 kb and showed a smooth distribution in size.

At present, there are far more P-element insertions of the sort suitable for producing deletions by means of male recombination. The *P{wHy, w^+ y^+}* strategy, however, may be more useful for cases in which one wants to produce a very fine-grained set of nested deletions. Given that the vast majority of the functions of predicted genes from the Fly Genome Project are unknown, it may well turn out that such fine-grained analysis will be the most efficient way to make lesions in them.

MAPPING BY DNA POLYMORPHISMS

The generality of the transposon-based mapping strategies just outlined depends on the density of available insertions. A rich and ubiquitous source of randomly distributed markers for mapping is endogenous DNA sequence variation. DNA polymorphisms are not easier to score than dominantly marked P elements, but they have little or no impact on phenotype, and they are likely to exist in strains in which it would be detrimental to the genetic background if P elements were introduced. An ever-expanding series of DNA sequence variations (mainly single nucleotide polymorphisms, SNPs) have been identified in various fly strains (Teeter et al. 2000; Berger et al. 2001; Hoskins et al. 2001; http://www.fruitfly.org/SNP/index.html). DNA polymorphisms have the advantage that they are dominantly detectable, but they are also at a disadvantage because they are sequences and must be **sequenced** to be scored (after PCR amplification) or else assayed for altered melting of heteroduplex DNA by denaturing high-performance liquid chromatography (HPLC) (Nairz et al. 2002) or temperature gradient capillary electrophoresis (Zhai et al. 2003).

Studies of natural variation in phenotype rely on careful control of genetic background, the overall genetic constitution of the strains under study. Whether comparing strains brought in from the wild or strains subjected to multiple generations of phenotypic selection, the ability to map the loci that contribute to the aggregate phenotype demands maintenance of the integrity of the genetic constitution of the strain. Introduction of marker mutations or marker P elements is impossible under these conditions, and endogenous DNA sequence variation or endogenous retrotransposons become the markers of choice for the mapping of these quantitative trait loci (QTL). QTL mapping is a discipline onto itself. In recent years, at the molecular level, it has scored major successes in the identification of loci contributing to naturally occurring phenotypic variation (Mackay 2002). This capability will continue to be enhanced by further advances in molecular mapping techniques.

CHAPTER 4

Synthesizing Specific Genotypes

VIRTUOSO FLY GENETICS REACHED ITS PINNACLE WITH THE ART of synthesizing strains and generating new rearrangements. The Stradivarius of this art, Ed Novitski, set a standard that has yet to be equalled with his synthesis of an entire, diploid set of autosomes attached to a single centromere (Novitski et al. 1981). Not surprisingly, meiotic segregation was not a happy event in this strain and it did not survive well. But the principle was established.

For most purposes, the extent of complex stock construction only goes as far as putting a few chromosomes together in the same fly. This can be more complicated than it sounds and always requires some planning. Many times I thought I could whip off a new strain in a few crosses starting from stocks I happened to have on hand, only to find two generations later that I could not distinguish genotypes clearly and had to start over again.

The ability to distinguish genotypes unambiguously is the heart and soul of fly chromosome manipulation. This feature, which sets fly genetics apart from all others, is essential for exploiting the full potential of the organism.

Principles

The strategies for stock construction take advantage of the following facts, most of which have been said before but bear repeating. These three facts allow all chromosomes to be faithfully followed.

1. Homologous chromosomes segregate from each other reliably during meiosis, meaning that if a progeny received a particular chromosome from its mother, it did not also receive that chromosome's homolog.

2. The dominant markers and balancers for each of the chromosomes indicate the homolog from each parent that a progeny received, by virtue of either the marker's presence or its absence (see previous fact).
3. There is no recombination in males and a balancer chromosome effectively suppresses recombination with its homolog in females. Thus, a dominant marker that started out on a particular homolog will stay on that homolog through meiosis and thus serve as a reliable marker for it.

Designing schemes for fly matings is a little like undertaking problems in organic chemistry. You must determine how to combine the available starting materials using the most economical and reliable steps to reach a final product, and you must be able to "purify" the product of each step before proceeding to the next. It is often helpful to work backward from the final product. (If the organic chemistry analogy summons up bad memories, do not worry; fly mating schemes are more fun and ultimately more satsifying to carry out. In addition, you can work on them while passing the time during boring seminars or laboratory meetings.)

SIMPLE MANIPULATIONS OF A SINGLE CHROMOSOME

This is the baseline of fly manipulations, and it is essentially the same as that outlined in Chapter 2 in the section on isolating mutants and performing complementation tests. The principle of identifying progeny by absence of dominant markers is illustrated in the following complementation test between *hunchback* (*hb*) and a new third-chromosome lethal:

$$\frac{TM3,\ Ser}{\ell} \times \frac{TM6,\ Ubx}{hb}$$

$\frac{\ell}{hb}$ Survival of this class of progeny that are non-*Ubx* and non-*Ser* indicates complementation.

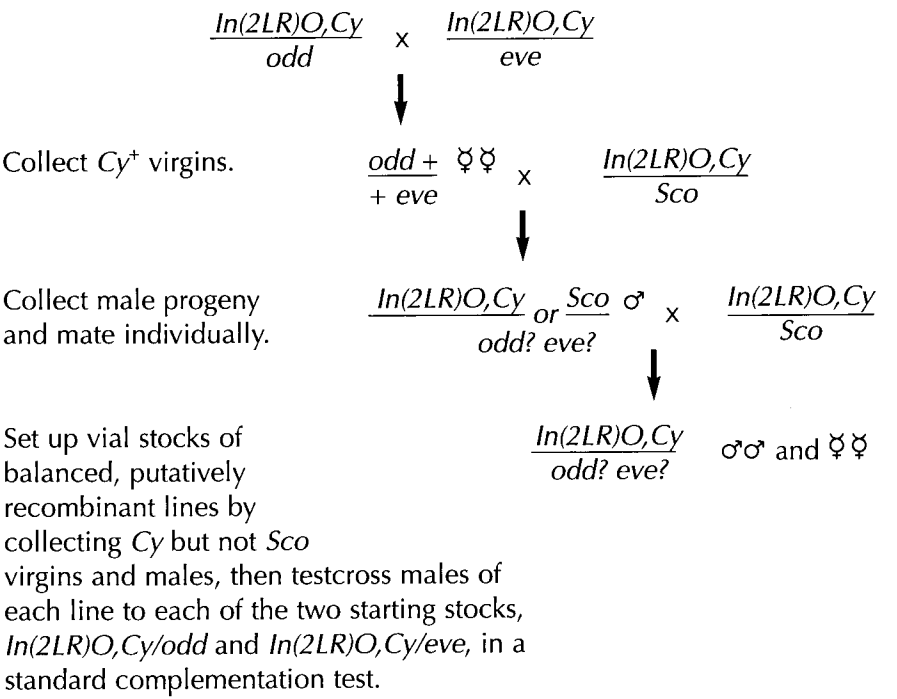
The key feature of this cross is that each genotype is distinguished by a unique combination of markers—always a goal to be aspired to.

Linking Mutations

Another common, slightly more involved, manipulation is recombining two mutations (or a P-element insertion and a mutation) onto the same

chromosome. This is often necessary for mapping mutations by P-element-induced male recombination (see Chapter 3), carrying out double mutant tests (see Chapter 5), and linking markers to mutations for mosaic analysis (see Chapter 6).

In its simplest form, the process requires females that are doubly heterozygous on the same chromosome for the two mutations to be linked. One then recovers individual, putative recombinant chromosomes in the progeny of these females and tests them for the presence of both mutations. An example of making a double mutant of *odd-skipped* and *even-skipped* on the second chromosome is shown below.



Immediately raised is the question of how many vial stocks of putatively recombinant chromosomes to set up. The answer depends on the distance between the two mutations. The further apart, the more likely it is that you will recover your desired product. In this case, *odd* and *eve* are very far apart (approximately 50 map units), which is equivalent to being unlinked. Therefore, you will probably recover your product in 10 lines, but do 20 to be on the safe side.

For loci that are closer together, you need to use correspondingly higher numbers of lines to be certain of recovering your recombinant. The expectation is a simple function of map distance: 5 map units between loci yields a 5% chance of recombination. You might recover it in 100 lines, but it would be wiser to do 200.

In addition, to be more rational about this, you can use Mather's (1951) formula for calculating the amount of progeny (N) you need for a 95% probability (p) of obtaining a recombinant if f is the expected fraction of progeny that will be recombinant: $N = -\log(1-p)/\log(1-f)$. If the expected recombination frequency is 1%, you need to score approximately 300 progeny to be 95% certain of obtaining the desired recombinant.

As mentioned earlier, exchange is temperature sensitive. Both high and low temperatures increase exchange (go figure!), so the probability of obtaining a recombinant chromosome for two closely linked markers can be somewhat increased (approximately twofold) by raising the doubly heterozygous females at 30°C (Plough 1917). It is crucial that these double heterozygotes are **raised** at high temperature, rather than simply placed there as adults for the next cross, because the critical period for this effect does not extend into adulthood. On the other hand, if they are raised at 30°C for their entire life cycle, their fecundity (egg laying) will be reduced approximately twofold. Therefore, the best compromise is to shift the bottles to 30°C for most of the larval period, i.e., begin when you see the food churning up and end when you see pupae forming on the sides of the bottle. The high-temperature effect is most pronounced near the centromere and the tips of the chromosome arms.

Another condition that will increase recombination is the presence of a heterozygous inversion on another chromosome. This "interchromosomal effect" increases recombination anywhere in the genome outside the rearranged regions.

Problem 7 Work out how to recombine a *P[w⁺h⁺]* (*white*⁺, *hairy*⁺) insertion element located on *3L* in cytological region 71A (meiotic map position of approximately 3-43) with a mutation at the *hairy* (*h*) locus (3-26.5).

Sometimes it helps to use additional, visible markers to aid in the recognition of recombinants for invisible, recessive mutations. This is very easy to do if, for instance, you have carried out meiotic mapping of your mutation and therefore already have a chromosome with your invisible mutation and some visible markers on it.

MANIPULATING TWO CHROMOSOMES

To control the genotype on two different chromosomes at once, you must start planning your mating schemes. Here you will make use of balancers, dominant markers, the lack of recombination in males, and the reliable segregation of homologs. As an example, we consider how to make a stock to produce double mutant embryos for *ftz* on the third chromosome and *eve* on the second chromosome.

Most mutation-bearing stocks contain the mutation and a balancer for that chromosome, e.g., *TM3, Ser/ftz*. This is the healthiest way to keep them, since the presence of other dominant markers and balancers on other chromosomes only detracts from the stock's viability. To manipulate two chromosomes at once, it is necessary to introduce additional balancers and dominants without losing track of the chromosome you started with. For this reason, it is useful to keep a stock with two chromosome's worth of balancers and markers, such as

$$FM7a;\ \frac{TM6,Ubx}{Sb}\ \text{or}\ \frac{In(2LR)O,Cy}{Sco}\ ;\ \frac{TM3,Ser}{Sb}\ \text{or}\ \frac{In(2LR)O,CyO}{Sco}\ ;\ \frac{TM6,\ Ubx}{Sb}$$

However, the deleterious effects of multiple balancers on female meiosis indicate the necessity to use males of these genotypes whenever possible. The trick is to mate your starting mutant stock with another that uses different markers. That way, you can exploit the obligatory segregation of homologs to trade markers. In this first step, you build out from one chromosome onto another.

Cross 1

$$\text{♀♀}\ \frac{TM6,Ubx}{ftz}\ \times\ \frac{In(2LR)O,Cy}{Sco}\ ;\ \frac{TM3,Ser}{Sb}\ \text{♂♂}$$

$$\downarrow$$

F1 ♀♀ $\dfrac{In(2LR)O,Cy}{+}\ ;\ \dfrac{TM3,Ser}{ftz}$

ftz is now recoverable in the same flies with the balancer *In(2LR)O,Cy* in the *Ser, Ubx*$^+$ progeny. However, these are not particularly healthy and happy females because of the presence of two balancers.

At the same time, make an analogous genotype from the other mutant line:

Cross 2

♀♀ $\frac{In(2LR)O,Cy}{eve}$ x $\frac{In(2LR)O,Cy}{Sco}$; $\frac{TM6,\ Ubx}{Sb}$ ♂♂

↓

F1 ♂♂ $\frac{Sco}{eve}$; $\frac{TM6,\ Ubx}{+}$

eve is now recoverable heterozygous with the dominant *Sb* in the *Sco, Cy*$^+$ males. However, the flies of this genotype that are used in any subsequent cross must be male to prevent any exchange on the second chromosome that could place *eve* and *Sco* on the same homolog.

Now the F1 virgins from cross 1 and the F1 males from cross 2 can be mated together to yield a stock for producing double mutants.

♀♀ $\frac{In(2LR)O,Cy}{+}$; $\frac{TM3,\ Ser}{ftz}$ x $\frac{Sco}{eve}$; $\frac{TM6,\ Ubx}{+}$ ♂♂

↓

$\frac{In(2LR)O,Cy}{eve}$; $\frac{TM6,\ Ubx}{ftz}$

Collected as males and virgin females (*Cy, Sco*$^+$ and *Ubx, Ser*$^+$), these flies will constitute a true-breeding (albeit unhealthy) stock, 1/16 of whose progeny would be doubly homozygous for *eve* and *ftz*. If, perchance, this *eve* mutation suppressed the lethality of this *ftz* mutation (see Chapter 5), even only 1% of the time, it could be detected and scored unambiguously by the markers in this stock. A unique combination of markers permits each genotype to be distinguished at each step.

If, on the other hand, you wanted to produce doubly mutant embryos, these adult markers would be useless. The desired result could have been obtained much more simply and with better viability by crossing the two original stocks as follows:

$\frac{In(2LR)O,Cy}{eve}$; $\frac{+}{+}$ x $\frac{+}{+}$; $\frac{TM6,Ubx}{ftz}$

↓

$\frac{eve}{+}$; $\frac{ftz}{+}$

Collect *Cy*$^+$, *Ubx*$^+$ males and virgins, mate them to each other, and collect embryos, 1/16 of which will be doubly homozygous for *eve* and *ftz*.

The drawback of this simple approach is that you do not know if the double mutant phenotype is different from either of the single mutant phenotypes. To solve this problem, you can make use of balancer chromosomes that have P elements expressing a ubiquitous marker (such as green fluorescent protein [GFP]) in them. When you collect progeny from the stock consisting of *In(2LR)O,Cy P[ActGFP]/eve ; TM6B, P[Ubi-GFP.S65T]PAD2, Tb1/ftz,* score them under a fluorescent microscope. Only those progeny failing to fluoresce will be double mutants.

The value of markers becomes apparent whenever you want to score viability, detect small numbers of progeny of a given genotype, and recover individual progeny of known genotype.

An additional tool that is helpful for simultaneously manipulating the second and third chromosomes is a reciprocal translocation between *In(2LR)O,Cy* and *TM9* called *T(2;3) CyO; TM9.* Since it is a reciprocal translocation between both balancers, it ensures that all pieces of the translocation must be present in a fertilized egg for the egg to be viable; if not, the zygote will be aneuploid and die. Because it contains complete sequences for both chromosomes, it effectively balances all of chromosomes *2* and *3* and allows you to keep a stock such as

$$\frac{T(2;3)\ CyO;\ TM9}{eve\ ;\ ftz}$$

It is necessary to manipulate two chromosomes when producing P-element transformants to see if a genomic clone rescues a mutant phenotype. You can then use the visible marker in the P element (e.g., w^+) as a dominant marker, provided that the *X* chromosomes in the scheme all carry a mutant allele of *white.*

For example, if you have produced a transformed line carrying a $P[w^+, odd^+]$ insert on the third chromosome and you want to construct flies that are homozygous for *odd* on the second chromosome and also carry this P element on their third chromosome, it helps to start with stocks that are mutant for *w.* Generally, this will already be true of the P-element-containing stock, since it is likely to have been made with *w* in it:

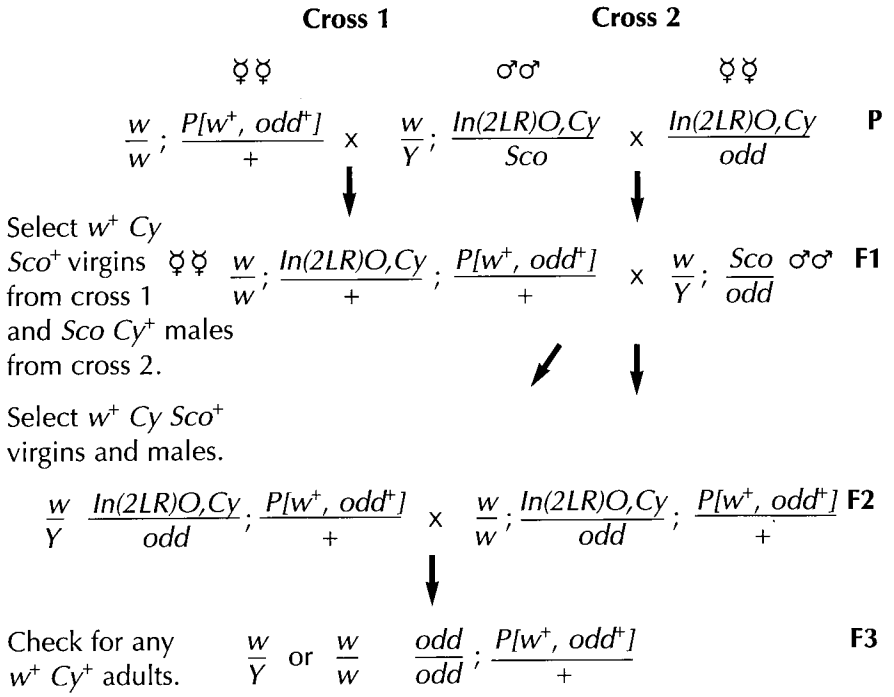
$$\frac{w\ ;\ P[w^+,\ odd^+]}{+}$$

However, this will need to be made for *odd* as follows:

$$\frac{w\ ;\ In(2LR)O,Cy}{odd}$$

Similarly, all the balancer stocks used in the scheme must be *w*. You would generally have these are stocks on hand anyway to do linkage analysis of newly derived transformants (see Chapter 3).

Since *P[w⁺, odd⁺]* will be scorable as dominant on a *w* background, the scheme is somewhat simpler than the previous example for making a double mutant:



The first line of this scheme is shorthand for setting up two crosses simultaneously in the parental (**P**) generation, using males of the same genotype for each. It also shows only the relevant chromosomal genotypes, since all others are assumed to be wild type. Thus, the virgins on the left show only the *X*- and third-chromosome genotypes (since the *P[w⁺, odd⁺]* insert is on chromosome *3*), whereas the males in the middle and the virgins on the right show the *X*- and second-chromosome genotypes. The last line shows that you get both males (*w/Y*) and females (*w/w*) that test the efficacy of your P-element rescue. If *odd/odd; P[w⁺, odd⁺]/+* flies survive, then your insert rescues the mutation.

Problem 8 Construct a stock for testing the phenotype of four doses of *P[w⁺, odd⁺]* on an *odd⁻* background, starting with P inserts on the *X* and third chromosomes. (Note: a *P[w⁺, odd⁺]* insert on the *X* creates some problems when using *w* to score the presence of the P element.)

Pitfalls

How Many Flies?

The viability problems that result from using multiple dominants and balancers have been alluded to already. In general, the more that are piled into the same fly, the sicker the fly. The sicker the fly, the more you need to start out with at the beginning of the scheme. Otherwise, you may find yourself at the end of a three-month, multigenerational scheme, only to be left with one male of the correct genotype who turns out to be sterile.

It is possible to get a rough idea of how hard it will be to obtain the flies you need by figuring out how few of the progeny from each cross are the ones you want. Usually, the amount is theoretically 1/8 or 1/16. Poor viability of markers and nonhomologous segregation of multiple balancers further reduce the number. Unlike cloning, you cannot amplify the products at each step, so you must start with enough to get you through—much as in organic synthesis.

A simple rule of thumb is to aim for enough flies to do at least one full bottle cross for the last generation of a scheme. Given the viability problems discussed, a bottle cross will require roughly 40–50 virgins. Working backward, this suggests that the initial crosses in a scheme for manipulating two chromosomes should be started with several bottles each, thus requiring 100–150 virgins.

Which Sex?

The choice of sex at each step of a scheme is influenced by several factors. One already mentioned is the lack of exchange in males. This makes it possible to use a dominantly marked chromosome as if it were a balancer, a technique commonly used in the examples in this book (e.g., *Sco/odd*). A second factor is the trouble associated with multiple balancers in females, to which males are oblivious (so what else is new?).

A third factor in choosing the sex at each step is the problem of nonvirginity. Despite our best efforts, nonvirgins do sometimes sneak

through our fine net. The presence of a few nonvirgins in a cross can totally subvert the scheme since a given set of markers can apply to more than one genotype. By taking a careful look at each cross, it will be clear that in some cases nonvirginity will not be a problem; the desired progeny will be uniquely marked, whether or not all of the mothers are virgin.

This is not an argument against collecting virgins for your crosses, since there is still the matter of how tiny a fraction of the progeny is the one you desire. It is simply another consideration for making the schemes work in the end. Take an example from an earlier cross (p. 82):

$$\text{♀♀}\quad \frac{In(2LR)O,Cy}{+}\,;\,\frac{TM3,\ Ser}{ftz}\quad\times\quad\frac{Sco}{eve}\,;\,\frac{TM6,\ Ubx}{+}\quad\text{♂♂}$$

$$\downarrow$$

$$\frac{In(2LR)O,Cy}{eve}\,;\,\frac{TM6,\ Ubx}{ftz}$$

To determine whether you are at risk, consider the markers you seek in the desired progeny (*Cy* and *Ubx*) and see if it is possible to get the same combination of markers from a nonvirgin. You must consider the cross that produced the virgins and what rogue males might have been present with them in the bottle. Could the *In(2LR)O,Cy/+; TM3, Ser/ftz* virgins have mated with males carrying *TM6, Ubx* such that you could potentially get *Cy* with *Ubx* in the progeny of a nonvirgin? In this case, the earlier cross was

$$\text{♀♀}\quad\frac{TM6,Ubx}{ftz}\quad\times\quad\frac{In(2LR)O,Cy}{Sco}\,;\,\frac{TM3,Ser}{Sb}\quad\text{♂♂}$$

$$\downarrow$$

$$\text{♀♀}\quad\frac{In(2LR)O,Cy}{+}\,;\,\frac{TM3,\ Ser}{ftz}$$

It is clear that the virgins could have mated with *TM6, Ubx* males, thus producing misleading progeny with the appropriate markers but the wrong genotype on other chromosomes. This shows that you are at risk and should be scrupulous about ensuring virginity.

MANIPULATING THREE CHROMOSOMES

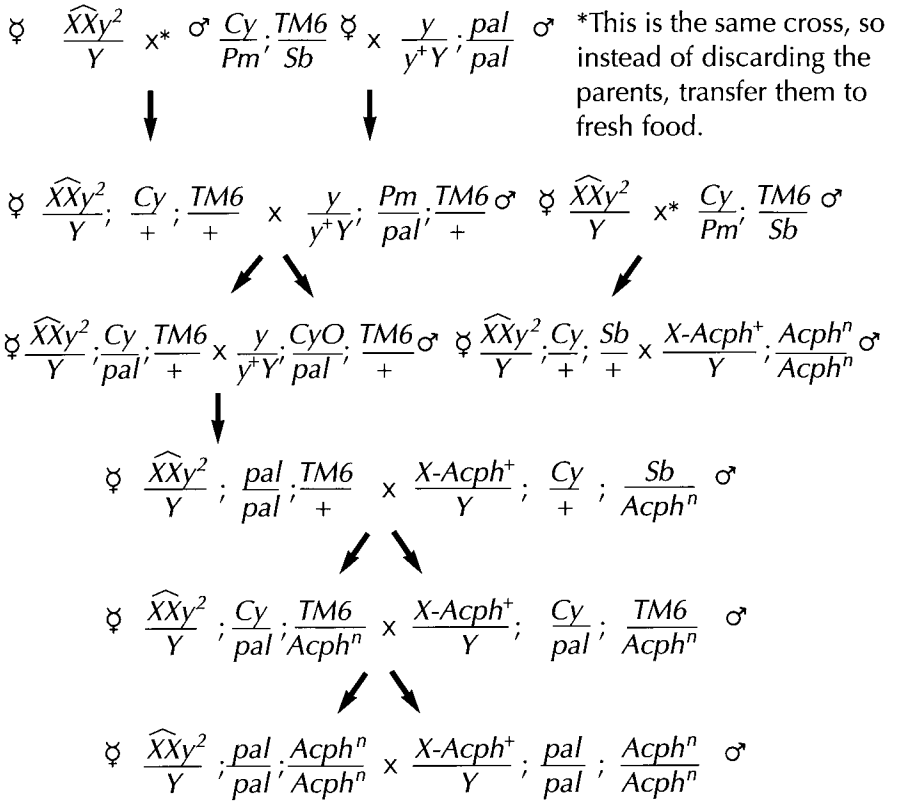
Just as in juggling, where there is a major gap in going from three balls to four, so in chromosome manipulation is the great divide between two chromosomes and three. Fortunately, three chromosomes are rarely needed. Even rarer, in fact, nearly unheard of, is the need for anyone to maneuver all four chromosomes at once. The principles are the same as those in two-chromosome schemes, but the problems are magnified. It gets harder to make genotypes that are uniquely distinguishable, and the likelihood of ending up with one sterile male (at most) is greater. If you are unlikely to need such esoteric techniques, or are uninterested in such matters, you may want to skip to the next section.

In the past, such elaborate schemes were mainly needed for certain kinds of mosaic experiments—the generation of individuals with a mixture of mutant and wild-type cells (see Chapter 6). The stocks thus generated were used to produce gynandromorphs (*X*-chromosome mosaics) under the influence of an autosomal mutation that induces chromosome loss. In addition, such stocks make use of a duplication on the *X* to cover the mutation being studied, which is recessive and lies on an autosome. Thus, when *X*-chromosome mosaicism is induced, the individual is also mosaic for the autosomal locus duplicated on the *X*. Kankel and Hall (1976) used this kind of system in their fate map study of the nervous system with an enzyme marker, *Acid phosphatase* (*Acph*). They had to make a challenging stock consisting of the following:

$$\frac{X\text{-}Acph^{+}}{\frac{\widehat{XX}y^{2}}{Y}} \; ; \; \frac{pal}{pal} \; ; \; \frac{Acph^{n}}{Acph^{n}}$$

The designation $X\text{-}Acph^{+}/\widehat{XX}y^{2}/Y$ indicates that males in the stock have the $X\text{-}Acph^{+}/Y$ and females have $\widehat{XX}y^{2}/Y$. $Acph^{n}$ is the mutation being made mosaic. A mutation in the enzyme *Alkaline phosphatase* (*n* is for null, actually, allele number *n11*), it served as a histochemical marker for lineage analysis during development. $X\text{-}Acph^{+}$ is an *X* chromosome with a duplication of the *Acph* locus on it and *pal* is the mosaic-producing mutation *paternal-loss*. To produce mosaics, males of this stock were mated to *y*; $Acph^{n}$ females (see Chapter 6 for details). For the sake of posterity, the

mating scheme Jeff Hall used to generate it is included here. It is long and almost as difficult to understand as it is to carry out. Consider it a challenge and a test of your growing fly genetics acumen. If you are still baffled after studying it, do not despair—schemes of this complexity are like a path to Zen.



Pm denotes the eye-color mutation *Plum*, which was subsequently shown to be a dominant allele of *brown*, *bw*.

MAKING REARRANGEMENTS

There have been a few virtuoso practitioners of chromosome rearranging in the history of *Drosophila* genetics. H.J. Muller was the first (in this, as in so many other procedures), Ed Novitski the grand master, and Loring Craymer the most recent (see Ashburner et al. 2004 for details and references). Deletions, duplications, and translocations are the classes of

rearrangement that you are likely to make, and of these, deletions are the most commonly needed. In general, rearrangements can be made from scratch (i.e., normal chromosomes) or from preexisting rearrangements. Usually the process requires using some kind of agent, such as radiation or chemical mutagens. In some cases, they can be made by exchange events between rearrangements. Above all, it is important to consult the Red Book (Lindsley and Zimm 1992), FlyBase (FlyBase Consortium 2003), and the stock collections to ensure that the desired chromosome does not exist already. The universal rule for making rearrangements is that it is always easier to start from some preexisting rearrangement.

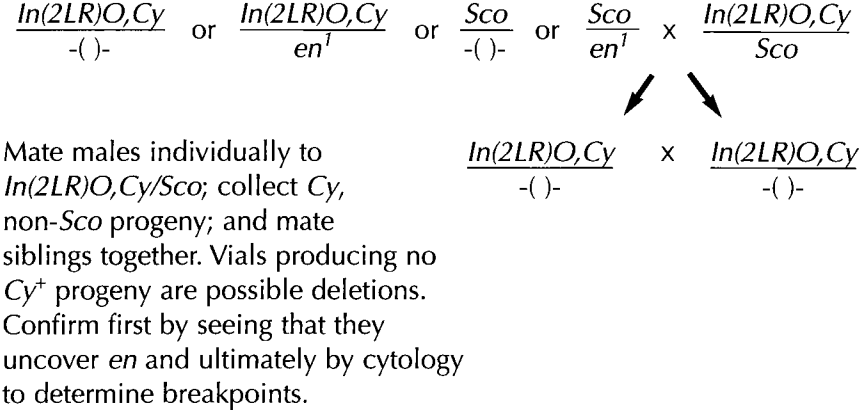
Inducing Deletions

The best way to induce deletions is to e-mail the Bloomington Stock Center and request that they be sent to you. When this is not possible, producing a deletion by radiation or chemical mutagenesis is very much like inducing a new recessive allele of a gene (see Chapter 2): Carry out a complementation test of the treated chromosomes. If you start with a wild-type chromosome and want to delete a region containing an easily scored, visible marker, simply treat normal males with the agent (radiation or chemicals), mate to females homozygous for the marker, and recover progeny displaying the marker phentoype.

Mutagenized males $\frac{+}{+}$ x $\frac{en^1}{en^1}$ en^1 is a viable allele of the engrailed locus, a locus that can mutate to lethality.

$\frac{en^1}{-(\ )-}$ Any progeny that look engrailed will have a newly induced allele or deletion symbolized by -( )-. If it is a deletion, it is likely to be homozygous lethal, testable by separating the two chromosomes, testing each for homozygous lethality, and retesting for failure to complement engrailed. For many loci, including *en*, new alleles are likely to be lethal themselves and can be handled the same way.

$\frac{en^1}{-(\ )-}$ x $\frac{In(2LR)O,Cy}{Sco}$



The scheme outlined above takes a shortcut that was eschewed in Chapter 2, namely, screening in the F1 generation. The rationale for this here is that deletions spanning more than one locus do not arise with great frequency and are almost always homozygous lethal. Thus, you can afford the trouble of separating out the chromosomes in the F2 generation because it allows you to throw out so many of the F1s. You may need to screen 10,000 or more mutagenized chromosomes. The limitation is that this approach only works with visible markers, but since deletions are hard to get, it is worthwhile trying to find a way to do it with visible markers.

A dominant allele at or near the locus you wish to delete works just as well. Here you screen for reversion of the dominant phenotype. This can also be done as an F1 screen. (Warning!! Not all dominant mutations are revertable. Dominants resulting from haploinsufficiency [e.g., *Ubx* or the class of mutants known as *Minutes*] are not. Those that are revertible represent gain-of-function mutations, hypermorphs, neomorphs, or antimorphs, discussed in Chapter 5.) Note that all *P[w+]* inserts are dominant if the fly is mutant at the *w* locus. Since these inserts are just about ubiquitous, it is now possible to "revert a dominant" almost anywhere.

Of the mutagens described in Chapter 2, radiation (specifically, X rays) has been most commonly used to induce deletions and is generally preferable to chemical agents in its reliability, though the frequency of recovering such deletions is on the order of 1–5/10,000 using 4000r. Up to half of the recovered X-ray-induced alleles at a locus are multilocus deletions (Pastink et al. 1987, 1988). Large ones are rarer than small ones. As in other kinds of mutagenesis, mature sperm are the most sensitive. EMS mutagenesis, traditionally thought to be a point mutagen, has been found over the years to produce many deletions as well. Many of these are small

and intragenic, an ideal way to generate a null allele (see Chapter 5) without taking out other genes.

P-element excision has also been widely used for producing small deletions. Imprecise excisions produce deletions that are generally small (up to 2 kb) and thus usually intragenic, but larger deletions also occur (see Ashburner et al. 2004 for references and details). The frequency of producing such deletions is as unpredictable as any P-element transposition event, depending in large part on the site of insertion.

Excision schemes are set up to detect loss of the wild-type allele of an eye-color marker such as w^+ in the P element, following the same general principles outlined earlier for induction of new mutations by P elements. For a lethal, w^+ insertion on chromosome *3*, the scheme would proceed as follows:

$$\frac{w}{Y}\ ;\ \frac{P[w^+]}{TM3,\ Ser} \times \frac{w}{w}\ ;\ \frac{Sb\ P[ry^+\ \Delta 2\text{-}3]}{TM6,\ Ubx}$$

↓

Select *Ser*$^+$ *Sb* males.

$$\frac{w}{Y}\ ;\ \frac{P[w^+]}{Sb\ P[ry^+\ \Delta 2\text{-}3]} \times \frac{w}{w}\ ;\ \frac{TM6,\ Ubx}{Sb}$$

↓

Select *w*, *Sb*$^+$, *Ubx* males.

$$\frac{w}{Y}\ ;\ \frac{-(\ )-}{TM6,\ Ubx} \times \frac{w}{w}\ ;\ \frac{TM6,\ Ubx}{Sb}$$

↙ ↘

Mate to balancer stock, select heterozygous excision progeny (*w*, *Sb*$^+$, *Ubx*) and mate to test for lethality.

$$\frac{w}{Y}\ ;\ \frac{-(\ )-}{TM6,\ Ubx} \times \frac{w}{w}\ ;\ \frac{-(\ )-}{TM6,\ Ubx}$$

The frequency of obtaining such P-element excisions depends on both the sequences in the P element and the site of insertion. The ratio of precise to imprecise excisions, which will influence the recovery of deletions, also varies but tends to favor those that are imprecise. Of those that are imprecise, however, most are not deletions but rather excisions that have left behind a piece of the P element. Thus, the induction of new deletions may be as rare as 0.1% and as common as 10% of chromosomes screened. The frequency of imprecise excisions can be increased if the chromosome bearing the P element is unable to pair with its homolog and use it as a template for repair (Engels et al. 1990). The virtue of the genet-

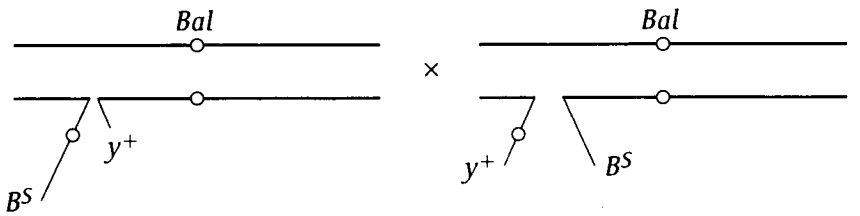
ic screen outlined above allows you to score simultaneously for excision (loss of w^+) and lethality.

Your ability to recover a deletion will depend on the loci being uncovered. If the region you wish to delete contains a haplolethal locus, you are in big trouble. If the region contains some other kind of haploinsufficient locus, such as sterility, you will also have difficulty. Even if it only contains a locus that is unhealthy in one dose, such as a *Minute* locus, you will have a hard time. (*Minutes* are a class of loci, encoding ribosomal proteins, sprinkled around the genome, that have a similar hemizygous phenotype: thin bristles, retarded development, and small size. In addition to being haploinsufficient, they are also recessive lethal. See Ashburner et al. 2004 for a detailed discussion.)

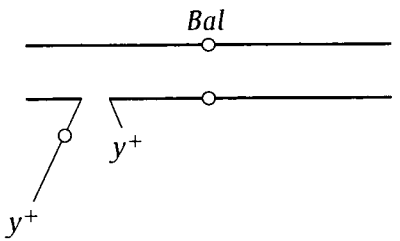
Synthesizing Stable Deletions from *T(Y;A)*s

In keeping with the principle that it is better to make rearrangements from preexisting ones, the *T(Y;A)*s described above (see Synthesis of Duplications and Deletions in Chapter 3) are sometimes a good starting point for making deletions.

One generates flies carrying a deletion by crossing two *T(Y;A)* stocks bearing different breakpoints (different points along the autosome and different arms of the *Y* so that the y^+ and B^s markers are oppositely arranged) as follows:

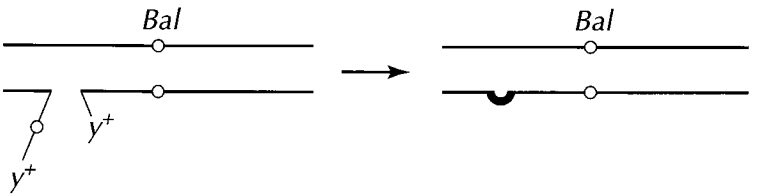


Both of these stocks look y^+ and B^s. The deletion-bearing progeny will lack B^s and will have two doses of y^+.



It is possible to collect males and virgin females of this y^+ B^+ genotype and set up a stock. However, such a stock does not thrive because many aneuploid progeny are produced. Since you usually want a deletion for screening new mutations or for some other use that requires large numbers of flies, the poor viability of the stock is a liability.

With irradiation, it is possible to reattach the autosomal pieces in this stock to produce an intact, stable deletion. An event of this sort will occur much more frequently than induction of a deletion de novo because the arms of the *Y* present a large target and you are inducing an exchange event between them. Since it is necessary to have both autosomal pieces of the *T(Y;A)* present in the cell that receives the radiation, this is done in females and at a much lower dose than that used in males, 1500r, to reduce damage to the oocyte.



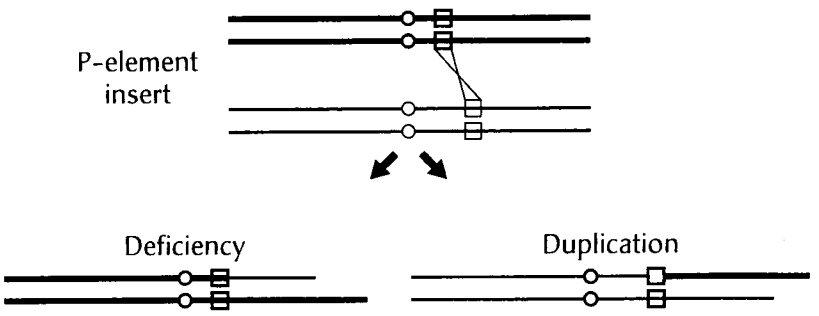
It is sometimes easier to recover such reattachments by using a selection scheme based on the restoration of fertility with the reattached chromosome to an otherwise sterile genotype when it is unattached (Lyttle 1984).

Select reattachment easily by scoring loss of y^+ in flies otherwise mutant for *yellow* on the *X* (which is already true for the *T(Y;A)* stocks). Simply collect females from the unhealthy, synthetic deletion stock, irradiate them, mate them, and look for *y* progeny. Since the stock is balanced anyway, the new chromosome comes out balanced. It can then be tested to confirm that it is truly reattached by mating to one of the parental *T(Y;A)* stocks and seeing that no aneuploids are recovered, i.e., no separation of y^+ and B^s occurs.

Synthesizing Deletions from P-element Insertions

P elements have brought a new level of rationality to the generation of deletions. Because their chromosomal position can be accurately determined, they provide identified targets for rearrangement events. Since they can be mobilized to insert at new sites easily by a simple cross (see Mutagenesis, Chapter 2), new insertions can be readily obtained. More importantly, there is a nonrandom probability that transposition will occur

to a site relatively close to the original site. This means that if a mechanism existed to catalyze chromosome breakage and rejoining at these P-element insertions, a technique could be developed for the rational synthesis of deletions and duplications with known end points by unequal crossing-over.



Golic (1994) devised a strategy for inserting P elements into sites of interchromosomal recombination. He introduced target sites for the yeast flip recombinase target (FRT) sequence into P elements and made transformants carrying these sequences at various sites. (The use of this technique for analyzing time and place of gene action is discussed in Chapters 5 and 6.) He made use of the fact that when an inserted P element is transposed, it has a high probability of moving to a new site in the same general vicinity of the chromosome. When two homologs have such inserts in the same vicinity and the FLP recombinase is induced, it can catalyze an unequal recombination event between the homologs. The result is a deletion and the reciprocal duplication with known end points. (Induction of the recombinase is accomplished by heat shock of the *hsp70* promoter driving the *FLP* gene.)

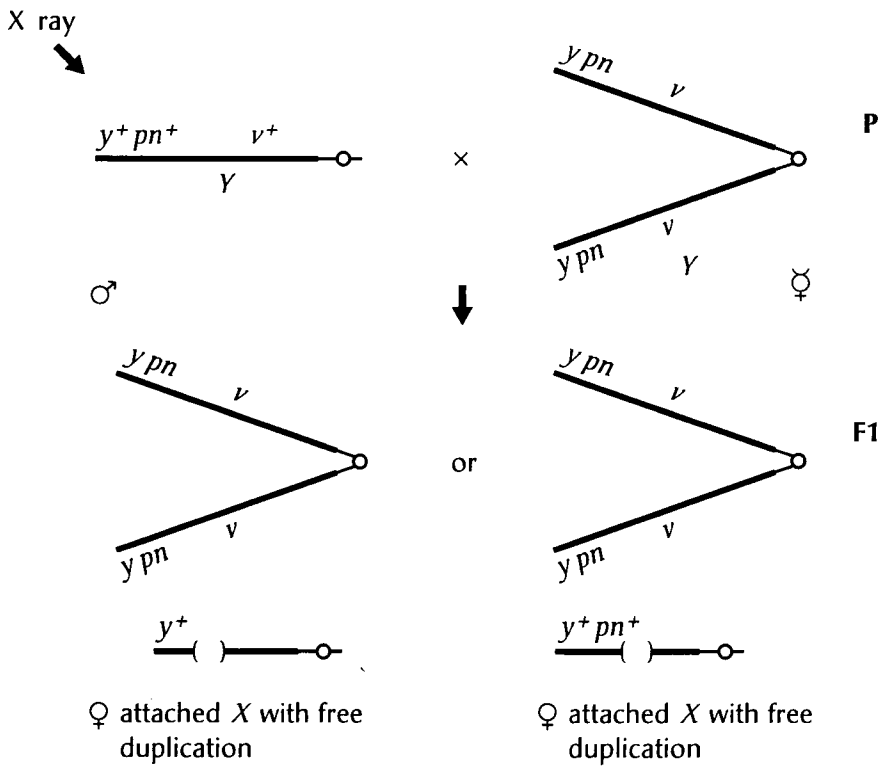
The frequency of obtaining transpositions varies with the location and composition of the starting insert. Golic obtained a transposition rate ranging from 18/97 G_1 males (F1 progeny of the cross between the initial P element and the *Δ2-3* transposase) to 97/104 G_1 males. Of those, 86% went to nearby regions of the chromosome. When set up as heterozygotes with inserts at different but nearby sites (approximately 30 bands apart), the frequency of obtaining deletions and duplications was 0.7%. Although not overwhelming, it is significant and has the advantage of producing rearrangements with known, nearby breakpoints.

Techniques for producing new deletions using P-element-induced male recombination and *hobo*-mediated transposition are described in Chapter 2 as part of the discussion of mapping with deletions.

Inducing Duplications

Stable duplications sometimes arise when a piece of chromosome is cut out during X-ray induction of deletions. The excised fragment is sometimes inserted into a site on another chromosome (or elsewhere on the same one). When the chromosome with the inserted piece segregates away from the excised chromosome, a reciprocal duplication and deletion forms. This approach, however, is not one to use when looking deliberately for a particular duplication.

Free duplications of the *X* are the easiest to induce. These are small pieces of the *X*, retaining its centromere and the distal tip with the y^+ locus on it. The technique is basically to chop out a piece of the *X* to create a freely segregating minichromosome detectable by the dominance of y^+ on a *y* background (i.e., in a female with $\widehat{XX}$ *y pn v*). By retaining this marker, it is easy to select for such "free duplications" since flies better tolerate large duplications of the *X* chromosome, especially in females, than of autosomes. The basic idea is illustrated below.



Two of many possible outcomes are a y^+ duplication and a y^+pn^+ duplication; each represents possible female genotypes in progeny. The free duplications survive if they are not full-sized *X* chromosomes. They can then be assayed for other loci they cover by mating to males with a multiply marked *X*.

Selection schemes can be designed for duplications based on their ability to rescue the phenotype of a haploinsufficient locus. A balanced haploinsufficient locus will appear in all nonbalancer progeny unless another wild-type dose of the locus is present. Thus, irradiating wild-type flies and mating to a balanced haploinsufficient locus will reveal the presence of a new duplication in any nonbalancer, non-haplo-insufficient progeny.

Tandem duplications—side by side on the original chromosome—can be obtained with reasonable probability (~1/10,000) by irradiating normal chromosomes in females instead of males with a lower dose of radiation, 1500r (Ashburner et al. 2004). This presumably results from the induction of an unequal exchange event between the two homologs, both of which are present in the irradiated oocyte.

The technique of P-element-induced male recombination is an alternative strategy for making new duplications (Preston et al. 1996). These duplications are produced in the same way as the corresponding deletions described in Chapter 2, retaining one of the P element's end points.

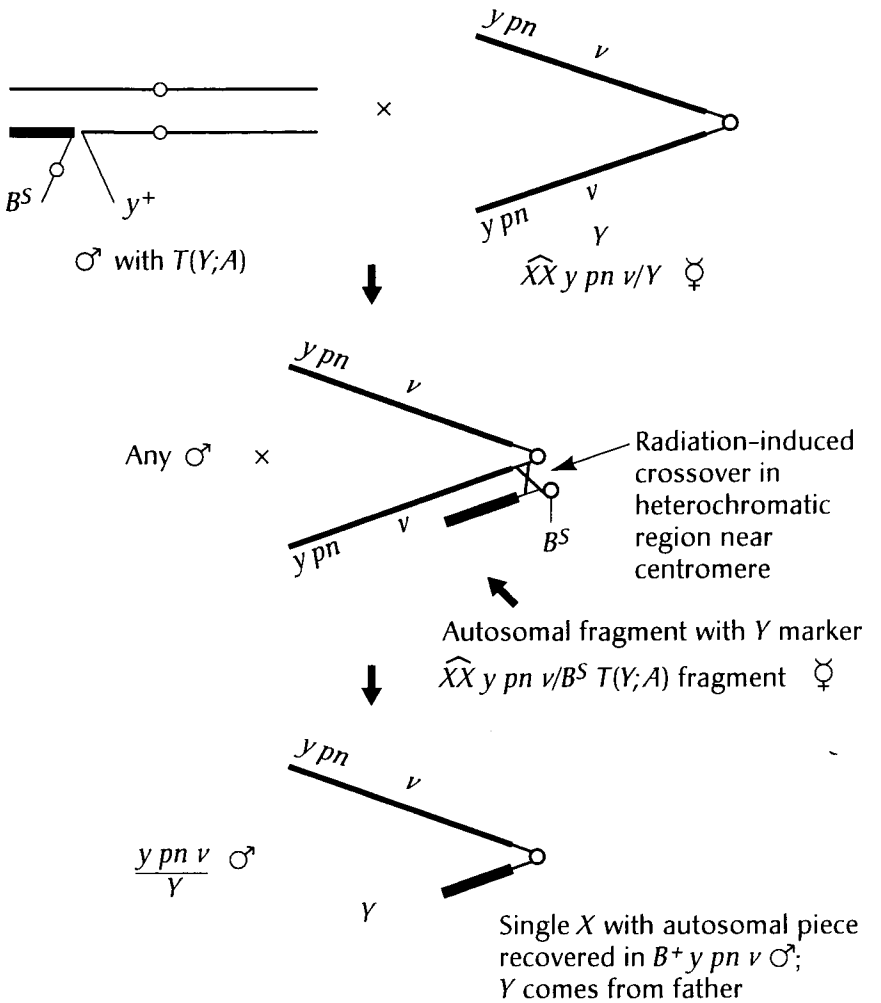
Synthesizing Stable Duplications from *T(Y;A)*s

For duplications, no direct counterpart to the resealing of *T(Y;A)* pieces works as well as for deletions. However, *T(Y;A)*s whose breakpoints are relatively near the end of a chromosome arm can be used to attach an autosomal piece onto the *X* chromosome in a fairly reliable manner.

This technique detaches an attached-*X*, an exchange event between the *Y*-derived heterochromatic portion of a *T(Y;A)* and the heterochromatic regions near the centromere of an attached-*X* chromosome, helped along by a little radiation. Heterochromatic regions have rough homology with one another and will recombine with a low probability (~1/1000) that can be boosted by radiation.

Most attached-*X*s are not suitable for this maneuver, since they are generally deleted or rearranged for most or all of their centric (centromere-linked) heterochromatin. There is good reason for this: Since attached-*X* stocks are usually kept with a free *Y* chromosome, they would detach spontaneously if heterochromatic pairing and exchange could

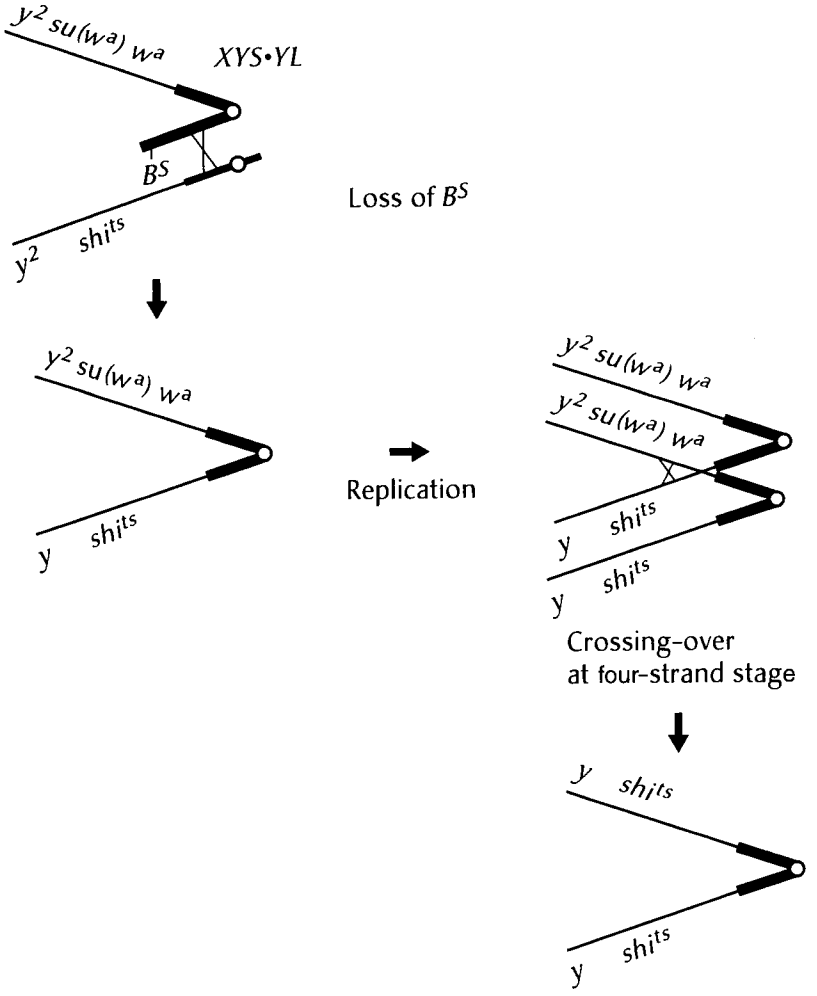
occur. The particular attached-*X*s suitable for this technique retain their centric heterochromatin, and are thus not appropriate for normal stock maintenance, e.g., *C(1)RM, y pn v*. You simply cross the *T(Y;A)* into that attached-*X* stock, irradiate females, and mate them to unmarked males.



The resulting product is an *X* chromosome linked to the terminal fragment of an autosome. It is recognizable because males will not receive the recessive markers on the attached-*X* unless there has been a breakdown. Thus, one looks for a male with *y pn v* but not B^S (or y^+, depending on the *Y* breakpoint). The size of duplication that will be viable depends on the autosomal arm from which it comes (see above).

Synthesizing Attached-*X*s

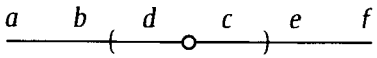
A new attached-*X* can be made in the same way just described for taking one apart. You would do that if you wanted to make an attached-*X* with, for instance, a temperature-sensitive lethal mutation on it so that the stock could be made to conditionally produce only males (the opposite of a virginizing stock). The strategy is to start with an *X* chromosome with the desired mutation on it and then allow it to recombine with an *X* joined to a *Y* marked with B^s (called *XYS* • *YLB*^{s} and carrying y^2 *su*(w^a) and w^a).



The loss of B^s is easily monitored in individual female progeny who are then used to start separate strains. An attached-*X* with arms heterozygous for different alleles is inherently short lived, since crossing-over takes place after replication and will occur freely, making various homozygous combinations of the differing markers. Here, you would again start strains from individual female progeny and test them for presence of the temperature-sensitive paralytic mutation, *shibire-temperature-sensitive* (shi^{ts}).

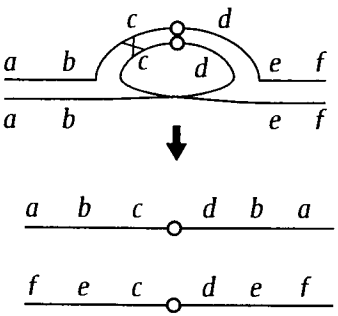
Autosynaptic Chromosomes and the Joys of Gibberish

One of the more esoteric backwaters of fly genetics is the synthesis of duplications and deletions from that class of inversions spanning the centromere, known as pericentric inversions.



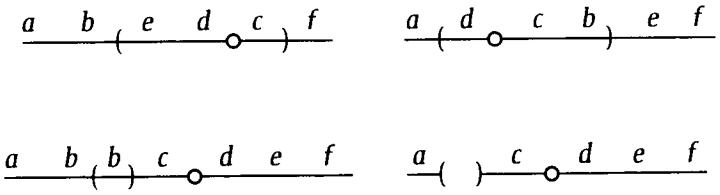
The meiotic behavior of inversion chromosomes has held a strange fascination for chromosome mechanics aficionados ever since Sturtevant and Beadle's (1936) classic and voluminous study of crossing-over in inversion heterozygotes.

Pericentric inversions are relevant to the construction of aneuploids because exchange in a heterozygote produces reciprocally duplicated and deleted products.



If one has access to more than one pericentric inversion of this sort, where breakpoints of one are somewhat displaced from those of the other, it is

possible to generate tandem duplications and deletions of the region of chromosome that is displaced between the two.



This occurs by a series of exchange events, the details of which are spelled out in Craymer (1981, 1984) and Ashburner et al. (2004). The technique has the virtue of being rational and deliberate, but it is limited by the availability of appropriate pericentric inversions. They too can be synthesized (see the same references), but not with great ease.

CHAPTER 5

Analysis of Mutations I. Characteristics of the Allele

ONE OF THE MAIN REASONS for doing fly genetics is, after all, the analysis of mutants. With this in mind, two main kinds of genetic tests are carried out: The first tests the phenotypic nature of the mutant defect and the second tests the cellular site of action of the gene. The first is dealt with in this chapter, the second in Chapter 6. An obvious prerequisite to the analysis of alleles is that you must have one or more of them. In fact, as you will see, the more the better.

FORMAL CLASSIFICATION OF MUTATIONS

The history of mutational analysis begins, as do so many aspects of fly genetics, with H.J. Muller. Decades before anyone had a clue regarding the physical basis of the gene, Muller realized that much could be inferred about the nature of gene action from comparing the phenotypes and interactions of different alleles. In the late 1920s and early 1930s, when he was developing these ideas, the primary goal was to understand what a gene actually was. In the 1950s and 1960s, this approach became a mainstay of microbial genetics and had a particularly important role in early studies of gene regulation in bacteria. Today, the same analysis turns out to be extremely powerful in analyzing how a gene acts and has been used repeatedly to unravel developmental pathways in flies and worms.

Principles

The overall strategy is based on examining the range of phenotypes displayed by a series of alleles. Genes can mutate to either "loss-of-function"

or "gain-of-function" phenotypes. Under ideal circumstances, the available alleles span the range from complete deletion of the gene (the ultimate loss of function), through varying degrees of partial function, to alleles that confer new and different phenotypes. One does not necessarily know a priori which of these are which, but straightforward genetic tests can characterize each type of allele.

An obvious requirement of this approach is that the phenotypes of the various classes of mutations be distinguishable in terms of either severity or qualitative effect. Comparisons can then be made by means of complementation tests and varying the dosage (number of copies) of the gene.

This kind of analysis does not magically disclose everything you may want to know about the gene that you are studying. There are few hard and fast rules for the molecular correlates of a particular, formal class of mutations. Much depends on the actions and interactions of the protein in question. However, the analysis can tell you quite a bit about the realm in which a gene acts. For example, if the only alleles of the *even-skipped* gene that existed were those that completely eliminated the gene's function (a "gene knockout," as mouse investigators are wont to say), it would never have been called "*even skipped.*" Read on.

Null Alleles (Amorphs)

A mutation that completely eliminates a gene's function is a "null" allele or, in Muller's original parlance, an "amorph." In molecular terms, this can be caused by any lesion that completely blocks trancription or translation of the gene, as well as many nonsense and missense mutations. Null alleles are generally the most likely kind of mutation to be isolated, presumably due to the fact that there are many more ways to wreck a protein's ability to function or block its expression than there are to make subtle alterations in it. In organisms in which it is easy to isolate multiple alleles, such as the nematode, the phenotypic class of mutants that is recovered most frequently is tentatively designated as the null phenotype. (This assumes that the mutant screen is not biased in favor of a particular kind of allele, such as demanding viable alleles of a gene that is lethal when null.)

A deletion of the locus is, of course, the quintessential null allele. Deletions are characterized on the basis of cytologically determined breakpoints in the chromosome and thus they become the basis for an objective genetic test against which any allele can be compared. This provides a solid standard for all subsequent characterizations and does not

begin by depending on any particular phenotype.

The formal definition of a null allele is that it behaves no differently from a deletion of the locus in complementation tests. "Behavior" in this context refers to the phenotypes produced when combined with other alleles. A homozygous null allele should give the same phenotype as the null allele over a deletion (i.e., hemizygous):

$$\frac{null\ allele}{null\ allele} = \frac{null\ allele}{Df} = \frac{Df}{Df}$$

These should be no different from the deletion homozygote, provided the deletion does not also uncover a lethal or another gene with an obvious mutant phenotype. ("Uncover" in this context is fly jargon for failing to complement.) Homozygous deletions are usually lethal and therefore this particular comparison usually requires that the mutation itself is lethal. What they all have in common is the lack of any functional gene.

You can be misled in other ways in this kind of analysis. For example, you would mistakenly conclude that your mutant is not null if you obtained a more severe phenotype with the deficiency because of a second mutation present on the *null*-bearing chromosome that interacts dominantly with the deficiency (stranger things have occurred). Alternatively, if the phenotype of a null allele is indistinguishable from the phenotype of an allele with 10% of normal activity ("Hypomorphs"; see below), there is no way to tell the difference.

It is not always true, however, that a missense mutation resulting in an inactive protein will be equivalent to a deletion of the locus. If the protein is a subunit in a multimer, the presence of dead subunits can sometimes "poison" the multimeric complex—a situation that would not occur with a deletion of the gene. This serves only to underline the fact that the formal characteristics of a mutation depend on the actions and interactions of the protein in question.

Hypomorphs

Mutations that produce a partial loss of function are called "hypomorphs." At the molecular level, these usually consist of lesions that reduce the level or efficiency of the gene product. This can occur at the level of transcription; transposable element insertions frequently reduce transcription as a consequence of their disruption of 5′ untranslated regions (Ashburner et al. 2004). Similarly, a reduction can occur at the level of protein func-

tion as a consequence of missense or nonsense mutations that reduce protein activity or increase turnover.

Hypomorphs produce a graded series of phenotypes with increasing copy number. That is, the homozygous state is somewhat less severe than the hemizygous state (hypomorph over deletion), and the hemizygote is somewhat less severe than the homozygous null allele (or deletion). The order of phenotypic severity is thus

$$\frac{hypomorph}{hypomorph} < \frac{hypomorph}{Df} < \frac{Df}{Df}$$

or

$$\frac{hypomorph}{hypomorph} < \frac{hypomorph}{null} < \frac{null}{null}$$

These correspond to two, one, and zero doses of the allele, respectively. In some instances, it is possible to construct a duplication containing the mutant allele (see Chapter 4) and so construct flies with three and four doses of the hypomorph to demonstrate the increased rescue of the phenotype with additional doses. This can be done most readily with P elements carrying the wild-type locus.

In most instances, hypomorphic mutations produce phenotypes that differ only in degree from the null. The simplest and earliest examples are eye-color mutations. A more interesting contemporary example is the *Toll* maternal-effect locus in which the null allele results in a fully dorsalized embryo and various hypomorphs produce a graded series of partially dorsalized embryos. Sometimes, however, as in the case of *even-skipped*, a hypomorphic phenotype is qualitatively distinct from the null phenotype. Null alleles of *eve* produce an unsegmented lawn of denticles, whereas the phenotype of missing even-numbered segments, originally so aesthetically pleasing, is a hypomorphic phenotype. Thus, even qualitatively distinct hypomorphs can still represent a graded expression of the gene, especially if there is a threshold for the transition from one phenotype to another.

Temperature-sensitive mutations are usually hypomorphs. A case in point is the heat-sensitive allele of the *no-receptor-potential-A* (*norpA*) locus. At restrictive temperature, the $norpA^{H52}$ mutant produces no electrical activity in the eye (Deland et al. 1980). At permissive temperature, there is detectable electrical activity but, as revealed by intracellular

recording, the normally synchronous electrical potentials are splayed out over a longer time course. This illustrates that the gene product is involved in an intermediate step of phototransduction, as opposed to the initial light absorption or final channel opening. Partial-function alleles can thus be more informative than null alleles in some instances, but it is always necessary to know the null phenotype as a baseline. Temperature-sensitive alleles are always rare and thus it is necessary to isolate a series of alleles.

A temperature-sensitive hypomorphic phenotype does not always mean altered protein structure due to missense mutation. Read-through of a nonsense codon can be a temperature-sensitive process (Samson et al. 1995), depending on the sequence immediately following the stop codon (Chao et al. 2003). Also, depending on the thermal sensitivity of a particular biological process, a reduced level of a particular protein can produce a temperature-sensitive phenotype. The sodium channel gene *paralytic* (*para*) illustrates this principle. The original heat-sensitive allele turns out to be a transcriptional hypomorph that produces a reduced level of gene product, in which electrical conduction fails at high temperature (Kernan et al. 1991). This is presumably a result of an alteration in the balance between the opposing ion currents that produce depolarization and hyperpolarization in the neuron. In the same manner, an insertional mutation can be temperature sensitive, such as the case of the original *bithorax* allele, bx^{34e} (isolated by Calvin Bridges in *34e,* the fifth month, May, as symbolized by the fifth letter, e, in 1934). Temperature-sensitive nulls also exist (e.g., *csp*; Zinsmaier et al. 1994). These are more properly referred to as temperature-sensitive phenotypes rather than temperature-sensitive alleles.

Germ-line transformants often produce hypomorphic phenotypes because it is difficult to achieve fully normal expression for many cloned and reintroduced genes. The hypomorphy is generally due to the lack of particular *cis*-acting controlling elements, resulting in reduced transcriptional efficiency or missing domains of expression. The lack of such flanking sequences can also make the insert more sensitive to "position effects"—influences of surrounding sequences and controlling elements on its expression. Many transformants for the period locus, governing biological rhythms, produce a "long-day" phenotype with a free-running circadian rhythm of up to 27 hours instead of 24 (Hamblen et al. 1986). These resemble both heterozygous deletions of the locus as well as the per^{long} alleles isolated in standard mutant screens and shown by tests over a deletion to be hypomorphs.

Hypermorphs

Mutations that produce an excess of the normal gene product or a hyperactive version of the protein represent one kind of gain of function and are called "hypermorphs." The molecular phenotype of such mutations is the converse of hypomorphy. Transcription may be increased or a missense mutation may increase the efficiency of a protein's function.

The diagnostic test for a hypermorph is that it can be corrected, or at least ameliorated, by being placed heterozygous with a deletion or with a null allele. The order of phenotypic severity would thus be

$$\frac{hypermorph}{hypermorph} > \frac{hypermorph}{+} > \frac{hypermorph}{Df}$$

When the hypermorphy is not extreme, the phenotype is sometimes approximated by duplications of the wild-type locus. The *Confluens* (*Co*) "allele" of the *Notch* locus falls into this category. *Co* is similar to a N^+ duplication, producing thickened wing veins; *Co/Df* is approximately wild type. *Co* actually turned out to be a tandem duplication of the *Notch* locus, correctable by unequal crossing-over to restore euploidy.

Neomorphs

Mutations that produce a novel function are called "neomorphs." Inconsistency is common in kinds of molecular lesions that produce this second type of gain of function. In contrast to hypermorphs, these mutations are most easily defined by the fact that they are not ameliorated when placed hemizygous with a deletion. Nor can they be fully rescued by addition of extra doses of the wild-type allele. They are distinguished by their novelty.

Neomorphy can be produced by ectopic expression of a gene, such that it appears at times or in places in which it does not belong. Such transcriptional lesions usually result from chromosome rearrangements that juxtapose new promoters or enhancers with the gene in question. The original *Antennapedia* mutation was the result of a rearrangement that induced expression in the head of a transcript normally found in the thorax (Schneuwly et al. 1987). Unregulated activity at the protein level can also produce neomorphy. This is the case for many human oncogenes and for mutations such as the constitutively active mitogen-associated protein (MAP) kinase of the *rolled*^{Sevenmaker} allele, which produces multiple R7 photoreceptors (Brunner et al. 1994).

Complementation tests for neomorphic mutations are problematic. Since they are not ameliorated (or worsened) when combined with a deletion, it is often impossible to see any effect when the homologous chromosome carries another mutant allele of the gene. They are, however, usually dominant for some phenotype. Thus, a good strategy for obtaining null alleles is to revert the dominant phenotype. The result is almost always a null allele (especially when done with radiation) that is thus easier to map by testing for complementation with other alleles.

In cases for which the null phenotype of the locus is not already known, reversion of a neomorph can be extremely informative, as shown with the aforementioned *Antennapedia* mutation. When subject to chemical (EMS) mutagenesis, revertants of the dominant *Antennapedia* allele produce recessive lethal alleles that show a reciprocal phenotype: transformation of leg tissue into antenna (Struhl 1981). (Since the new alleles are lethal, their effect on leg phenotype had to be studied in mosaics. See Chapter 6.)

An informative exception to the rule of obtaining nulls from reverting dominants is the case of *Sex-lethal* (*Sxl*), a gene that occupies a key place in the sex-determination hierarchy. The original allele, $Sxl^{f\#1}$, which proved to be a null, is lethal when homozygous in females, whereas a dominant allele, $Sxl^{M\#1}$, which proved to be constitutively active, is lethal to males (Cline 1978). Reversion of the dominant by chemical mutagenesis yielded several new alleles whose properties were crucial for making sense of *Sxl*. These alleles, recessive but not null, dissociated the initiating function that *Sxl* plays in setting off the female pathway of development from its autoregulatory function in maintaining that determined state in cells (Cline 1984).

Problem 9 Design the genetic crosses to recover revertants of the neomorphic *Contrabithorax*, a third-chromosome, dominant, viable variant of the *Bithorax* complex. Be sure that you can reliably recover reverted chromosomes even if they have become recessive lethal or confer no obvious phenotype at all.

Antimorphs

An "antimorph" is a mutation that actually antagonizes the wild-type allele. It is distinguished from a neomorph by the fact that it can be rescued, at least in part, by additional doses of the wild-type gene. "Poison" subunits, mentioned earlier as an exception to the equivalence between null alleles and deletions, fall into this category.

Some of the *Abruptex* (*Ax*) alleles of the *Notch* locus are antimorphs in which homozygotes are viable and heterozygous combinations of alleles are lethal. *Ax* produces shortened wing veins, short and thin wings, and clumped bristles. For some alleles, the defect is corrected in *Ax/Df* but not exactly approximated by extra doses of N^+. This makes sense in the context that the Notch protein is thought to act as a cell-surface receptor for signal transduction and the *Ax* mutations behave as if constitutively activated (Kelley et al. 1987). Extra wild-type proteins would not be expected to act this way.

Gain Versus Loss of Function

Not all genes are capable of mutating to all possible "morphs." Some will never produce obvious gain-of-function phenotypes and others will never produce obvious loss-of-function phenotypes. It all depends on the mode and site of action of the gene's product.

Genes that are present in multiple copies (i.e., more than one per haploid genome) may be detectable only as gain-of-function mutants. This may be the only way to stand out from the crowd. Such hypermorphs or neomorphs, when reverted, may result in the infamous "no phenotype" phenotype (cf. Hall 1994), since they are well buffered by their cognate comrades. Conversely, of the many extant eye-color genes of the fly, most of which are enzymes of pigment synthesis and deposition, few have ever been mutated to gain-of-function phenotypes.

Gain-of-function alleles often show phenotypes reciprocal in some fashion to those of loss-of-function alleles. When they do, it usually means that the gene product plays a regulatory role. The molecular nature of these gene products can range from receptor-like proteins, such as torso (Klingler et al. 1988; Sprenger et al. 1989) or Notch (Palka 1990; Lyman and Young 1993), to transcription factor-like proteins such as Antennapedia (Struhl 1981; Levine et al. 1983), or splicing factor-like proteins such as Sex-lethal (Cline 1984; Bell et al. 1991).

Intragenic Interactions

Back in the Cambrian period of premolecular genetics, much attention was paid to anomalous patterns of complementation as perhaps important clues to the nature of the gene. These "complex loci" were so called because of a characteristic complementation between some allelic combinations but not others.

For example, $\frac{m^1}{m^2}$ and $\frac{m^2}{m^3}$ failed to complement, but $\frac{m^1}{m^3}$ did complement.

Three of these loci, the *bithorax* region (Lewis 1963), now known to be the *Ubx* transcription unit; *Notch* (Welshons 1965); and *rudimentary* (Carlson 1971), were studied in great genetic detail, including fine structure (recombinational mapping of alleles within the gene). These studies succeeded in grouping some of the mutant phenotypes into particular regions of the gene, in some cases showing that alleles that complemented one another were nonetheless separable by recombination. This apparent paradox—inexplicable at a time when genes were thought of as units of structure as well as of function—was termed pseudoallelism but offered no compelling explanation of the phenomena. Cloning these loci and identifying their gene products revealed that each one was a single, large transcription unit, although it also turned out that the *Bithorax* complex was authentically complex and consisted of two more, adjacent transcription units (Lewis 1985). The complex complementation patterns resulted from separable functions within the unit or even within the protein.

"Negative complementation" was another anomaly much puzzled over in the precloning days of genetic analysis. Here, a heteroallelic combination of mutations produced a more severe phenotype than either homozygote.

For example, $\frac{m^1}{m^2}$ was worse than either $\frac{m^1}{m^1}$ or $\frac{m^2}{m^2}$.

Behavior of this sort was observed for the *Abruptex* alleles of the *Notch* locus (see above and Welshons 1965) and was interpreted as resulting from the interaction of subunits.

INTERACTING LOCI

So far, all of our examples of gene analysis have pertained to interactions between different alleles of the same locus. Some of the most interesting doors that genetic analysis can open are those pertaining to the interaction between loci, assayed in double mutants. The construction and testing of double mutants is a tried and true technique originating from the early days of microbial genetics for determining if an interaction between two genes or gene products occurs. In its simplest form, you construct a strain producing individuals mutant at two loci (see Manipulating Two

Chromosomes in Chapter 4) and compare the phenotypes of each mutant separately with that of the double mutant. If you are careful to control for genetic background and you can measure an accurate quantitative phenotype, more subtle effects of gene interactions can also be revealed in double heterozygous combinations of mutations (Fedorowicz et al. 1998).

The nature of the interactions detected from any of these approaches can vary from direct physical contact between protein products to indirect contributions to a common end point. For this reason, such interactions must be interpreted carefully. Criteria such as allele specificity (i.e., the interaction is only apparent between certain alleles) are one way of verifying the directness of an interaction.

"Epistasis," a term frequently used in studies of gene interaction, means different things to different people (Phillips 1998). Early in the history of genetics, it signaled that when two mutants with distinguishable phenotypes were combined in a double mutant, one mutation masked the effect of the other. Later on, during the development of quantitative genetics, it meant that when two mutations were combined, usually tested as heterozygotes, their effect on the quantitative phenotype was nonadditive.

Ordering Genes in a Pathway

The pathway analogy is a popular metaphor for describing the actions of genes involved in development. The paradigm for this model is the classical biochemical pathway, in which a set of gene products (enzymes) carries out sequential transformations of metabolic precursors. Metabolic pathways played a pivotal role in the history of theories on the nature of the gene, leading to the elaboration of the "one gene–one enzyme" hypothesis from work on biochemical mutants in *Neurospora*.

With the growth of studies in signal transduction, second messengers, and their membrane and cytoplasmic components, the pathway analogy has taken on a new incarnation. Interactions between growth factors, their tyrosine kinase receptors, and their intracellular targets have been routinely described in terms of biochemical pathways, and genetics made the critical connection between these elements and development. Interactions between mutations affecting the same developmental event made it possible to order the steps defined by each mutation—first in the nematode *Caenorhabditis elegans* and later in the fly (Avery and Wasserman 1992; Goldstein and Fyrberg 1994).

The most straightforward test is for the interaction between gain- and loss-of-function mutations in different genes of the same pathway. If a

step, such as a kinase, is normally active only when stimulated by another gene product, then a gain-of-function mutation in the kinase relieves it of the stimulus requirement and makes it constitutively active. Loss-of-function mutations of genes that act before the kinase have no effect on its constitutive activity; the kinase mutation is epistatic (from the Greek for "stand above") to the prior-acting mutation. On the other hand, loss-of-function mutations of genes that act subsequent to the kinase block the gain-of-function phenotype by preventing the constitutive signal from reaching the end of the pathway. These, in turn, are epistatic to the kinase mutation.

Examples of this kind of interactive behavior have been obtained in studies of cell-fate determination in photoreceptor R7 (i.e., *sevenless* and its friends; e.g., Brunner et al. 1994) and in the establishment of the terminal patterning system in the embryo (*torso* and its colleagues; e.g., Ambrosio et al. 1989). In many cases, the discovery of the interacting locus is the result of mutant screens for "suppressors" or "enhancers" (e.g., Simon et al. 1991). These names refer to cases in which a newly isolated mutation in one gene ameliorates the phenotype of a previously isolated mutant (suppressor) or exacerbates the original phenotype (enhancer). In other instances, interactions are discovered simply by making double mutants between different loci affecting the same ultimate phenotype or process.

Screens for suppressors or enhancers are simple if one screens for new dominant mutations. Because of their dominance, the new mutations appear in the F1 generation. This makes their recovery and propagation easier, while you are waiting to map them (see Chapters 2 and 3). For example, the screen that turned up a constitutive MAP kinase in the *sevenless* pathway (Brunner et al. 1994) started with a strain mutant for the *bride-of-sevenless* (*boss*), the extracellular ligand that activates the *sevenless* pathway (Reinke and Zipursky 1988).

Mutagenize ♂♂ w^{118}, $boss^{3991}$ x w^{118}, $boss^{3991}$ ♀♀

Progeny of this cross were tested in a behavioral assay requiring intact R7 photoreceptors. The resulting mutation, *Sevenmaker*, turned out to be a dominant allele of the *rolled* locus (rl^{Sem}) and was subsequently found to encode a MAP kinase (Biggs et al. 1994). Since the mutant screen demanded viability from the very start, the researchers obtained a viable allele of *rl*, the locus for which nulls are lethal (Hilliker 1976).

Recessive enhancers or suppressors are more difficult since they

require homozygosing of mutagenized chromosomes in the presence of the starting mutation.

Problem 10 Design a screen for recessive suppressors of *Sco* (35B2).

Dosage-dependent Interactions

The genetic strategy of identifying interacting loci as a way of defining additional important contributors to a biological process has broader applicability than just pathway analysis. The pathway analogy, although of unquestioned heuristic value, fails to incorporate the nonlinear nature of many gene interactions. Gene interactions, on the other hand, occur in all sorts of situations and offer a way to begin unraveling mechanisms that might otherwise be intractable.

Trans-regulatory genes fit this description. Transcription factors that must form complexes or that compete for DNA sites with other such factors may be sensitive to dosage (defined as changes in copy number of a wild-type locus). If dosage alteration causes no overt phenotypic change, it can often sensitize the fly to dosage changes at other loci. By definition, these other loci interact with the first (though not necessarily in a direct fashion).

Botas et al. (1982) used this approach to identify the *extra macrochaetae* (*emc*) locus on the basis of its interaction with genes of the *Achaete-scute Complex* (*AS-C*). Years later, the emc protein was found to antagonize the binding of an AS-C protein complex to specific DNA sites (Van Doren et al. 1991). The existence of emc was revealed by mutagenzing flies that carried extra wild-type doses of AS-C (an insertion into the second chromosome of the *X*-chromosome region containing the locus *Dp[1;2] sc*^{19}) and screening for progeny with extra bristles. The idea was that extra doses of the normal *AS-C* genes would throw the system off balance, making it more sensitive to alterations in the quantity (dosage) of interacting gene products. The strategy worked: The new *emc* mutation was recessive for its effects on a normal genetic background and dominant on a background of extra copies of *AS-C*. The specifics of the cross were

Mutagenized males $\quad \frac{y}{Y}\,;\,\frac{Dp(1;2)\,sc^{19}}{Dp(1;2)\,sc^{19}} \quad \times \quad \frac{y}{y}\,;\,\frac{Dp(1;2)\,sc^{19}}{SM5}\,;\,\frac{TM1}{TM2}$

One of the virtues of this scheme is that any new autosomal mutations become automatically balanced in the F1 generation, revealing any dominant phenotypes in that generation.

CONDITIONAL ALLELES

Many of our favorite analytical tools in contemporary fly genetics trace their origins to the days during which the nature of the gene was the premier question. Recombination mapping, Muller's classification of "allelomorphs," and even mutagenesis itself all served the same goal. Conditional mutations share the same history. They emerged as a focus of study to prove the "one gene–one enzyme" hypothesis of the 1940s and became a mainstay of microbial genetics. In the late 1960s, David Suzuki brought the technique to *Drosophila* and launched a major campaign to isolate temperature-sensitive mutations in a variety of different kinds of genes (Suzuki et al. 1976).

Temperature sensitivity is the most common of several kinds of conditional mutation, and sensitivity to heat is more common than sensitivity to cold. Such mutations are often a result of amino acid substitutions, although occasionally they can be caused by insertions that make transcription conditional. (Temperature-sensitive alleles must be distinguished from mutations that reduce transcription levels of thermolabile processes. Thus, in the case referred to above for the sodium channel locus *para*, reduced numbers of sodium channels in the membrane produced a heat-induced failure of action potentials—a function of the kinetics of sodium and potassium channel opening [Stern et al. 1990].) Alternatively, the removal of a subunit from a heteromultimer can also produce a thermolabile phenotype. In such a case, as seen with the *cysteine-string-protein* (*csp*) mutant, the mutation can be null and still produce a temperature-sensitive phenotype (Zinsmaier et al. 1994).

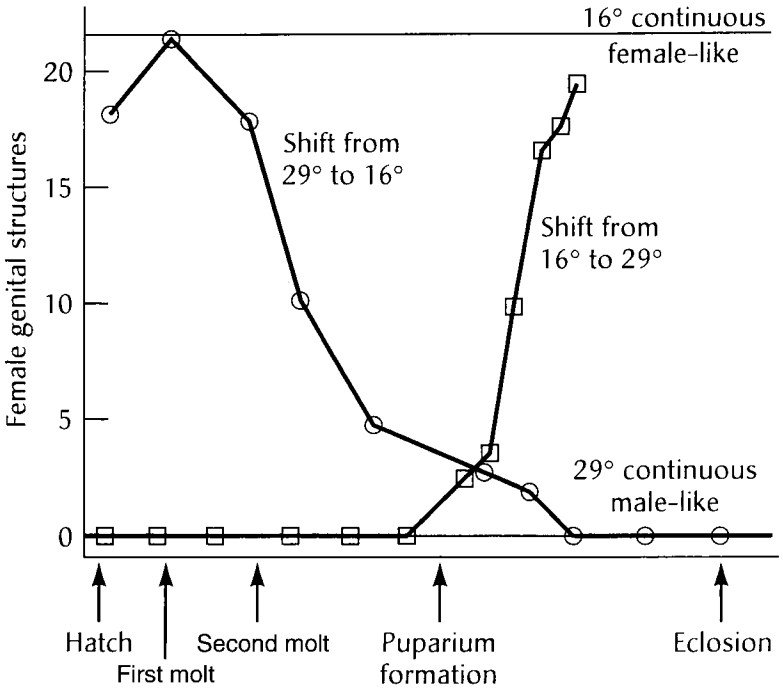
Conditional alleles are an important developmental tool because they provide temporal control over the gene's function. This is particularly important for genes used for multiple functions or at multiple times—characteristics that are not at all uncommon (Hall 1994). Depending on the "leakiness" of the mutation, it may be possible to turn the gene completely on or completely off by means of shifting flies from one incubator to another at any time in the life cycle. For lethal mutations, this means that the gene's action can be assayed at stages of the life cycle later than the usual lethal phase, as has been done extensively with the temperature-sensitive *Notch* allele N^{ts1} (e.g., Cagan and Ready 1989). Even if lethality is not bypassed, a later phenotype can be revealed that would have been overshadowed by a more dramatic earlier one. Such a case occurs in the use of the temperature-sensitive *even-skipped* allele eve^{ID19} to study the gene's role in neuronal wiring after its role in segmentation of the epider-

mis has been completed (Doe et al. 1988). For behavioral mutations, in which it is important to distinguish effects on development from those on acute physiology, conditional alleles permit abrogation of the gene's product posteclosion, as with neurotransmitter mutations such as *Choline acetyltransferase* (Greenspan 1980). Because they are so useful, it is wise to check new mutations for temperature sensitivity. Even when a mutant screen is not specifically designed to yield conditional alleles, sometimes they do, as in the case of as eve^{ID19} referred to above.

Temperature-sensitive alleles also permit the defining of critical periods for particular phenotypes. For genes that are expressed throughout the life cycle, such as those involved in sex determination (Belote and Baker 1982), this is not otherwise a simple matter to determine, although mosaic techniques give some of the same information (discussed in Chapter 6). The strategy for defining a temperature-sensitive period (TSP) is to set up a series of timed cultures, half at permissive temperature and half at restrictive temperature. At regular intervals during the life cycle, shift cultures from one temperature to the other. In the end, score the percentage of individuals with mutant or wild-type phenotypes. Each set generates a curve that produces reciprocals of one another. The point at which they move off their plateaux defines the boundaries of the TSP, and the point of intersection is taken as its midpoint (see figure, facing page).

Some genes are refractory to the induction of temperature-sensitive alleles, presumably a result of the extraordinary thermostability of their protein products. When this occurs with proteins that form homomultimers, conditional genotypes can sometimes be synthesized by appropriate combinations of alleles that are nonconditional as homozygotes. Only in this fashion could heat-sensitive genotypes be obtained at the *Acetylcholine esterase* (*Ace*) locus (Greenspan et al. 1980). As a rule of thumb, if one screens for mutants at high temperature (29°C), roughly 10% of the alleles recovered at a locus will be normal or less severe at low temperature.

Cold-sensitive mutations, although rarer, have also been recovered from mutant screens. They afford the same ability to define TSPs and turn the gene on and off at will. They have an advantage in that heat is often more disruptive to wild-type processes than cold, particularly in some behavioral assays. They also provide interesting and unusual phenotypes, as in the cold-sensitive allele of the *Acetylcholine esterase* gene, Ace^{ji29}, which differs from all of the heat-sensitive genotypes in that it is conditionally lethal, but retains enzyme activity even at restrictive temperatures (Greenspan et al. 1980).



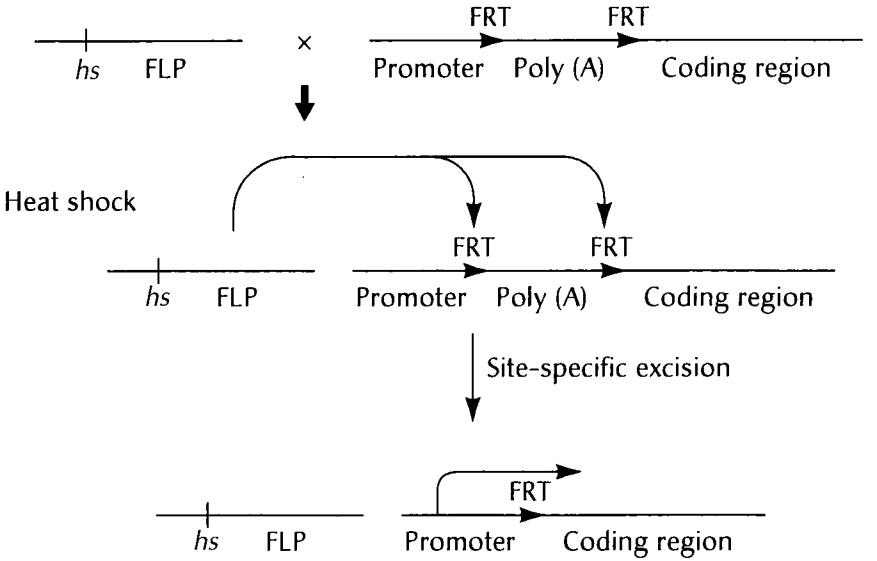
TSP for a temperature-sensitive allele of transformer 2. (Redrawn, with permission, from Belote and Baker 1982.)

Inducible Promoters

The use of transgenes driven by heat-shock promoters serves the same purpose as conditional alleles, similarly permitting temporal control over gene expresson. Heat shock has the advantage of being well defined as a phenomenon and, because a transgene is being induced, the parameters of induction, persistence, and decay can be accurately determined. It is limited, however, by the inability to give sustained expression (the induction temperature of 37°C is too high for long exposures) and by being able only to turn on, not turn off, a gene.

Stable induction has become possible with the introduction of the FLP-FRT system derived from yeast (Golic and Lindquist 1989). This technique involves a site-specific excision by an enzyme (FLP) that is placed under heat-shock control and acts on its target sequences (flip recombinase targets, FRTs) to unblock the expression of a transgene (Struhl et al. 1993). The strategy is to take a 3′ transcriptional termination sequence,

flank it with FRTs, and introduce it into a clone of the gene you wish to control. This construct is then introduced into embryos to derive germ-line transformants. These flies do not express the blocked gene until a FLP enzyme is expressed in those cells to excise the FRT-flanked blocker. To accomplish this, one only needs to perform a cross to combine hs-FLP with the FRT-blocked gene.



The advantage of the FLP-FRT system over plain heat shock is that once a gene is activated, it stays activated.

In the initial study, a wild-type white gene was excised (the symbol is $>w^{hs}>$ for a FRT-flanked sequence) in a fly otherwise mutant at the *w* locus (Golic and Lindquist 1989). Pupae roughly halfway through the pupal period were heat-shocked for either 60 or 80 min at 37°C or 38°C. When scored as adults, 100% of the individuals showed some *w* eye tissue. The later the heat shock, the smaller the proportion of excisions in the eye. After cells in the eye disc cease dividing, no additional excisions could be induced, presumably because of the requirement for division to complete excision. In the non-heat-shocked siblings, 8% showed some *w* eye tissue, presumably a result of low-level expression of the uninduced hs-FLP.

Ligand-regulated promoters responding to tetracycline/doxycycline or RU486 have also been adapted for use in the fly. They have the advantage of being temporally controllable and are able to go on and off and be

maintained in the on state for as long as one wishes. In contrast, they are rather sluggish when going on or off, because of the slow pace of the internal chemical concentration increase and decrease when it is fed to flies. Precautions must also be taken to ensure the absence of leaky transcription or that any leakiness is below the level that will affect the phenotype under investigation. Usually this can be accomplished by mobilizing the construct to find a "good" spot in the genome to avoid position effects. In addition, it can sometimes be ameliorated by adding insulator sequences around the transgene (Stebbins et al. 2001).

The tetracycline system makes use of the bacterial *tetO* regulatory sequence placed upstream of the transgene to be induced, as well as an activator protein that binds to it. Two versions of the system can be used, depending on the form of activator protein: the original tTA protein to produce a "Tet-Off" system, in which presence of the drug blocks transcription (Bello et al. 1998), and a mutated form (*rtTA-M2-alt*) that has an opposite mode of action called "Tet-On" (Bieschke et al. 1998; Stebbins et al. 2001). So far, rearing flies in the continuous presence of drug has not produced noticeably deleterious effects.

The RU486 system employs a yeast transcription factor, GAL4, that has been made chimeric with the human progesterone receptor, making it dependent on binding of the receptor ligand RU486 to be active (Osterwalder et al. 2001; Roman et al. 2001). GAL4 (described in more detail in Chapter 6) binds to a specific target sequence (UAS, for upstream activating sequence) and activates transcription downstream if there is a transcriptional start site present. Thus, you can hook up the gene to be induced to a UAS and introduce it into the germ line. Separately, you either fuse the modified RU486-dependent GAL4 with a tissue-specific promoter (Osterwalder et al. 2001) or make into an enhancer-trap vector (Roman et al. 2001; see also Chapter 2). The combination of the *GAL4* progesterone receptor transgene with the *UAS* transgene produces gene expression if RU486 is present.

MAINTENANCE OF MUTANT PHENOTYPES

Mutations are defined as stable changes to a gene, so it may seem silly to question how to maintain mutant phenotypes. But it is one of the endless fascinations and frustrations of genetics that mutant stocks can lose their phenotypes over time. In a *Drosophila* meeting in the early 1970s that has since entered into the folklore, one investigator began his talk by saying,

"I would like to announce that *Hyperkinetic* is now a recessive" (J.C. Hall, pers. comm., but he was not the speaker). That is, the strain no longer showed a dominant mode of inheritance for the mutation, as reported originally.

The problem is most acute when mutation-bearing chromosomes are kept homozygous. Selection pressure in the culture for any other variations in the population, no matter how subtle and otherwise invisible, will ameliorate the effects of the mutation you are trying to maintain. In the extreme, the mutant phenotype is lost altogether. The fly jargon for this is "accumulation of modifiers"—a description that makes it sound as though we know what is happening. (The trivial explanation for loss of a mutant phenotype is contamination of the stock. If you have kept the mutation on a chromosome that also carries an obvious recessive marker, then you can tell instantly if this is the source of the problem.)

Outcrossing is the standard remedy for retrieving a strong phenotype, since it presumbly undoes the fixing of "modifiers." The simplest form of outcrossing is to mate the flies carrying the mutation to a stock with a balancer for that chromosome, pick up heterozygous males and females, and mate them to rehomozygose the mutation. This works well if the "modifiers" are not on the same chromosome as the mutation. If the modifiers are linked, then it is necessary to allow the mutation-bearing chromosome to recombine freely with a wild-type chromosome and then reisolate the mutation, a technique described in Chapter 2 for cleaning up a mutagenized chromosome.

The problem can sometimes be prevented simply by keeping stocks heterozygous (balanced). If the mutation is viable, however, this requires some effort since you will need to select heterozygous balanced progeny each generation.

GENETIC BACKGROUND

The foregoing discussion of modifiers and maintenance of mutant phenotypes is a special case of the more general issue of genetic background. Many mutations, especially those affecting behavior, are notoriously sensitive to variations in genetic background—the natural, genetic heterogeneity in laboratory stocks (which are the self-same entities as "modifiers"). Whereas quantitative geneticists have long been aware of this ever-present variable, single-gene practitioners, in general, have not (Greenspan 2004).

Where background influences are obvious, as in the study of learning mutants (deBelle and Heisenberg 1996; Dubnau and Tully 1998), experiments have been conducted in which mutants and controls are on the same background. But lack of "obviousness" is not a sufficient test to allow you to ignore background effects. Genetic background can affect the phenotype of a mutation in ways that are not obvious, but nonetheless relevant to the issue of genetic mechanism. A graphic example of the range of these effects was shown in a study of modifiers of the now-famous *sevenless* mutation in *Drosophila*, a mutant originally isolated as part of a genetic dissection of phototaxis behavior (Harris et al. 1976) and subsequently studied in great depth for its role in cell-fate determination in photoreceptors (Tomlinson and Ready 1986). When a moderate allele of *sevenless* (roughly midway between the most severe and wild type) was placed on a range of different genetic backgrounds, phenotypes were found that ranged from fully wild type to more severe than the most effective enhancer mutations previously isolated (Polaczyk et al. 1998).

Caveat pecuarius!

CHAPTER 6

Analysis of Mutations II. Mosaics

MULTICELLULARITY BEGS A WHOLE SET OF QUESTIONS about gene action with which practitioners of phage and bacterial genetics never had to contend: What are the consequences for mutant analysis of differential gene expression in the various cells of the organism? Where does a gene act? Which cells must be mutant to produce a mutant phenotype? Is the mutant phenotype a direct result of aberrant gene action in the affected cell(s) (i.e., cell autonomous) or the indirect result of cell interactions? Questions of this sort have become increasingly prominent as studies of development and behavior have progressed. The genetics-related way to address these questions involves the use of mosaic analysis.

Mosaicism occurs when the normal mitotic process of parceling out the same genetic material to each cell is subverted. The result is an individual whose cells are not all identical in genotype. If the difference between the two populations of cells is that of mutant versus normal, you have an experimental situation in which the kinds of questions listed above can be asked. In addition, mosaics also permit the tracing of cell lineages and the analysis of lethal mutations at late stages of the life cycle when lethality would already have occurred.

Principles

Genetic mosaicism occurs in nature as part of normal life. *X* inactivation in female mammals and the switching of immunoglobulin and T-cell receptor genes in lymphocytes are the best-known examples. Mosaicism that is not part of normal life also occurs in various human chromosome

disorders (e.g., Down's or Turner's syndrome), in which the individual is aneuploid in some tissues but not in others. These mosaics are the result of aberrant chromosome segregation during mitosis, such that all cells do not receive the same chromosomes.

Mosaics can be produced experimentally by inducing chromosome loss during mitosis, mitotic recombination between heterozygous homologs, or excision of a DNA sequence that regulates a gene's expression. In all cases, a preexisting genetic difference must be present that is highlighted by the loss of a dominantly acting allele or synthetic transgene. You can also produce expression mosaicism by tissue-specific control of expression of transgenes.

The ability to recognize mosaics is just as important as the ability to induce them. In particular, whether doing an experiment to determine the cells that must be mutant to produce a defect or tracing a lineage, it is crucial that the genetically different populations of cells be clearly marked. It is equally crucial that the marker be as benign as possible so that the only effects on phenotype are a result of the mutation under study. There are physical methods for making mosaics as well, such as tracer injection (Technau 1986) and nuclear mixing (Lawrence and Johnston 1986), which are outside the purview of this book (see Goldstein and Fyrberg 1994; Ashburner et al. 2004; Sullivan et al. 2004).

The power of the technique is derived from the fact that no two mosaics are ever alike (almost), whether induced by chromosome loss, in which the orientation of early cleavages in the fly embryo is not uniform, or by mitotic recombination, in which random cells are singly affected. As a consequence, many different juxtapositions of dividing lines between mutant and normal cells are possible.

CHROMOSOME LOSS

Induced loss of the *X* chromosome has been one of the most commonly used strategies for mosaic analysis in the fly, beginning (as with so many other things) with Alfred Sturtevant's studies of the *claret* mutation in *Drosophila simulans* (Sturtevant 1929). Since chromosome loss produces gross aneuploidy, one can only obtain viable mosaics after loss of an *X* chromosome in females, a marked *Y* chromosome in males, or a chromosome *4*. Loss of an *X* in a female zygote produces a gynandromorph—part male, part female—which is perfectly viable as long as the loss event occurred early enough in development (i.e., during preblastoderm cleavage stages) to avoid lethality problems associated with lack of dosage

compensation of *X*-linked genes in the islands of *XO* cells. Loss of a chromosome *4* is viable because it is so small, although haplo-*4* flies exhibit a *Minute* phenotype that affects growth rate.

Uses of Chromosome Loss Mosaics

Chromosome loss is the method of choice to produce mosaics with very large clones (~20–50% of the animal) and to produce clones very early in development (preblastoderm stages), before any zygotic gene expression.

Fate Mapping

The initial use of the mosaic technique was to construct a fate map of the fly embryo. Sturtevant (1929) applied the same principle to map the distance between primordial cells on the blastoderm as he had to map the recombination distance between genes on the chromosome: The more mosaic the dividing lines that fall between two structures, the farther apart must they be. The assumption was that mosaic dividing lines cut across the blastoderm at random (an assumption that may hold truer for mosaic dividing lines than for recombinational events on chromosomes). Two structures that never differ in their marker genotype must be derived from a common precursor cell on the blastoderm. This idea, originally developed in Sturtevant's studies of *D. simulans*, was expanded and applied to *D. melanogaster* by Garcia-Bellido and Merriam (1969) for the adult cuticle and subsequently for internal adult (Kankel and Hall 1976) and larval (Janning 1978) tissues. In honor of Sturtevant, and by analogy to the centiMorgan unit of recombinational mapping, the map unit of fate mapping was dubbed the "sturt."

The fate map was constructed by scoring the presence or absence of a marker for each structure (y^+ versus y for the adult cuticle; enzyme staining versus no staining for internal tissues) and then triangulating the distances of all structures from one another and from the midline. The resulting formal fate map bore a striking resemblance to the histologically derived fate map originally determined by Poulson (1950) based on sections of embryos. The map has subsequently been confirmed by focal ablation of cells on the blastoderm with a hot needle (Bownes and Sang 1974).

On a more local level, lineage relationships have been established between cells in the same structure, such as the ommatidia of the compound eye, for which it was first shown that neither the ommatidium nor

even the eight photoreceptor cells were clonally derived (Ready et al. 1976). Lineage relationships are more commonly studied by means of mitotic recombination (see below).

Focus of Gene Action

The random nature of mosaic dividing lines produced by chromosome loss and the fact that the loss event occurs before any zygotic gene expression make it ideal for assessing the cellular focus for developmental mutations, that is, identifying the cells that need to be mutant for the abnormal phenotype to appear. From this, one infers the cells that must normally express the wild-type gene so it can carry out its developmental function.

The technique has been used to make embryonic mosaics for mutants in the segmentation gene *runt* (Gergen and Wieschaus 1985, 1986) and the cell-fate-determining gene *Notch*, with both external and internal markers (Hoppe and Greenspan 1986, 1990), as well as to produce adult mosaics for mutants in genes such as *sevenless* (Harris et al. 1976) and *bride-of-sevenless* (Reinke and Zipursky 1988). In each case, the autonomy of the mutation was determined whether or not the gene has its primary action in the cell type that is chiefly affected in the mutant phenotype.

Gynandromorphs have played a central role in genetic studies of behavior. In general, the large size and the contiguity of the clones ensure that significant numbers of neurons in any given area will be simultaneously affected. More specifically, the fact that they are mixtures of genotypically male and female cells has made them well suited for the identification of brain regions that mediate sex-specific behaviors (Hall 1977, 1979). The analysis essentially consists of counting the number of times each brain structure is male or female and correlating it with the amount of male behavior the fly performs. It also contains an additional formal component, derived from Sturtevant's original fate map calculations and designed to localize behavioral foci when you do not know where to look or when the focus appears to involve large or multiple portions of the nervous system (Flanagan 1977; Arnold and Kankel 1981).

For physiological mutations that affect function in many or all neurons, mosaics provide a way of studying the behavioral effects of localized disruptions. Since many of these mutations are also embryonic lethals, gynandromorphs can survive to adulthood while retaining mutant brain regions, as was done for mosaics of *Acetylcholine esterase* mutations (Greenspan et al. 1980).

Methods for Inducing Chromosome Loss

A variety of mutations that affect mitotic and meiotic segregation produces genetic mosaics. This was the starting point for Sturtevant when he studied the *claret* mutation in *D. simulans*, a homolog of which also exists in *D. melanogaster*. The effects of these mutations are not confined to a given chromosome, and the proportion of mosaic progeny is low for all. A principal technique is the unstable ring-*X* chromosome $R(1)w^{vC}$ (also known as $In(1)w^{vC}$ because it contains an inversion), a rearrangement that is efficiently lost from early mitotic divisions.

Ring Loss

The unstable ring *X* has been the method of choice whenever appropriate, because the stock construction for its use is so simple and a relatively large proportion of progeny are actually mosaic. It limits the user to mosaicism for *X*-linked genes because it is difficult (although not impossible) (Gailey et al. 1987) to transpose or translocate autosomal loci onto it. The markers to be scored must also be *X* linked.

In a female embryo heterozygous for the unstable ring *X* and an *X* mutant for *yellow* (*y*) and *white* (*w*), mosaicism is clearly visible on the cuticle and in the eye. This is the result of loss of the ring *X* during early cleavage divisions and subsequent expansion of the haplo-*X* clone.

$$\frac{y\ w}{Y} \times \frac{R(1)w^{vC}}{In(1)dl\text{-}49,\ y\ w\ lz}$$

$$\downarrow$$

$$\frac{y\ w}{R(1)w^{vC}} \Big/ \frac{y\ w}{0}$$

Mosaic notation designates the genotype of the starting embryo on the left, separated by a slash from the genotype of the mosaic tissue on the right, where *0* indicates the lost chromosome.

The ring *X* is best introduced from a female, since it has poor viability in hemizygous males. If a ring *X* has attained good viability in a stock, which can happen over time, it usually means that it has also become more stable and thus less useful.

The chromosome *In(1)dl-49* is routinely used to balance ring-*X* stocks since it carries markers that reveal mosaicism (*y w lz*) but will not recombine with the ring because of its own inversion. It is important to be able

to score the presence of mosaicism because ring instability must be selected to maintain it in the stock. Presumably this lability of phenotype is due to the unhealthiness of chromosome loss. Unlike other examples of the infamous accumulation of "modifiers" (see p. 118), loss of ring instability seems to be associated with the ring-*X* chromosome itself. Thus, selection is the only remedy.

Selecting gynandromorphs to maintain the stock may seem challenging, given the intersexual nature of these flies. The rule of thumb is that if you collect all such mosaics, at least some of them will be fertile (those that have female reproductive organs and enough of a female brain to mate with males). The recovery of mosaic progeny can be at least 20% of the potential mosaic class (i.e., *R(1)w^{vC}/dl-49*) in a well-selected stock. The size of mosaic patches averages 50% of the fly, but smaller patches (resulting from late loss) and larger ones (resulting from multiple loss events) occur with reasonable frequency (Hall et al. 1976).

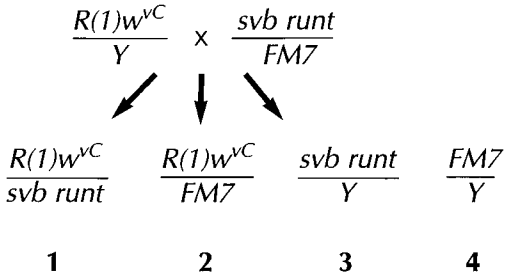
Harris et al. (1976) used ring loss gynandromorphs to demonstrate that the now-famous *sevenless* (*sev*) mutation acts in retinula cell R7 to produce the mutant phenotype. That is, the *sev* phenotype (loss of R7) is cell autonomous. Since *sev* is *X* linked, the ring carries its wild-type allele. By recombining the eye pigment mutation *w* onto the same chromosome as *sev*, and taking avantage of the fact that the retinula cells contain pigment granules that are unpigmented in cells mutant for *w*, this establishes a situation in which cells that lose the ring *X* are simultaneously mutant for both. They showed that all R7 cells that were recovered in mosaics were pigmented, i.e., *sev*$^{+}$. A *sev*$^{-}$ R7 was never found.

The same approach was later used by Reinke and Zipursky (1988) to demonstrate that the *bride-of-sevenless* (*boss*) mutation, which also causes loss of R7, is **non**autonomous to R7. It acts in R8, as shown by the fact that many *boss*$^{-}$ R7 cells were recovered, but whenever an ommatidium had a *boss*$^{-}$ R8, it was also missing R7.

The ring *X* works at a high enough frequency to be used in embryonic studies. Mosaic frequency makes a big difference here, since it is relatively easy to recognize rare mosaic adults by the presence of small patches of *y* cuticle, but much more difficult (almost impossible) to pick out rare embryos. Gergen and Wieschaus (1985, 1986) used the *X*-linked denticle marker *shaven-baby* (*svb*) to study the cell autonomy of *X*-linked segmentation mutants in the embryonic epidermis. For this study, they linked the *svb* marker to chromosomes mutant for each of the segmentation loci *runt, armadillo, fused, giant,* and *unpaired.* Promoter fusions, such as *armadillo-lacZ* (Vincent et al. 1994) or poly-ubiquitin-GFP (Davis et al. 1995) can also serve as effective mosaic markers.

Sometimes the gene you wish to study can serve as its own marker. Hoppe and Greenspan (1990) used antibodies to the protein product of the *X*-linked *Notch* (*N*) gene as a marker of neuroblasts in embryonic *Notch* mosaics. Histochemistry for the enzyme *Acetylcholine esterase* was used to mark mosaics for *Ace* mutations (Greenspan et al. 1980).

The crosses to produce embryonic ring loss mosaics require somewhat different handling than those for adults. It is very helpful if some of the non-mosaic progeny classes can be easily recognized, so that one does not spend even more hours looking for tiny mosaic patches in already tiny embryos that do not have a prayer of being mosaic. To achieve this end, the ring *X* should be introduced from the father and the mutation to be uncovered should be introduced from the mother. The class of potential mosaics is still 25%, but now there are also classes of hemizygous mutant progeny, namely, those that receive the mutation from the mother and the father's *Y*:



Class 1 is the potential mosaic class. All class 3 embryos appear entirely mutant for both genes and are thus recognizable. Counting the number of class 3 embryos gives a reliable estimate for 25% of fertilized eggs and can thus be used as the denominator to determine embryonic mosaic frequency in class 1. Moreover, class 3 embryos need not be examined in the same excruciating detail as the others for small patches of mosaicism. Granted, mosaics do occur that are almost entirely mutant as a result of multiple ring loss events, but these are few and irrelevant to many studies.

Chromosome Loss Mosaics for Autosomal Genes

The *X* is nice, but most of the fly's genes are on chromosomes *2* and *3* and, unfortunately, mitotic loss of a large autosome is lethal at a very young age (Wright 1970). To get around this problem, Lewis (1963) figured out that if you could get the wild-type locus of your gene onto the *X*, then you can make mosaics for that gene by losing the *X*. This works as long as the autosomal loci for that gene are also mutant in the mosaic individual.

In Lewis' case, he used a translocation of the *Bithorax* complex (*BX-C*) onto the *X* (*T(1;3)O5*), and crossed it onto a ring *X* by a double crossover event. This produced a ring *X* with *BX-C*$^{+}$ on it. (This was not the unstable ring *X*, *R(1)w*^{vC}, but rather a stable ring *X* that he induced to be lost by an unreliable technique that it is not worth mentioning here.) He then performed the following cross to determine whether *bithorax* produced its homeotic transformations cell autonomously:

$$\frac{R(1;3)\ O5}{Y}\ ;\ \frac{bx}{bx} \quad \times \quad \frac{y}{y}\ ;\ \frac{bx}{bx}$$

All of the progeny were homozygous for *bx* and thus all of the females were potential mosaics, heterozygous for the ring and for *y*. The outcome was that transformed cuticle was always *y* and untransformed cuticle was always *y*$^{+}$, indicating cell autonomy (Lewis 1963). (All right, I'll tell how he made the mosaics: He mated very old females to very young stable ring-*X* males, a technique that no one since has ever gotten to work—this author included.)

Sometimes, it is possible to transfer a wild-type autosomal locus onto the unstable ring *X*, *In(1)w*^{vC}. Gailey et al. (1987) did this by taking an *X* chromosome containing a P-element insertion of the wild-type locus for *Dopa decarboxylase* near the middle of the chromosome arm, and allowing it to recombine by double crossover with the ring *X*. In principle, the same thing could be done by mobilizing a P element and screening for its insertion into the ring *X*. The difficulty with the latter approach is that the *w*^{vC} allele on the ring *X*, which causes variegation of the *white* gene, makes it difficult to score the presence of either a *w*$^{+}$ or a *ry*$^{+}$ marker to detect the presence of the insert. More generally, the difficulty with any attempt to transfer a gene onto the ring *X*, given its poor viability, is the necessity to recover the new chromosome in a single fly and propagate a stock from it.

A more general (and workable) approach to making gynandromorphs with autosomal loci translocated or transposed onto the *X* is to use mutations that destabilize chromosomes. Three have been used successfully, two of which are known to encode components of the mitotic apparatus.

paternal-loss (pal)

Originally isolated for its meiotic phenotype, *paternal-loss* (*pal*) destabilizes paternally derived chromosomes (Baker 1975). It is one of the few paternal-effect mutants in the fly. Progeny of a *pal/pal* male will be mosaic for any of his chromosomes that they inherit. If chromosome *2* or *3* is

lost, the embryo will die. If a female zygote loses the paternally derived *X*, she will be a gynandromorph and if chromosome *4* is lost, the fly will survive and have a *Minute* phenotype (see Chapter 4) in the haplo-*4* tissue. Thus, the *X* carrying a wild-type autosomal locus must be introduced from a homozygous *pal* father:

$$\frac{X\text{-}Acph^{+}}{Y};\ \frac{pal}{pal};\ \frac{Acph^{n}}{Acph^{n}} \quad \times \quad \frac{y}{y};\ \frac{Acph^{n}}{Acph^{n}}$$

$$\downarrow$$

$$\frac{X\text{-}Acph^{+}}{y};\ \frac{pal}{+};\ \frac{Acph^{n}}{Acph^{n}} \quad / \quad \frac{X\text{-}Acph^{+}}{0};\ \frac{pal}{+};\ \frac{Acph^{n}}{Acph^{n}}$$

Acph is the structural gene for acid phosphatase, $Acph^{n}$ is a null allele of the locus, and $X\text{-}Acph^{+}$ is a translocation onto the *X* of the wild-type locus. The scheme for generating these males is outlined in Chapter 4.

The frequency of chromosome loss with *pal* is reasonable (1–5%) (Hall et al. 1976), and the stock does not need to be selected constantly. Mosaic patch size averages 30–40%. It works at a tenfold lower rate of loss on *Y* chromosomes or rearrangements with *Y*-derived centromeres. This matters if you have made an *X* with an autosomal duplication by detaching an attached *X* with a *T(Y;A)*, as described in Chapter 4. These will sometimes acquire the *Y* centromere as a result of the exchange event and, if so, will be less destabilized by *pal*.

claret-non-disjunctional (ca^{nd})

claret-non-disjunctional (ca^{nd}) is the *D. melanogaster* homolog of the *D. simulans* gene that Sturtevant discovered. It has since been shown to be comprised of two separate genes, one affecting eye pigment (*claret*) and the other encoding a kinesin-like component of the spindle (Walker et al. 1990). In some ways, this mutant is the converse of *pal*. Chromosomes inherited from a mother homozygous for ca^{nd} are destabilized in the earliest mitotic divisions after fertilization. Thus, the *X* carrying a wild-type autosomal locus must be introduced from a homozygous ca^{nd} mother.

The *D. simulans* mutation was used in early fate-mapping and lineage-tracing studies (Garcia-Bellido and Merriam 1969). Whereas the proportion of progeny with mosaicism can be as high as 20%, with patch sizes averaging 50%, the mutation has a serious drawback: The homozygous females are so severely impaired in meiotic segregation that their fer-

tility is very poor (Hall et al. 1976). As a consequence, few potential mosaics are produced.

Care of Mosaic-producing Stocks

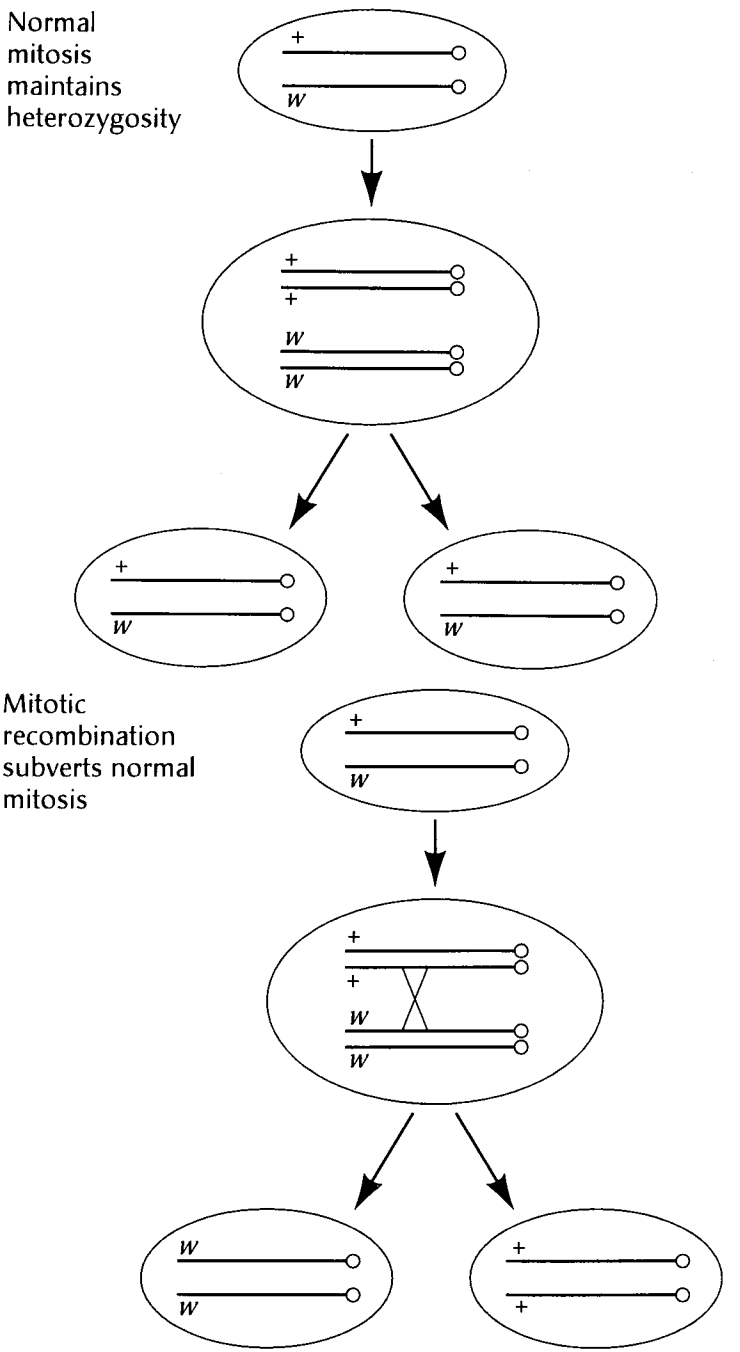
Most of the variants used to destabilize chromosomes are themselves unstable. Ring-*X* stocks tend to lose their mosaic-producing ability if simply transferred generation after generation. *pal* and ca^{nd} have less of a problem. To maintain the instability of ring-*X* stocks, it is necessary to select and wise to maintain the chromosome $In(1)w^{vC}$ balanced over *In(1)dl-49, y w lz* so that mosaicism for cuticle and eye pigmentation is readily visible in every generation. (This balancer is viable in males and homozygous sterile in females, precluding the stock from losing the ring *X* altogether.) One can then recognize and select gynandromorphs, choosing those with female genitalia, and configure cultures with these as the females. A superb ring-*X* stock produces 30% gynandromorphs out of the potentially mosaic class of progeny. A ring-*X* stock that has been allowed to languish may have fewer than 5%. When this is the case, the rescue operation must be carried out in vial cultures.

In contrast, *pal* stocks that have been left homozygous for decades in stock collections still retain their mosaic-producing prowess. Given the fact that *pal* has no effect on viability, in the end one could obtain as many mosaics in the same period of time as with the ring *X*. ca^{nd} stocks, which must be balanced or they become extinct, do not seem to degrade over time either.

Problem 11 Design a scheme to produce mosaics by *X*-chromosome loss for the *X*-linked mutation *Hyperkinetic* (*Hk*) using ca^{nd} (3-100.7) as the mosaic producer and *y* as the marker. Start from the simplest stocks ($TM6/ca^{nd}$ and *Hk/Hk*) and use any other balancers or markers that you wish.

MITOTIC RECOMBINATION

Loss of a wild-type or dominant allele can also be brought about by recombination between heterozygous homologs during mitosis. Recombination can occur between chromosomes in somatic cells at a very low spontaneous rate or it can be induced by radiation or other techniques (see facing page). Here, in a cell heterozygous for *w*, the wild-type (dominant) allele is made homozygous in one daughter cell, whereas the mutant allele is made homozygous in the other.


Normal
mitosis
maintains
heterozygosity
+
w
+
+
w
w
+
w
+
w
Mitotic
recombination
subverts normal
mitosis
+
w
+
+
w
w
w
w
+
+

The homozygous wild-type cell will be phenotypically indistinguishable from the rest of the fly's cells, but the homozygous mutant cell will be *white* if present in eye tissue. The resulting daughters of the *w/w* cell will inherit the mutant phenotype and produce a clone. Daughter cells and their resultant clones can be marked if one includes another recessive marker in *trans*. Thus, irradiating a fly doubly heterozygous for *w* (1-1.5) and *roughest* (*rst*; 1-2.2), *w/rst*, will produce "twin spots" of either non-pigmented (*w/w*) or roughened (*rst/rst*) ommatidia. Twin spots are useful for lineage-tracing studies.

The event is a random one and thus can occur in any dividing cell of the fly. Detecting the clone depends on whether the mutation has a phenotypic effect on that cell and its progeny. If the *white* mutation is made homozygous in a bristle precursor cell, you will not see it. A distinction needs to be made here between marker mutations (such as *white*) used for detecting mitotic recombination events and for mapping lineage, and "interesting" mutations whose effects are being assessed by means of mosaic analysis.

Marker mutations are chosen for their cell autonomy and for their gratuitousness, i.e., lack of effect on the developmental and behavioral processes that one uses mosaics to study. Detecting a patch of mosaicism by means of a marker thus depends on whether that gene is normally expressed in the cell type that has undergone mitotic recombination. The marker must also lie close or proximal to the mutation it is marking. ("Proximal" means closer to the centromere.) This ensures that you will never have a situation in which the marker is made homozygous without simultaneously making the interesting mutation homozygous. Mitotic recombination can occur at any point along the chromosome arm, but (fortunately, for this kind of analysis) tends to occur nonrandomly near the centromere.

Whether interesting mutations produce mutant phenotypes in mosaics depends on two criteria: The mitotic recombination event must occur in the right place and the timing of the event must be early enough to make a difference. The "right place" refers to a precursor of those cells that are the focus of the mutation's effect and "early enough to make a difference" refers to the time of critical expression for the gene.

Developmental timing is crucial to the use of this technique. Some of the key findings on cell lineage restrictions in the embryo depend on carefully timed clone induction (Wieschaus and Gehring 1976). Consequently, the accurate age of these progeny must be known. You can obtain synchronized cultures by collecting freshly laid eggs over a short time span.

Females that lay eggs continually fertilize them just before laying, thus time of deposition is a good measure of initiation time of zygotic development.

Synchronizing Age of Cultures

There is as much folklore about how to coax females to lay eggs at a brisk rate as there is about anything else in the fly world. It all boils down to the fact that if they are content, they lay lots of eggs. The best way to make them happy is to keep them well fed in clean bottles that are not too crowded. The easiest way to do this is to place approximately 50 virgins in a fresh bottle with a large amount of yeast paste at the bottom (use dried baker's yeast mixed with just enough water to thicken it to the consistency of thin peanut butter). After 2 or 3 days, change the bottle and add males of the appropriate genotype. After another day or two, you are ready to collect eggs.

Collections are best carried out for an hour at a time if you will be implementing irradiations and analyses on embryos or early larvae. Longer collections are permissable for later times, since asynchronies will appear as development proceeds. The collection container can be a piece of desk blotter paper saturated with vinegar and yeast paste, agar plates with a thin paste of yeast, or culture bottles. The choice depends on the stage at which mitotic recombination is induced and when the mosaics are to be collected. Blotter paper is fine for embryos, agar plates for larvae, and culture bottles for pupae and adults.

The first egg collection or two is usually unsuccessful. By the third, eggs are typically being produced at full blast. (The problem with collecting eggs when the females are not laying at full tilt is that they harbor the fertilized eggs, thus confounding your efforts to synchronize progeny age.) The collected eggs are then incubated for the requisite period of time, usually sometime during the larval stage, and then irradiated or heat-shocked, depending on the technique being used to induce mitotic recombination.

For careful staging of the pupal period, it is more accurate to collect white prepupae. These are recognizable as milky looking, immobile rods, the size of third-instar larvae (which they recently were) that have taken up positions on the side of the bottle. They can be carefully picked off the side of the culture and transferred to a vial in which they will complete pupation and metamorphosis. Even greater accuracy in identifying wandering larvae can be obtained by placing them on food colored with blue dye and then choosing only those larvae that do not ingest any dye.

Uses of Mitotic Recombination Mosaics

Mitotic recombination is the method of choice for producing small clones, controlling the time of clone induction, and noninvasively tracing lineage. (It is also called somatic recombination, to distinguish it from the recombination that occurs in the germ cells during meiosis.)

The first use of the technique was genetic—to demonstrate that recombination could occur in somatic cells. At the same time, it was used to address an early formulation of the developmental question of determining which cells must be mutant to give a mutant phenotype. In Curt Stern's original formulation (1968), the question was whether you could distinguish between genes affecting "pattern" versus "prepattern," a concept corresponding, to some extent, to current notions of autonomy and nonautonomy.

Lineage Analysis

Mitotic recombination provides the only noninvasive means of marking cell lineage. As such, it allows clones to be marked at virtually any time in development, no matter how inaccessible the tissue. The success of the technique depends on the ability to limit mitotic recombination to a single cell in the primordium being studied. Fortunately, when radiation is used to induce the recombination event, it can be calibrated so that the probability of obtaining single clones is extremely high.

Lawrence and Green (1979) introduced a clever technique for ensuring that a marked clone represents a single event by exploiting the rarity of recombination between two mutations in the same gene. They took two different alleles of the *white* gene, which give a white-eyed phenotype in the double heterozygote. The rare mitotic recombination events that occurred between the two mutations produced a normal, restored w^+ gene as one daughter and a doubly mutant *white* gene as the other. The restored w^+ phenotype has the virtue of appearing as a red-colored clone on an otherwise white background, making it easier to score even if small and rare.

Lineage tracing by mitotic recombination has been used most commonly on external tissues, which contain easily recognizable pigment mutations to mark the cuticle and the eye. The experiment is done by performing a cross to produce progeny that are heterozygous for a recessive marker mutation. The resulting heterozygous progeny are the appropriate targets for inducing clones. Mitotic clones have been used to establish lineage relationships in the wing (Bryant and Schneiderman 1969), thorax

(Wieschaus and Gehring 1976), eye (Lawrence and Green 1979), and thoracic muscles (Lawrence 1982).

The size of a clone depends on how many divisions the affected cell has yet to go through. The relative proportion of the structure (e.g., a wing) that the clone occupies depends on the amount of precursor cells present at the time of clone induction. The discovery of compartments, one of the pivotal findings in fly development, arose from the use of a technique for increasing clone size by increasing the division rate of the clone relative to its neighbors. This is accomplished through the use of *Minutes*, a class of mutations in ribosomal proteins that slows development and is characterized by thin bristles. Fortunately for the purpose at hand, *Minutes* also act dominantly and autonomously (Morata and Ripoll 1975). This means that if a fly starts out as *M*/+ and has a +/+ clone induced during development, that cell and its progeny will outstrip their *M*/+ neighbors and occupy a greater proportion of the final structure than the progeny would have otherwise. In the course of such studies, some of the inflated clones were found to bump up against a set of boundaries in the wing disc. These boundaries, dubbed "compartments," divide the primordium along anterior-posterior and dorsoventral axes (Garcia-Bellido et al. 1973).

Focus of Gene Action

Any mosaic can be used to answer questions about a gene's site of action; mitotic recombination mosaics are no exception. This was Stern's original experiment when generating clones of mutant tissue for the mutation *extra sexcombs* (*esc*) on the legs of male flies. In the original *esc* allele, male mesothoracic and metathoracic legs produced ectopic sex combs as a result (as we now know) of being homeotically transformed. Tokunaga and Stern (1965) induced clones in *esc*/+ heterozygotes to create small patches of mutant tissue in the legs.

As in all mosaic experiments, clones must be marked to be observable. For mitotic recombination, the marker must be on the same chromosome arm as the mutation being studied, thus an event that makes a mutation homozygous will also make the marker homozygous. You have leeway in one direction: It is tolerable to have the mutation homozygous without the marker, since you will simply miss it. However, you are limited if you make the marker, but not the mutation, homozygous: This is akin to scoring a false positive. The best way to ensure that you are not misled by the marker is to choose one that lies proximal (i.e., closer to the centromere) to the mutation that you are studying.

Tokunaga and Stern used flies heterozygous for a translocation of the wild-type locus for *yellow* (y^+) onto the tip of the left arm of the second chromosome (*2L*). As long as these flies were mutant for *y* on their *X* chromosomes, any induced recombinations of *2L* were detectable as *y* clones. To link this marking system with the generation of *esc* clones, they needed only to generate progeny heterozygous for the *esc* mutation on the other homolog from the y^+ translocation. As a result, whenever a mitotic recombination event made *esc* homozygous, it also made y^+ homozygous. However, as just described, this arrangement produces *y* clones that are not *esc* whenever the recombination occurs between the *esc* locus and the tip.

A cleaner experiment can be accomplished by using a wing cuticle marker *pawn* (*pwn*) to mark clones that are mutant for the *engrailed* (*en*) gene, key to establishing the importance and role of compartments (Morata and Lawrence 1975). By choosing a marker that is proximal to *en* (closer to the centromere), you ensure the absence of falsely marked clones (i.e., pwn^- but not en^-).

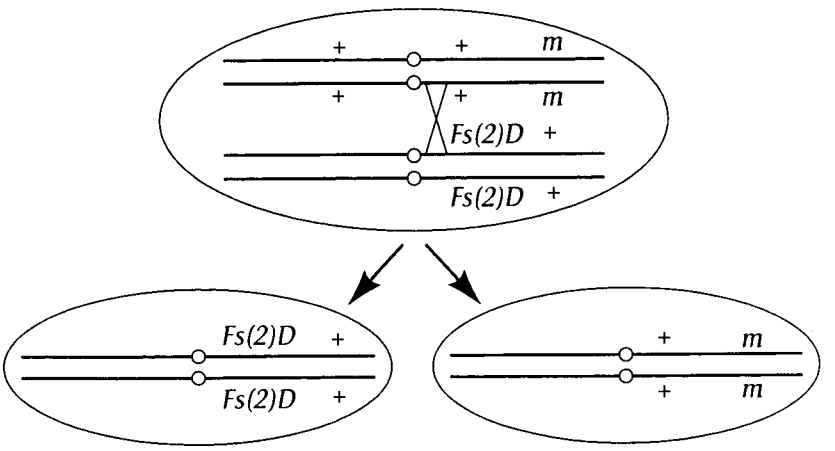
Time of Gene Action

Determination of the critical period of a gene's action was discussed earlier in the context of temperature-sensitive mutations. But not all of the genes that you wish to study have temperature-sensitive alleles. In such cases, it is nonetheless possible to determine the end point of a gene's action by eliminating the wild-type allele at any desired stage by means of mitotic recombination. When clones are induced at successively later stages, at some point a mutant phenotype no longer results. This marks the point after which the normal gene product is no longer needed. Said timing is subject to the persistence (or "perdurance," the term coined by Antonio Garcia-Bellido) of the normal gene product after the elimination of the wild-type allele.

Some genes, such as *bithorax* (Morata and Garcia-Bellido 1976) and *Sex-lethal* (Cline 1984), have been shown by this technique to be continuously required throughout development. In the case of *Sex-lethal*, this has been explained by reason of the gene's autoregulation (Cline 1984; Bell et al. 1991).

Germ-line clones. A special case when using mitotic recombination to study the time of gene action is distinguishing between maternal and zygotic effects of mutations. That is, many genes essential for oogenesis are also essential for zygotic and adult viability, making it difficult to

obtain homozygous females to test for maternal effects. Wieschaus (1980) developed a clever way around this problem using dominant, female-sterile mutations. A female heterozygous for such a mutation is, by definition, sterile. If a mitotic recombination event occurs in her ovaries during oogenesis, then a homozygous oocyte will sometimes arise.



Viable, fertile egg

One daughter cell product of this event will have lost the dominant *female-sterile* mutation (*F(2)D*) and will thus complete oogenesis normally. The other daughter cell product of this mitotic recombination event will acquire two copies of the domimant *female-sterile* mutation (*F(2))D/F(2)D*) and will not complete oogenesis. Hence, only those eggs that have had a mitotic recombination event induced in them and that have lost the dominant *female-sterile* mutation will successfully complete development. When another mutation (*m*) is present on the chromosome arm that has become homozygous, the resulting egg will develop in the absence of that gene's product. If this gene is required for some function in the early embryo, a phenotypically abnormal embryo will result.

A clear prerequisite for this approach is that the dominant *female-sterile* mutation must act autonomously in the oocyte (i.e., germ line). This can be determined to a first approximation by using the same technique, since clones of somatic ovary cells likely comprise such a small proportion of the ovary that they are unlikely to rescue sterility that is somatic in origin. Confirmation can be determined independently by germ cell transplants, in which mutant germ cells are placed into a wild-type background, and vice versa by pole cell transplantation (van Deusen 1977).

Methods for Producing Mitotic Recombinant Clones

Radiation. Ionizing radiation was the first method used to induce mitotic recombination, just as it was the first agent of mutagenesis. It is a simple method because all that you need is an X-ray machine or radioisotope source and some heterozygous larvae. The dose-response relationship has already been well worked out. The standard rule-of-thumb dose used to maximize recombination events and minimize lethality is 1000–1500r.

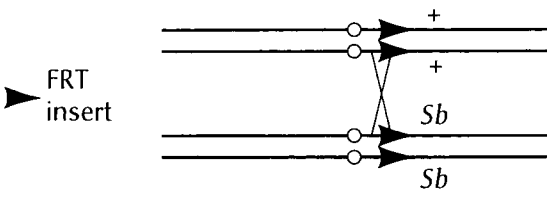
The disadvantage of this method is that radiation is unhealthy to cells and other living things. Some individuals are killed outright and those that survive suffer some degree of tissue damage. Subsequent development of the tissue undergoes some degree of cell destruction and regeneration quite apart from the effects of any mutations made homozygous. Sometimes, the regeneration can produce pattern duplications that have nothing to do with the mutation that you are trying to study.

Radiation can be delivered at almost any stage of the life cycle. The only stages that are totally sensitive are those that occur during embryogenesis. Even so, some windows of time during embryogenesis allow radiation to be tolerated well enough to yield some viable flies with mitotic clones: These occur at 3 hours and after 7 hours postfertilization, with flies reaching full survival after 10 hours (Wieschaus and Gehring 1976). Synchrony of developmental age is clearly very important when using this technique on embryos.

The FLP-FRT System. A kinder and gentler method for creating mitotic clones makes use of the site-specific recombinase from yeast, FLP, and its target sequence, FRT (Golic 1991). The system, introduced earlier in the context of creating deletions and testing for the time of gene action during development, has also been adapted for the induction of mitotic recombination. Heat shock is the triggering event that activates the recombinase. It can thus be induced whenever heat shock can be induced, i.e., essentially at any time except during stages of preblastoderm embryos.

This system has the virtues of being more benign than radiation (not difficult to accomplish) and ensuring that the recombination event will always occur at a predefined site on the chromosome. Since these FRT insertion sites are chosen to be close to the centromere, it obviates the need to worry about whether the marker mutation is proximal to the mutation being studied. In addition, it offers the double-edged advantage that clones can be induced with high probability, but the likelihood of inducing single clones is correspondingly reduced.

The key to the method is the presence of FRT sites in identical positions on both homologs.



It is essential that the FRT sequences on both chromosomes be oriented in the same direction, since the recombination event will only occur between identically oriented FRTs. If they are oppositely oriented, they may still recombine but produce a dicentric chromosome and an acentric fragment.

This method involves delivering heat shock for 60 minutes at 38°C at both the end of the larval period and beginning of the pupal period. The frequency of individuals with mitotic clones depends on the presence of single or tandem copies of the FRT on each homolog.

	Frequency of mosaics (%)	
FRT genotype	No heat shock	Heat shock
FRT-FRT / FRT-FRT	97.0	99.5
FRT-FRT / FRT	31.1	58.5
FRT / FRT	1.3	43.7
FRT-FRT / +	–	0.3

Data from Golic (1991).

As this table shows, considerable spontaneous recombination occurs without heat shock when more than one FRT is present on one of the homologs. Xu and Rubin (1993) generated many strains of flies carrying double FRT insertions near the centromeres of each chromosome arm.

The FLP-FRT system also works well for the production of germ-line clones (Golic 1991). A standard treatment (1 hr at 38°C) during the mid-

dle to late pupal period produces ample progeny from females heterozygous for dominant, sterilizing mutations (see above).

Markers for Internal Tissues

Mosaic markers for internal tissues were generally difficult to use before the adaptation of green fluorescent protein (GFP) for marking (Wang and Hazelrigg 1994). Early attempts used endogenous enzyme mutants in the fly (Kankel and Hall 1976; Greenspan et al. 1980), followed by the introduction of bacterial *lacZ* fused to various promoters (Vincent et al. 1994) or hopped around to produce various expression patterns (Bier et al. 1989; Wilson et al. 1989), and by an antigenic marker using a domain of the Myc protein and a commercially available monoclonal antibody specific for that epitope (Xu and Rubin 1993). All of these approaches carried drawbacks caused by high background, the difficulty of having successful staining reactions every time without a hitch, and the requirement of sectioning to see the staining patterns clearly.

All of these problems have withered away with the advent of GFP and its derivatives, which include variants flourescing at different wavelengths, localizing to different parts of the cell, and persisting for different time periods (Brand 1999). When used in conjunction with confocal microscopy, its signal is clear, strong, capable of being visualized easily in whole-mount preparations in any tissue at any stage of development, expressible as a transgene off of any promoter, and self-sufficient (in the sense that no substrate or label is needed). The problem of scoring internal genotype has thus disappeared.

GAL4 AND EXPRESSION MOSAICISM

Promoter fusions in transgenic animals produce another kind of mosaicism based on intrinsic mechanisms of gene regulation to drive expression of introduced, cloned genes. Selective expression of the gene being studied is achieved either by fusion to a defined promoter sequence or activation by the yeast transcription factor GAL4, itself driven as an enhancer trap.

This approach differs from clonal mosaic techniques (chromosome loss and mitotic recombination) because the pattern of mosaicism is nonrandom, reproducible, and bilaterally symmetrical—all features of enhancer-driven transgenes. Reproducibility is a major boon to mosaic studies in which the phenotype is inconsistent (such as behavior) or the assay is difficult (as in some kinds of histology).

The GAL4–enhancer-trap technique offers the additional advantage of circumventing problems associated with insertion site effects on promoter fusions. Since the same GAL4–enhancer-trap insert can work independently on any other transgene that has an upstream-activating sequence (UAS), its pattern of expression can be assessed with a UAS-*lacZ* or UAS-GFP strain separately from its use for ectopic expression of a *UAS*-effector gene (Brand and Perrimon 1993). This technique has been used to study developmental effects of ectopic expression of *even-skipped* in embryos, *Raf* expression in oogenesis (Brand and Perrimon 1993, 1994), and behavioral effects of regional sexual transformation (Ferveur et al. 1995; O'Dell et al. 1995). Such GAL4 lines can also selectively trap enhancers of a complex gene, as exploited by Vincent et al. (1994) to separate temporal elements of *Ubx* regulation.

GAL4 has the additional advantage of partial temperature sensitivity, i.e., it is more active at 29°C (closer to yeast's optimal growth temperature) than at 18°C. On the other hand, its disadvantage is that some GAL4 strains show inconsistent patterns of expression or sensitivity to changes in genetic background. These problems can be controlled by assessing expression patterns in a suitable number of individuals for each strain and keeping genetic background consistent.

Inducible expression of GAL4 has been achieved by combining the GAL4 system with other systems. To place activation of the *UAS* transgene under temporal control, a UAS vector has been made that is blocked by a large sequence flanked by FRTs, analogous to the *FLP/FRT* transgene of Struhl et al. (1993) described in Chapter 5 (see p. 115). Heat-shock induction of hs-FLP excises this blocking sequence and turns on the transgene irreversibly (Sweeney et al. 1995). Similarly, as described in Chapter 5, the RU486-dependent variant of GAL4 confers temporal on-and-off control over any *UAS* transgene. Tetracycline/doxycycline similarly allows temporal control over on-and-off activation of any transgene placed downstream from the *tetO* control sequence (Bello et al. 1998).

A variation on the GAL4 system that can be used for subdividing expression patterns uses the yeast GAL80 transcriptional repressor (Lee and Luo 1999). GAL80 binds to the same UAS target sequence as GAL4 and blocks transcription of any transgene linked to it. A fly that carries a *GAL80* transgene causing expression in a pattern that differs from, but overlaps with, that of a *GAL4* transgene in the same fly will result in transcription off of the *UAS* transgene, but only in that portion of the GAL4 pattern that does not overlap with the GAL80 pattern (e.g., Kitamoto 2002). Davis (2003) incorporated a temperature-sensitive variant of

GAL80 into a transgene driven by the αTub84B promoter, allowing temporal control over the GAL80-induced inhibition of GAL4.

CELL ABLATIONS

Promoter fusions and GAL4-enhancer traps have been effectively used to drive genetic cell ablations (autonomous expression of cytotoxic gene products). This technique has helped to overcome a nagging sense of inferiority that fly investigators experience when listening to the nematode litany of "advantages of the organism." (Another is the ability to freeze the animals in liquid nitrogen, a technique that is equally possible in flies and worms, but with somewhat different outcomes.) Initial efforts with diphtheria toxin and ricin expressed under tissue-specific and temporal control (Moffat et al. 1992; Sentry et al. 1993) generally ended up on the rocks because of the problem of leaky expression of toxin. (It does not take much.)

The identification of the cell death genes *reaper* (*rpr*) (White et al. 1996) and *head involution defective/Wrinkled* (*hid*) (Grether et al. 1995), and their incorporation into UAS vectors, has made cell-specific ablation efficient and accurate (e.g., see Renn et al. 1999). Both genes act dominantly to kill cells in which they are expressed.

For neurobiological purposes, several UAS-driven transgenes that modify neuronal function produce a sort of physiological ablation. The light chain of tetanus toxin can block synaptic transmission in neurons and be expressed chronically under GAL4, or it can be activated by FLP-induced excision of a blocking sequence (Sweeney et al. 1995).

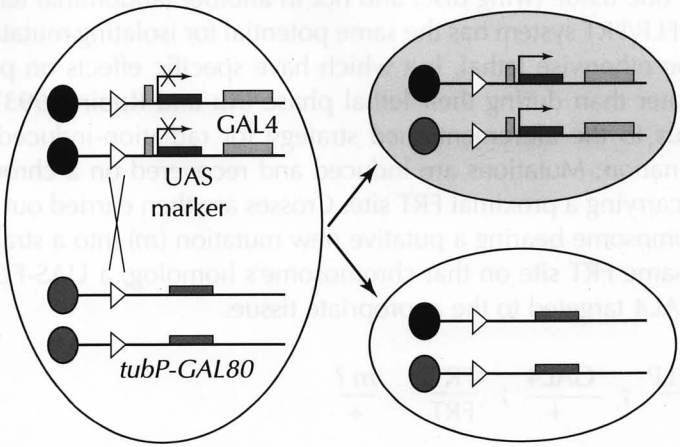
Transient blockage of neuronal activity by means of a tissue-specific conditional mutation has been very effectively achieved using a mutant allele of the *shibire* locus (shi^{ts1}) (Grigliatti et al. 1973), which encodes the fly version of *Dynamin* (Chen et al. 1991), incorporated into a UAS vector (Kitamoto 2001).

GAL4 TARGETED FLP/FRT RECOMBINATION

The tissue specificity of the GAL4 system has been merged with the site specificity of mitotic recombination of the FLP/FRT system to produce an efficient and targeted method of inducing mitotic clones. The key element is incorporation of the FLP recombinase into a UAS vector (Xu and Rubin 1993; Stowers and Schwarz 1999), allowing GAL4-induced expression of

FLP in a tissue-specific manner. It then operates on any homologous FRT sites in the targeted cells to initiate the exchange event. As in all cases of mitotic recombination, the cell in which it occurs must still be capable of dividing further for the event to produce homozygous clones.

The most elaborate effort to combine GAL4 with FLP-induced mitotic recombination involves the original use of GAL80 to restrict GAL4 expression patterns in the fly (Lee and Luo 1999). In contrast to most clonal marking strategies, in which the induction of a mitotic clone is detected by the *loss* of a cell marker, this strategy "uncovers" the marker in the induced clone by permitting its expression. This mosaic marking system, "MARCM," requires that a *GAL80* gene, expressed ubiquitously under the control of the β-tubulin promoter, be placed heterozygous with the desired GAL4, with the same proximal FRT inserts on each homolog. The *GAL80* blocks expression of any UAS construct present in the genome. Then, when a mitotic recombination event is induced on this arm by activating FLP, one daughter cell receives both GAL4s and no *GAL80*, thus releasing GAL4-induced expression of the UAS insert. (In the figure below, the UAS is shown as being on the same arm as the GAL4, but this is not a requirement.)



The MARCM system. Mitotic recombination between two FRT sites (*triangles*) results in loss of the repressor transgene (*tubP-GAL80*) in one of the daughter cells, releasing GAL4-dependent expression of the marker transgene (*UAS-mCD8-GFP*). (Redrawn, with permission, from Lee T. et al. 1999. © 1999 The Company of Biologists Limited.)

This technique permits a large degree of control over the clone size, down to single cells. When used with suitable transgene markers, the detailed morphology of individual neurons can be obtained. This would be inconceivable if the single cell was unmarked in a surrounding sea of marked cells.

Isolating Tissue-targeted Mutations Using Mitotic Recombination

Many genes that are vital to the fly have multiple roles in development and behavior and multiple stages in the fly's life span. Just as mosaics allow the lethal phase of a mutation to be bypassed to study its effects on later stages of development, they also permit the isolation of mutations targeted to specific tissues or phenotypes long after a whole-animal mutant would have died. The first use of this approach was directed at isolating cell lethal mutants in the wing disc (Ripoll and Garcia-Bellido 1973). In their scheme, *X*-linked lethal mutations were isolated on a chromosome already carrying markers and subsequently tested for cell autonomous lethality in clones produced by X-ray-induced mitotic recombination. Failure to recover clones of the homozygous marker indicated cell autonomous lethality. This permitted recovery of mutations that were lethal in one tissue (wing disc) and not in another (abdominal tergites).

The FLP/FRT system has the same potential for isolating mutations that would be otherwise lethal, but which have specific effects on particular tissues later than during their lethal phase (Xu and Rubin 1993). This is analogous to the aforementioned strategy for radiation-induced mitotic recombination: Mutations are induced and recovered on a chromosome already carrying a proximal FRT site. Crosses are then carried out to place this chromosome bearing a putative new mutation (*m*) into a strain carrying the same FRT site on that chromosome's homolog, a UAS-FLP insert, and a GAL4 targeted to the appropriate tissue.

$$\frac{\text{UAS-FLP}}{+} \; ; \; \frac{\text{GAL4}}{+} \; ; \; \frac{\text{FRT}}{\text{FRT}} \quad \frac{m\,?}{+}$$

A good exercise at this stage is to go through the scheme required to produce this genotype, paying attention to the possible problems associated with premature combining of GAL4, UAS-FLP, and FRT. The FLP/FRT system makes the process more efficient and directed than radiation-induced

mitotic recombination, producing a higher frequency of clones and limiting the clones to a particular tissue, stage, or both.

P-element Replacement for Exploitation of Existing Enhancer Traps

With all of the variations on multicomponent expression systems—GAL4, GAL80, GAL4-progesterone receptor, *tTA*, and *rtTA-M2-alt*—there is a clear need to be able to replace one kind of enhancer trap with another. Fortunately, if two P elements are in the genome and transposition is induced, a certain portion of the events will involve replacement of one by the other. If the two elements are on different chromosomes and distinguishably marked (e.g., *white*$^+$ and *yellow*$^+$), the replacement event can be detected and confirmed (Nassif et al. 1994; Lankenau et al. 1996; Sepp and Auld 1999). Detection initially involves showing that the linkage of the replacing element has changed from its original chromosome to that of the element to be replaced, with concomitant loss of the original element from that chromosome. Assuming that this has occurred, the accuracy of the replacement can be confirmed by PCR. Although the rate of such replacement is not high (0.3–1.4%), it beats rescreening expression patterns.

If a specific site in the genome is particularly attractive for repeated replacement events, an FRT-containing P element provides a landing platform for efficient and accurate insertion of sequences flanked by FRTs (Golic et al. 1997).

CHAPTER 7

Commencement

You have now completed the training and hazing process and are ready to go out into the world and begin pushing flies in earnest. It is hoped that by this time you have learned the principles at an intellectual level, but there is no substitute for doing crosses yourself with real flies in real time. That is the only way to get a proper feel for how it works and for its rhythms and pitfalls.

As a last reminder, several points bear repeating and remembering as you make your way through the maze of planning and executing genetic schemes:

- You will never overestimate the number of flies needed for a multigenerational scheme. Since it hurts to start over again after 3 or 4 months, even a tenfold excess over what you calculate as a minimum is not out of line for large schemes with many generations.
- If there are two ways to construct a strain, do both. Many schemes look great on paper but fail miserably (and sometimes unaccountably) in the bottle. If you will not know this for a couple of months, this step saves you from having to start over.
- Arrange your crosses to compensate for nonvirginity wherever possible. This is especially important if your social life should suddenly become complicated and you miss the odd virgin collection.
- Run small-scale pilots before undertaking large crosses. It permits you to test your stocks and avoid time-wasting failures.
- Do not be discouraged if you are not successful at first. You will acquire a feel for fly pushing with time. Moreover, the flies frequently require

that you do an apprenticeship on any important project—they will not start to perform until they are certain that you are serious.

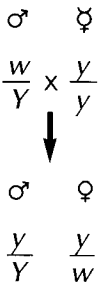
The final exhortation is best expressed by Shakespeare in *King Lear:*

The wren goes to't, and the small gilded fly
Does lecher in my sight.
Let copulation thrive...

Solutions to Problems

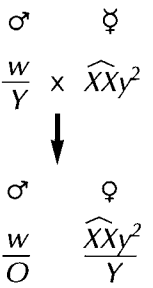
SOLUTION 1

Normal



Sons receive one of mother's *X*s and father's *Y*; daughters receive father's *X*.

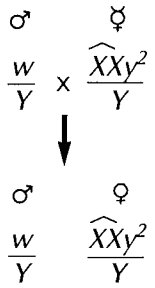
Attached-*X*



Sons receive father's *X* and daughters receive mother's attached-*X* and father's *Y*, producing *XO* sterile sons.

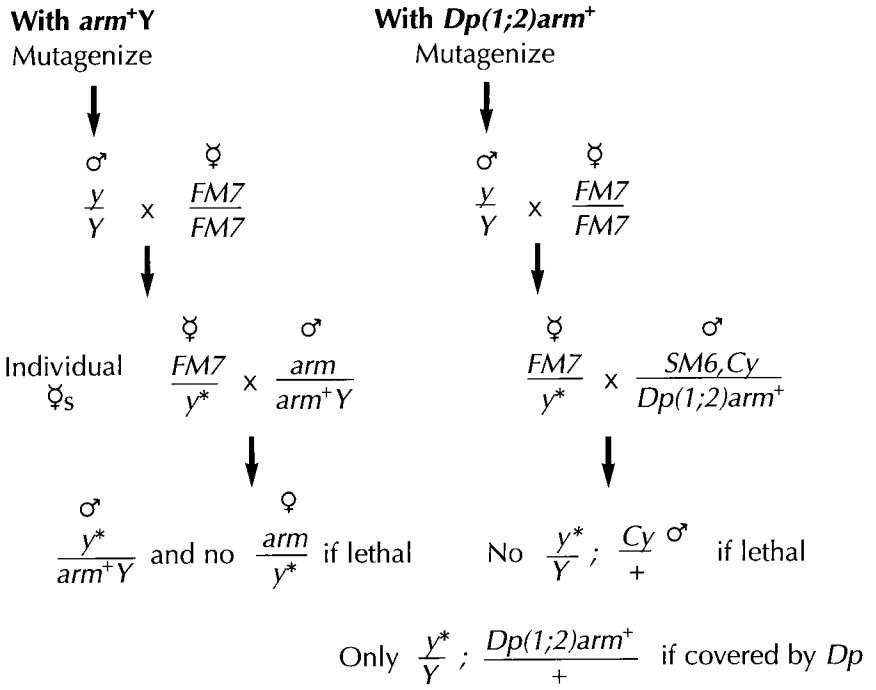
versus when the mother also carries a *Y*

Attached-*X* with *Y*



Sons and daughters both receive *Y*s, making sons fertile and having no effect on daughters.

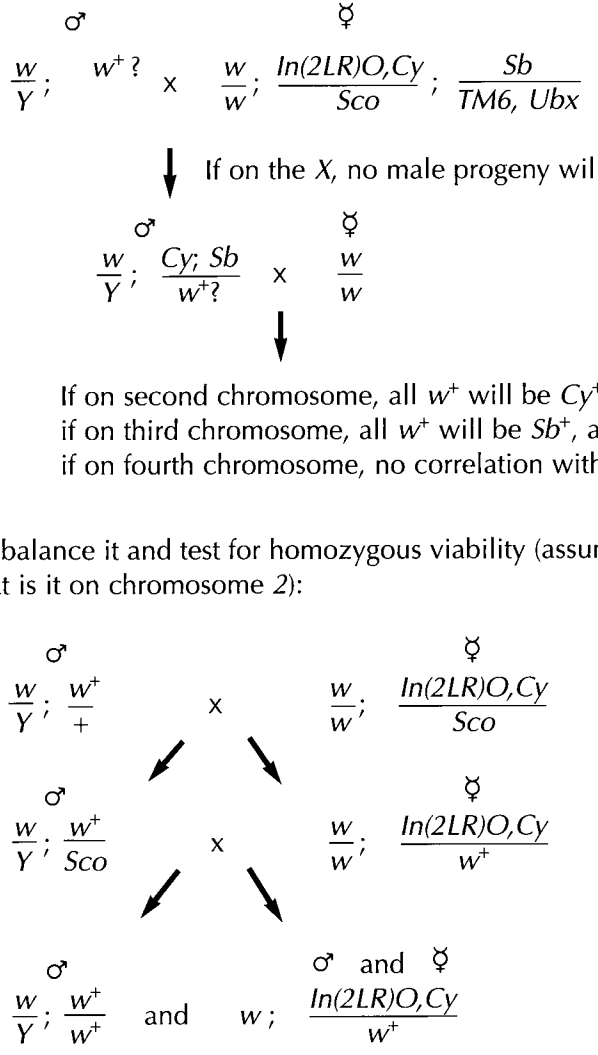
SOLUTION 2



SOLUTION 3

You are mutagenizing males hemizygous for an *X*, so any *X*-linked lethals in the stock would not appear in the adult males.

SOLUTION 4



To balance it and test for homozygous viability (assuming, hypothetically, that is it on chromosome *2*):

Viability test Balanced stock

SOLUTION 5

$\frac{v\ x\ ?}{FM7} \times \frac{\widehat{XX}\ yf}{Y}$ *x ?* symbolizes the new mutation introduced from ♀ in case it affects fertility

↓

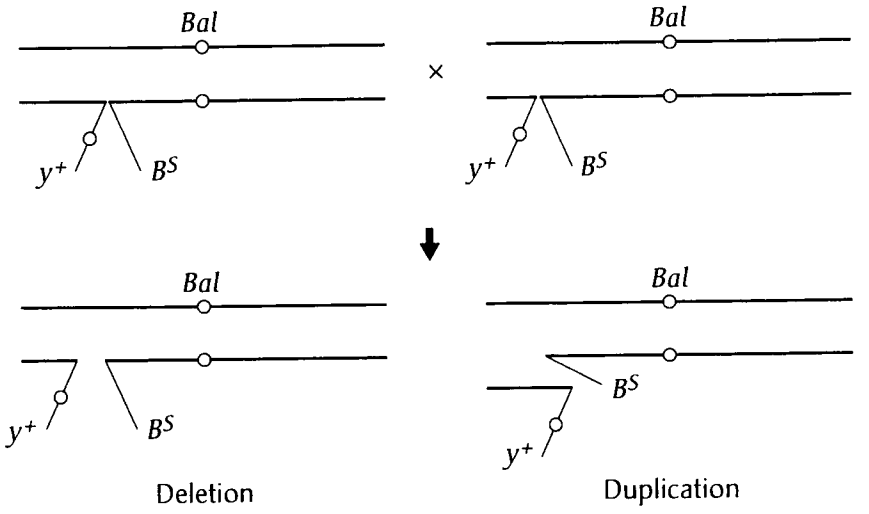
$\frac{v\ x\ ?}{yf} \times \frac{FM7}{Y}$

↓

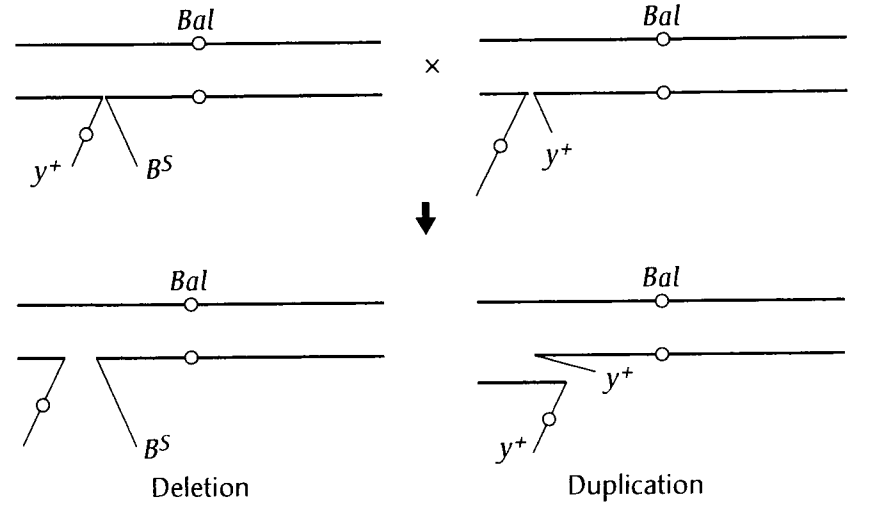
Individual ♂ progeny x $\frac{\widehat{XXy^2}}{Y}$ to start new stock from each

SOLUTION 6

When both *T(Y;A)*s have breakpoints in the same *Y* arm, then duplications and deletions look just like euploids: All have y^+ and B^s.



When breakpoints are in different *Y* arms, but one has lost B^s, as is the case with many of these stocks, duplications and deletions are distinguishable.



SOLUTION 7

$$\frac{w}{Y};\ \frac{P[w^+\ h^+]}{P[w^+\ h^+]} \times \frac{w}{w};\ \frac{TM6,Ubx}{h}$$ (For construction of this stock, see below.)

↓

$$\frac{w}{w};\ \frac{P[w^+\ h^+]}{h} \times \frac{w}{Y};\ \frac{TM3,Ser}{Sb}$$

↓

Individual ♂ $\frac{w}{Y};\ \frac{P[w^+\ h^+]\ h?}{TM3,Ser} \times \frac{w}{w};\ \frac{TM6,Ubx}{h}$

♂ and ☿

↙ ↘

$\frac{w}{Y};\ \frac{P[w^+\ h^+]\ h?}{h}$ and $w;\ \frac{TM6,Ubx}{P[w^+\ h^+]\ h}$

None of this class of progeny will survive if *h* is now linked to w^+.

Recover as balanced stock.

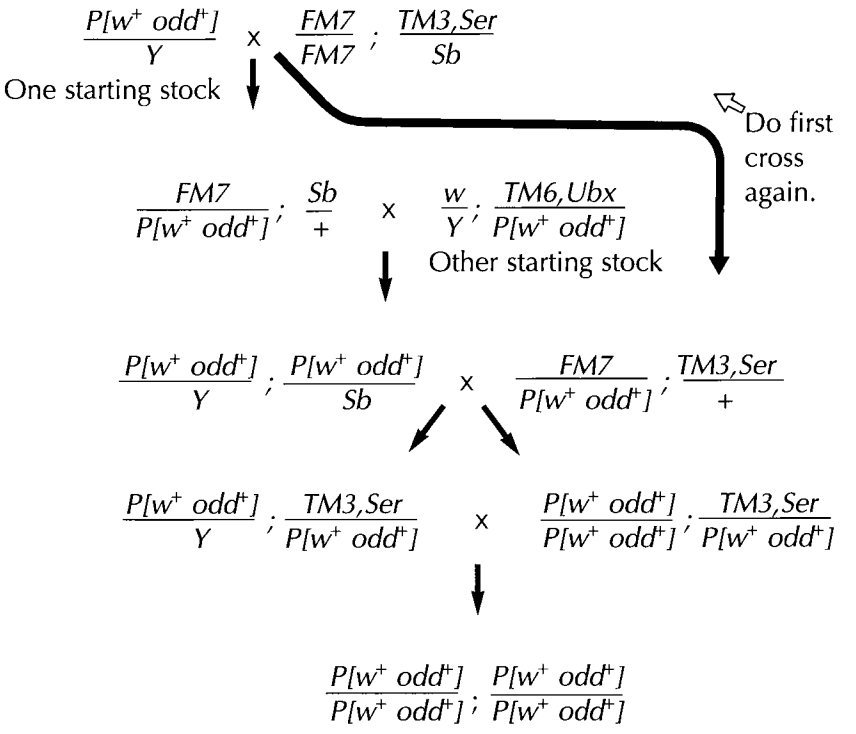
$$\frac{w}{w};\ \frac{TM3,Ser}{Sb} \times \frac{TM6,Ubx}{h}$$

↓

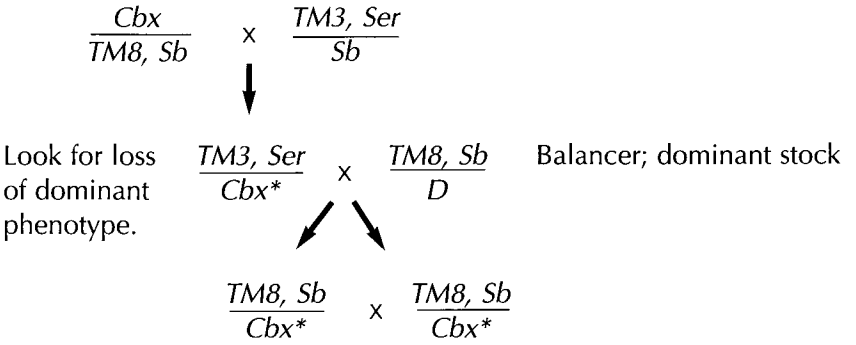
$$\frac{w}{Y};\ \frac{TM3,Ser}{h} \times \frac{w}{w};\ \frac{TM6,Ubx}{Sb}$$

$$w;\ \frac{TM6,Ubx}{h}$$

SOLUTION 8



SOLUTION 9



SOLUTION 10

On the *X*:

Mutagenize

$$\frac{y}{Y} \times \frac{\widehat{XXy^2}}{Y} ; \frac{In(2LR)O,Cy}{Sco}$$

$$\downarrow$$

$$\frac{y^*}{Y} ; \frac{Sco}{+}$$

On chromosome *3*:

Mutagenize

$$\frac{e}{e} \times \frac{TM6B,D}{Sb}$$

Handy stock to have $\frac{In(2LR)O,Cy}{Sco} ; \frac{TM6,Ubx}{Sb} \times \frac{e^*}{TM6B,D}$

$$\frac{In(2LR)O,Cy}{+} ; \frac{TM6B,D}{Sb} \times \frac{Sco}{+} ; \frac{e^*}{TM6,Ubx}$$

$$\frac{Sco}{In(2LR)O,Cy} ; \frac{TM6B,D}{e^*} \times \frac{Sco}{In(2LR)O,Cy} ; \frac{TM6B,D}{e^*}$$

$$\downarrow$$

$$\frac{Sco}{In(2LR)O,Cy} ; \frac{e^*}{e^*}$$

Cannot be done on chromosome *2* because *Sco* is homozygous lethal.

SOLUTION 11

The final, mosaic-producing cross will consist of the following:

$$\frac{Hk}{Hk};\ \frac{ca^{nd}}{ca^{nd}} \quad \times \quad \frac{y}{Y}$$

$$\downarrow$$

$$\frac{Hk}{y};\ \frac{ca^{nd}}{+} \quad \text{Potential mosaics}$$

To get there,

$$\frac{FM7}{FM7} \quad \times \quad \frac{TM3,Ser}{Sb}$$

$$\downarrow \qquad \searrow$$

$$\frac{Hk}{Hk} \times \frac{FM7}{Y};\ \frac{TM3,Ser}{Sb} \qquad \frac{FM7}{+};\ \frac{Sb}{+} \quad \times \quad \frac{TM6,Ubx}{ca^{nd}}$$

$$\downarrow \qquad\qquad\qquad\qquad \downarrow$$

$$\frac{FM7}{Hk};\ \frac{TM3,Ser}{+} \qquad \times \qquad \frac{FM7}{Y};\ \frac{Sb}{ca^{nd}}$$

$$\swarrow \quad \downarrow$$

$$\frac{FM7}{Hk};\ \frac{TM3,Ser}{ca^{nd}} \quad \times \quad \frac{Hk}{Y};\ \frac{TM3,Ser}{ca^{nd}}$$

$$\downarrow \qquad \searrow$$

$$\frac{Hk}{Hk};\ \frac{ca^{nd}}{ca^{nd}} \quad \text{and} \quad Hk;\ \frac{TM3,Ser}{ca^{nd}} \quad \text{♂ and ♀}$$

For mosaics For keeping the stock

Glossary

amorph a mutation that functionally inactivates a gene by producing either no product or a nonfunctional product (synonym: null allele)

aneuploidy individual, gamete or cell, that contains too little or too much genetic material

antimorph mutation that shows anomalous complementation such that the individuals are more severely affected when heterozygous with another mutation than when homozygous

balancer chromosome containing multiple inversions and markers that facilitate crossing schemes by their ease of detection and also by their suppression of recombination between homologs

complementation genetic test of allelism in which one mutation is placed heterozygous with another and scored for normal (complementing) versus abnormal (noncomplementing) phenotype

compound chromosome rearrangement in which both homologous arms of a chromosome are attached to the same centromere (also called "attached")

deficiency rearrangement in which a piece of a chromosome is excised and the remaining large pieces reattached (deletion)

deletion see "deficiency"

diploid containing the normal complement (two sets) of chromosomes (adjective: diplo-)

distal in the direction of the tip of a chromosome arm

duplication rearrangement in which an extra piece of chromosome is attached to or inserted into an ectopic site

enhancer (1) mutation or genetic variant at one locus that exacerbates the phenotype of a mutation at another locus; (2) regulatory DNA sequence that influences transcription of nearby gene(s)

enhancer trap technique in which a gene introduced into an ectopic chromosomal site is expressed in a restricted pattern because of its proximity to endogenous enhancers; also refers to the strain carrying such an insertion

euploid individual, gamete or cell, containing its appropriate and normal amount of genetic material

haploid containing a single set of chromosomes (adjective: haplo-)

haploinsufficient locus or chromosomal region in which a single dose of wild type does not produce a normal phenotype

hemizygous chromosome or chromosomal region present in only one dose

hypermorph mutation in which the gene product is produced at higher levels than normal or is more active than normal

hyperploid containing more than the normal amount of genetic material

hypomorph mutation in which the gene product is produced at lower levels than normal or is less active than normal

insertion ectopic presence of a DNA sequence or chromosomal region

inversion rearrangement in which part of the normal sequence of a chromosome is reversed

metacentric chromosome (or rearrangement) in which the centromere is flanked on both sides by major chromosome arms

mosaic individual whose cells are not all of the same genotype

neomorph mutation in which the gene product functions as if performing a new function, as opposed to no function (amorph), too little function (hypomorph), or too much function (hypermorph)

null see "amorph"

proximal in the direction of the centromere

suppressor mutation or genetic variant at one locus that rescues or mitigates the phenotype of a mutation at another locus

translocation rearrangement in which two different chromosomes are broken and rejoined such that they have exchanged pieces

transposition rearrangement in which a piece of a chromosome is excised and reinserted elsewhere in the same chromosome

triploid individual or cell containing three sets of chromosomes

Appendix: Fly Resources

BOOKS

Ashburner M., Hawley S., and Golic K. 2004. Drosophila: *A Laboratory Handbook,* 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

> The "Talmud" of fly genetics with referenced information on all aspects of fly genetics, biology, and molecular biology.

Sullivan W., Ashburner M., and Hawley R.S. 2000. Drosophila *Protocols.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

> The definitive laboratory manual of procedures for molecular, biochemical, and cellular studies on *Drosophila.*

FLYBASE

A computerized database for information on fly genetics, molecular biology, and related matters can be found at the web site

> http://flybase.bio.indiana.edu/

FlyBase includes the following:

- Information on genes and mutant alleles.
- Information about the expression and properties of transcripts and proteins.
- Information on the functions of gene products.
- Nucleic acid accession numbers linked from gene records.

- Protein sequence accession numbers linked from protein records.
- Information about natural and engineered transposons and other molecular constructs.
- Lists of genomic clones.
- Descriptions of chromosomal aberrations.
- Descriptions of *Drosophila* stocks held in stock centers and private laboratories.
- Images that illustrate *Drosophila* anatomy and development terms.
- A bibliography of *Drosophila* citations.
- An address book of *Drosophila* researchers.
- *Drosophila* genetic, cytological, and molecular map information.
- Berkeley Drosophila Genome Project data.
- European Drosophila Genome Project data.
- Allied databases.
- A searchable archive of bionet.drosophila postings.

FLY STOCK COLLECTIONS

Baylor Gene Disruption Project (BGDP)
Information about ordering P{GT1}(BG), P{SuporP}(KG), and P{Epgy2}(EY) lines from Baylor College of Medicine, Texas can be found at
http://flypush.imgen.bcm.tmc.edu/pscreen/

The Berkeley Drosophila Genome Project
http://www.fruitfly.org/

Bloomington Stock Center
A large collection of stocks at Indiana University can be accessed at
http://flystocks.bio.indiana.edu/

DrosDel Project
Information about ordering isogenic deficiency lines at the University of Cambridge, U.K. can be found at
http://www.drosdel.org.uk/

Ehime Drosophila Species Stock Center of Japan
A collection of wild-type strains of *D. melanogaster* and strains of some 30 species can be found at
http://kyotofly.kit.ac.jp/ehime/

Exelixis flyStation Stock Collection
P{EP} insertion lines at Exelixis, Inc., California can be obtained at
http://flystation.exelixis.com

FlyTrap Stock Collection
Green fluorescent protein (GFP) trap lines from the FlyTrap project can be obtained from Yale University at
http://flytrap.med.yale.edu/

FlyView Stock Collection
Enhancer-trap lines from the FlyView project can be obtained from the University of Muenster, Germany at
http://flyview.uni-muenster.de/

Japanese Laboratories
Stock lists from individual laboratories in Japan are available at
http://www.grs.nig.ac.jp/fly/CENTER.e.html/

Kyoto Stock Center
Information about ordering P-element insertion lines and deficiency or duplication kits from the Kyoto Institute of Technology, Japan can be found at
http://dbs.kit.ac.jp/stocks/

Szeged Stock Centre
Information about ordering P{lacW}, P{EP}, P{RS3}, and P{RS5} insertion lines at the University of Szeged, Hungary can be obtained at
http://gen.bio.u-szeged.hu/gen/

Tucson Stock Center
Approximately 270 different *Drosophila* species from the University of Arizona can be accessed at
http://stockcenter.arl.arizona.edu/

DROSOPHILA NETWORK RESOURCES (from FlyBase)

Atlases and Images

Electron micrograph maps of *Drosophila melanogaster* polytene chromosomes:
http://www.helsinki.fi/~saura/EM/index.html/

FlyBrain, an online atlas and database of the *Drosophila* nervous system:
http://flybrain.uni-freiburg.de/
http://flybrain.neurobio.arizona.edu/
http://flybrain.iam.u-tokyo.ac.jp/

FlyTrap, a GFP trap database:
http://flytrap.med.yale.edu/

Flytrap, a database of P{GAL4} enhancer traps and their expression in brains:
http://www.fly-trap.org/

FlyView, a *Drosophila* image database:
http://flyview.uni-muenster.de/

FlyMove, an online view of *Drosophila* embryonic development:
http://flymove.uni-muenster.de/

Patterns of gene expression in *Drosophila* embryogenesis:
http://www.fruitfly.org/cgi-bin/ex/insitu.pl/

Protein trap database, a GFP embryo expression database:
http://biodev.obs-vlfr.fr/gavdos/protrap.htm/

Scans of Bridges' original 1935 polytene drawings for *D. melanogaster:*
http://www.hawaii.edu/bio/Chromosomes/poly/poly.html/

Comparative Analyses and Genome Databases

CluSTr protein sequence similarity analysis of *Drosophila:*
http://www.ebi.ac.uk/proteome/DROME/clustr/table.html/

euGenes, genomic information for eukaryotic organisms, including *Drosophila:*
http://iubio.bio.indiana.edu/eugenes/

Homophila, human disease to *Drosophila* gene database:
http://homophila.sdsc.edu/

InterPro protein domain analysis of *Drosophila:*
http://www.ebi.ac.uk/proteome/DROME/interpro/stat.html/

The McGill *Drosophila melanogaster* genome project:
http://www.mcgill.ca/Biology/labs/MDGP/home.html/

Proteome analysis of *Drosophila:*
http://www.ebi.ac.uk/proteome/index.html?
http://www.ebi.ac.uk/proteome/DROME/

TaxoDros, the database on taxonomy of Drosophilidae:
http://taxodros.unizh.ch/

Whole-genome comparative analysis of *D. melanogaster* and *D. pseudoobscura:*
http://pipeline.lbl.gov/pseudo/

Sequence Analyses

cis-analyst, to search the *Drosophila* genome for clusters of binding sites:
http://rana.lbl.gov/cis-analyst/

Fly Enhancer, to search the *Drosophila* genome for clusters of binding sites:
http://www.flyenhancer.com/

Gene finder for *Drosophila:*
http://www.softberry.com/berry.phtml?topic=gfind&prg=FGENESH/

Genie gene finder for *Drosophila:*
http://www.fruitfly.org/seq_tools/genie.html/

GenePalette, a tool for genome sequence visualization and navigation:
http://www.genepalette.org/

GRAIL gene finder for *Drosophila:*
http://compbio.ornl.gov/Grail-1.3/

McPromoter, promoter prediction for *Drosophila:*
http://genes.mit.edu/McPromoter.html/

Neural network promoter prediction for *Drosophila:*
http://www.fruitfly.org/seq_tools/promoter.html/

Pattern Search, to search for short sequences in *Drosophila* DNA:
http://www.fruitfly.org/seq_tools/patscan.html/

Splice site prediction for *Drosophila:*
http://www.fruitfly.org/seq_tools/splice.html/

Target Explorer, to identify clusters of transcription factor binding sites:
http://trantor.bioc.columbia.edu/Target_Explorer/

TransTerm, a translational signal database:
http://uther.otago.ac.nz/Transterm.html/

Miscellaneous

Drosophila Information Service:
http://www.ou.edu/journals/dis/

Interactive Fly, a guide to *Drosophila* genes and their roles in development:
http://flybase.bio.indiana.edu/allied-data/lk/interactive-fly/aimain/1aahome.html/

Jfly, a data depository for the fly and other insects, with an emphasis on serving the Japanese-speaking fly community:
http://jfly.iam.u-tokyo.ac.jp/

Tracheal database, information on tracheal development in *Drosophila:*
http://www.biozentrum.unibas.ch/affolter/trachea/

DROSOPHILA MATERIAL RESOURCES (also from FlyBase)

BAC genomic clones and filters
Three libraries of BAC clones are now available. These were all made from DNA of the same *y1; cn1 bw1 sp1* stock as was used for the Berkeley Drosophila Genome Project P1 clones.

- BACH and BACE clones (23,400 clones in pBeloBAC11; clone size ranges from 75 to 150 Kb) are available from MRC gene service at
  http://www.hgmp.mrc.ac.uk/geneservice/reagents/products/descriptions/dros_BAC.shtml/
- BACR clones (18,432 clones in pBACe3.6; average clone size is 160 Kb) are available from BACPAC Resources at
  http://www.chori.org/bacpac/dromel98.html/

cDNA (EST) clones and libraries

- BD Biosciences CLONTECH
  D. melanogaster embryo and larva 5′-STRETCH cDNA libraries can be found at
  http://www.clontech.com/products/cat/HTML/1186.shtml/
- Berkeley Drosophila Genome Project
  If you do not find the information you need, check the following for updates:
  http://www.fruitfly.org/about/materials/

- Brian Oliver Laboratory
  Testis cDNA clones and filters constructed by Justen Andrews in Brian Oliver's laboratory at the NIH are available from the MRC gene service in the U.K. Ordering information can be found at

  http://www.hgmp.mrc.ac.uk/geneservice/reagents/products/descriptions/dros_cdna.shtml/
- cDNA library aliquots
  Aliquots from a number of BDGP cDNA libraries are available from the laboratories on this list.
- Drosophila Gene Collection (DGC)
  For clones in DGCr1.0 or DGCr2.0, contact BACPAC Resources at

  http://www.chori.org/bacpac/drosocDNA.html/,

  Open Biosystems at

  http://www.openbiosystems.com/productPage.php?pageType=cdna.drosophila, or

  MRC gene service at

  http://www.hgmp.mrc.ac.uk/geneservice/reagents/products/cdna_resources/index.shtml/
- EST Project cDNA Clones
  BDGP cDNA clones from the EST collection are no longer available from ResGen/Invitrogen. If no DGC clone can substitute for the cDNA clone you need, requests for clones may be directed to the BDGP at

  http://www.fruitfly.org/about/materials/order.p1.html/
- Incyte
  Incyte offers cDNA clones from their LifeSeq Foundation database at

  http://www.incyte.com/control/researchproducts/tools/order/
- Novagen
  Novagen offers a *Drosophila* cDNA library at

  http://www.novagen.com/Products/ProductDetail_NVG.asp?catNO=69626/

Cosmid genomic clones and filters, European Drosophila Genome Project (EDGP)

EDGP cosmids are available from the MRC gene service at

http://www.hgmp.mrc.ac.uk/geneservice/reagents/products/descriptions/drosophila.shtml/

P1 genomic clones, Berkeley Drosophila Genome Project (BDGP)
Since the P1 library is largely obsolete, the BDGP discourages the use of P1 clones. If you have a compelling scientific reason that a corresponding BAC is not sufficient, you can direct requests for P1 clones to the BDGP at

http://www.fruitfly.org/about/materials/order.p1.html/

Sibling species genomic libraries

- Fosmid Libraries from BACPAC Resources
  Genomic fosmid libraries were constructed for the BDGP by Andreas Gnirke at Exelixis, Inc. (San Francisco, California) from *Drosophila erecta, D. pseudoobscura, D. littoralis,* and *D. willistoni.* The fosmid libraries are available from BACPAC Resources at

  http://bacpac.chori.org/drosofosmid.htm/
- Library from Novagen
  A genomic library in 1BlueSTAR vector constructed from *Drosophila virilis* is available at

  http://www.novagen.com/Products/ProductDetail_NVG.asp?catNO=69657&CatID=1478/

YAC genomic clones, Ian Duncan Laboratory
A complete set of YAC clones is maintained by Ian Duncan and clones may be requested from him. The contact for YAC clones is Ian W. Duncan, Department of Biology, Box 1137, Washington University, St. Louis, MO 63130-4899. Telephone: 314-935-6719; fax 314-935-4432; e-mail address: duncan@biology.wustl.edu/.

Arrays and Primers

- Affymetrix
  The Affymetrix GeneChip *Drosophila* genome array based on Release 1 annotations can be obtained at

  http://www.affymetrix.com/products/arrays/specific/fly.affx/
- Drosophila Genomics Resource Center (DGRC)
  DGRC produces and distributes DNA microarray slides. More information is available at

  http://cgb.indiana.edu/genomics/projects/dgrc/

- Eurogentec
  The Eurogentec ready-to-spot PCR sets for microarrays based on Release 2 annotations, DGC Release 1.0, and Sanger Center annotations can be obtained at

  http://www.eurogentec.be/code/en/page_02.asp?Page=377/

- FlyChip
  FlyChip is a publicly funded project to provide a nonprofit microarray resource for the U.K. *Drosophila* research community. More information is available at

  http://www.flychip.org.uk/

- ResGen
  ResGen MyArray DNA preestablished clone sets, either as ready-to-spot PCR products or clone glycerol stocks, are available at

  http://www.resgen.com/products/PEMAD.php3/

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