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CLONING THE RASPBERRY LOCUS OF *DROSOPHILA MELANOGASTER* : A LOCUS INVOLVED IN PURINE METABOLISM

BY

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A THESIS

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Date: May 1, 1986

"If I believe I cannot do something, it makes me incapable of doing it. But when I believe I can, then I acquire the ability to do it even if I didn't have it at the beginning..."

Mahatma Gandhi

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled CLONING THE RASPBERRY LOCUS OF *DROSOPHILA MELANOGASTER*: A LOCUS INVOLVED IN PURINE METABOLISM submitted by NORMA JEAN LEONARD in partial fulfilment of the requirements for the degree of Master of Science..

.....
(Supervisor)

.....

.....

.....

Date: May 1, 1986

To my family
(especially Vanessa)

ABSTRACT

Transposon tagging, generated by PM hybrid dysgenesis, was used to isolate a DNA segment from the cytological region 9E1-E4 on the X chromosome of *Drosophila melanogaster*. This region contains the raspberry complex locus, a locus involved in an unknown ma[illegible] purine metabolism in *Drosophila*. Within this region there exist three distinct complementation groups, the guanosine 1, purine 1 and raspberry loci. Guanosine 1 and purine 1 yield two kinds of purine nucleoside auxotrophic mutants; raspberry mutants interfere with pteridine synthesis. Many point mutations in this region produce a recessive lethal phenotype and display allelism to all three kinds of non-lethal mutation.

Five raspberry lethal *Drosophila* stocks were produced through PM hybrid dysgenesis. Four were shown to bear P-elements at the cytological region 9E by *in situ* hybridization. A recombinant DNA library was prepared from one such stock, NJ-5, using the vector EMBL4. Subsequent hybridization with a P element probe (pπ25.1) revealed a large number of P positive clones. *In situ* hybridization to P element free chromosomes confirmed that the recombinant bacteriophage, LF1, possessed the DNA segment homologous to the raspberry locus. The isolated DNA segment is a 19kb insert in the bacteriophage LF1 and possesses a minimum of 5 internal EcoRI sites. A tentative restriction map has been established utilizing four restriction enzymes (BglII, XhoI, EcoRI and HindIII). *In situ* hybridization of LF1 to an inversion stock, with a breakpoint in the raspberry gene, seems to indicate that the cloned DNA spans the

inversion breakpoint confirming that DNA homologous to the raspberry locus has been isolated. Further molecular analysis, starting from the raspberry clone LF1 should reveal the organizaton and function of the complex.

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LIST OF ABBREVIATIONS

AIRS	Phosphoribosyl aminoimidazole synthetase
AMP	adenosine 5' monophosphate (adenylate)
ADP	adenosine 5' diphosphate
ATP	adenosine 5' triphosphate
dADP	deoxyadenosine 5' diphosphate
dATP	deoxyadenosine 5' triphosphate
APRT	adenosine phosphoribosyl transferase
BSA	bovine serum albumin
CPM	counts per minute
CsCl	cesium chloride
DTT	DL-dithiothreitol
EDTA	ethylenediamine tetraacetate acid disodium salt
EtBr	ethidium bromide
FGARAT	phosphoribosyl formylglycineamidine synthetase
GARS	phosphoribosyl glycineamide synthetase
GART	phosphoribosyl glycineamide formyltransferase
GMP	guanosine 5' monophosphate
GDP	guanosine 5' diphosphate
GTP	guanosine 5' triphosphate
dGDP	deoxyguanosine 5' diphosphate
dGTP	deoxyguanosine 5' triphosphate
HCl	hydrochloric acid
HCO3	bicarbonate
HGPRT	hypoxanthine-guanine phosphoribosyl transferase
IMP	inosine 5' monophosphate (inosinate)
KCl	potassium chloride
LB	Luria-Bertani medium
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide
NaH_2PO_4	sodium phosphate monobasic
NaOH	sodium hydroxide
P_i	inorganic phosphate
PEG	polyethylene glycol
PR-	phosphoribosyl
SDS	sodium dodecyl sulphate
TE	tris-EDTA buffer
TM	tris-magnesium buffer
Tris	THAM - Tris (Hydroxymethyl) amino methane
XDH	xanthine dehydrogenase
XMP	xanthosine 5' monophosphate (xanthylate)
(1-32.8)	position of gene on chromsome 1, map position 32.8 map units

1. INTRODUCTION

Genetic analysis of well defined biochemical pathways in *Drosophila* promises insights into gene structure, gene action and regulation as they are adapted to the particular circumstances of multicellular eukaryotic organisms. The genetics of pyrimidine biosynthesis is already well-known. The more complex ramifications of purine metabolism suggest that its genetics will be even more informative.

Recently, it has been unequivocally demonstrated that a block in purine biosynthesis in *Drosophila melanogaster* yields an auxotrophic phenotype. It is also true that several less well defined auxotrophs are associated with an eye color defect, displaying reduced levels of pteridines, which are derived from purines.

One particular interesting instance is the *gua1-pur1-ras* complex in *Drosophila melanogaster*. This locus includes two different classes of purine nucleoside auxotrophs and an eye color mutant. The three are related in a complex manner in a gene complex that appears to be involved in purine metabolism and it's regulation. The biochemical origin of this complex is completely unknown. It is probable that the best means of analyzing the locus will be its molecular cloning and analyis.

I. PURINE METABOLISM

A. Purines

Purines are conjugated 5 and 6 membered heterocyclic rings that

function as intermediates in energy metabolism, components of group-transfer coenzymes and structural components within the cell (review in Henderson, 1972). In energy metabolism, the cleavage of the pyrophosphate bonds of ATP and GTP takes place in the cell. Cleavage of the pyrophosphate bonds frees energy used to drive many energetically unfavourable reactions. Purines function as group-transfer coenzymes transferring compounds such as sugars, sulfates, alkyl groups, acyl groups and hydrides. Structurally they form components of histidine, riboflavin, folic acid, thiamine and most importantly the nucleic acids RNA and DNA where they function as the basic unit of genetic information. Purines also act as substrates in the production of pteridines. Pteridines are of particular interest in *Drosophila* in that they produce imaginal eye pigments (Nash and Henderson, 1982).

Purines are found inside the cells predominantly in the form of nucleotides and polynucleotides (RNA and DNA).

B. Purine metaboism

i. *de novo* purine nucleotide biosynthesis

In most organisms, purine nucleotides can be produced in two general ways (Nash and Henderon, 1982). The first is *de novo* biosynthesis (Flaks and Lukens, 1963) diagrammed in Figure 1. Inosinic acid (IMP) is produced in ten steps. IMP is the common substrate for two diverging, two-step pathways leading to adenosine monophosphate (AMP) or to guanosine monophosphate (GMP).

Nucleoside monophosphates are available for phosphorylation to the respective di- and triphosphates. Deoxyribonucleoside diphosphates are produced by the reduction of ribonucleoside diphosphates by

FIGURE 1. The pathway of *de novo* purine biosynthesis.

The enzymes catalyzing each of the steps are as follows: The abbreviated symbols (in brackets) represent enzyme names (capital letters) or E.coli genes encoding each enzyme (lower case letters).

1. PP-ribose-P-amidotransferase (purF)
2. PR-gylcineamide synthetase (GARS) (purD)
3. PR-glycineamide formyltransferase (glycineamideribotide transformylase)(GART)
4. PR-formylglycineamidine synthetase (PR-formylglycineamide amidotransferase) (FGARAT) (purG)
5. PR-aminoimidazole synthetase (AIRS) (purI)
6. a carboxylase reaction (purE)
7. PR-aminoimidazolesuccinocarboxamide synthetase (purC)
8. adenylosuccinate lyase (purB)
9. PR-aminoimidazolecarboxamide formyltransferase (purH)
10. inosinate cyclohydrolase (purJ)
11. adenylosuccinate synthetase (purA)
12. adenylosuccinate lyase (purB)
13. inosinate dehydrogenase (guaB)
14. guanylate synthetase (xanthylate aminase) (guaA)

PR=phosphoribosyl

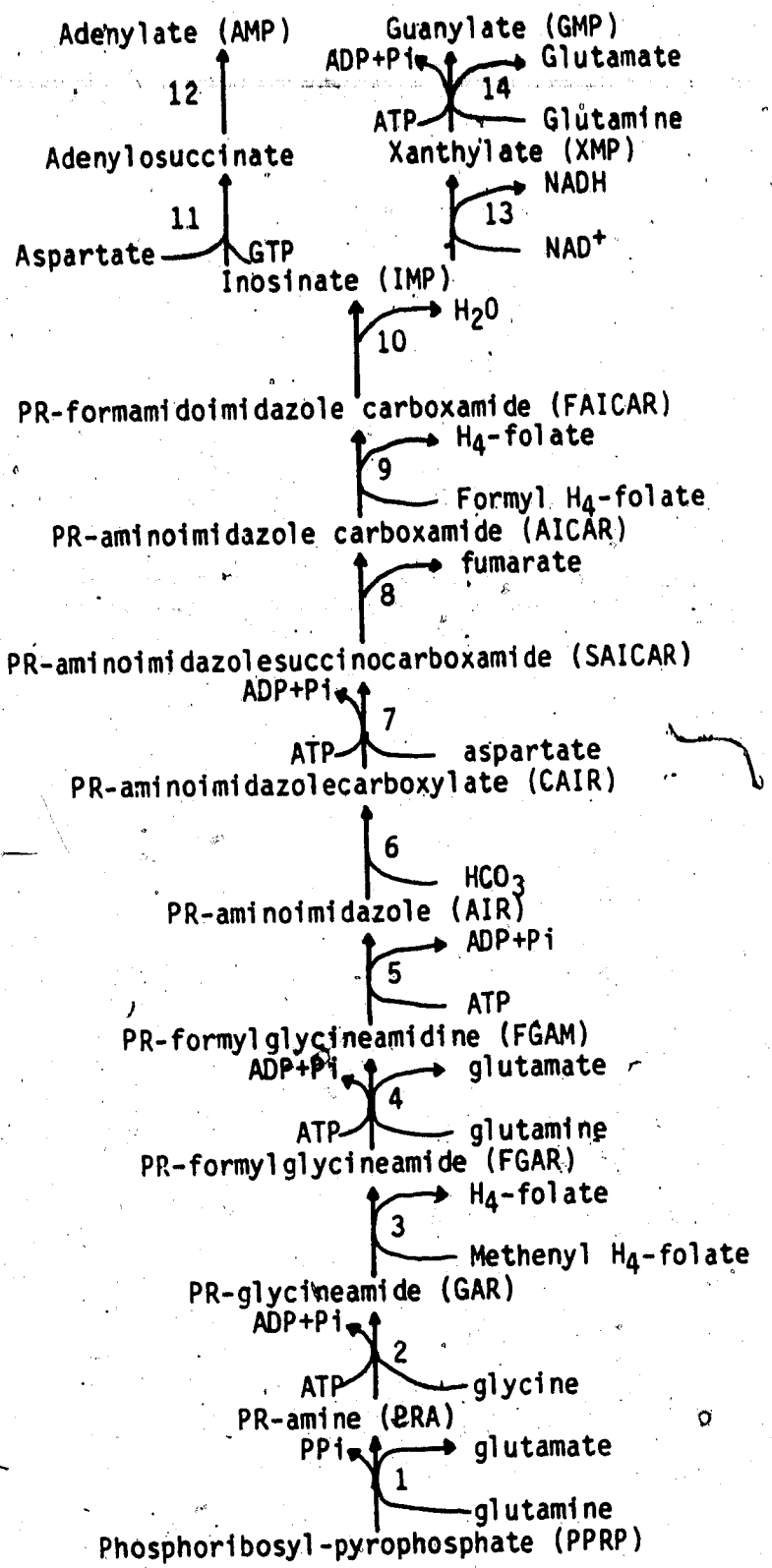


Figure 1. The pathway of *de novo* purine biosynthesis (Henderson, 1972)

ribonucleotide reductase. These pathways are illustrated only in Figure 2 for convenient use of space, but constitute a *bona fide* part of *de novo* biosynthesis as well as salvage synthesis.

ii. Purine salvage pathways

A second means for purine nucleotide production is a series of "salvage" reactions, utilizing purine bases and purine nucleosides to produce purine nucleotides (Figure 2). The substrates for these reactions are derived from nutritional sources or from breakdown of cellular nucleic acids or nucleotides.

iii. Interconversion of purines

There is interconversion of purine nucleotides between the purine nucleoside monophosphates (AMP, GMP and IMP) (Figure 2). Interconversion depends upon direct reconversion of AMP and GMP to their common precursor IMP. In addition, the potential for conversion of adenosine and adenine to inosine and hypoxanthine (Figure 3) provides secondary routes for conversion of AMP to IMP in some systems. No similar alternative routes are known for the conversion of GMP to IMP.

The enzyme responsible for the conversion of AMP to IMP is adenylate deaminase. In most organisms, GMP is converted to IMP by guanylate reductase activity. This enzyme however, may not be present in *Drosophila melanogaster* as will be discussed later (Sec. 1, I, C, iv.), so that interconversion is restricted in this species.

iv. Catabolism of purines

Cellular purines and purine derivatives are broken down to produce

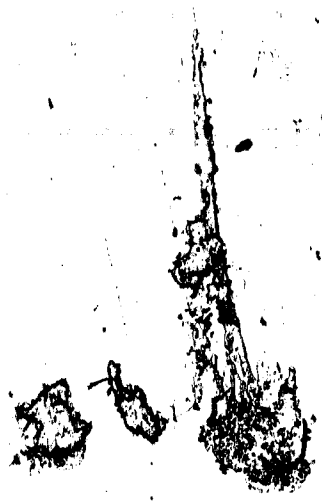


FIGURE 2. Salvage pathways and Interconversion of purine nucleotides
The names of the enzymes catalyzing each of the numbered steps are as follows: The abbreviated form (in brackets) are symbols used in the text

1. hypoxanthine-guanine phosphoribosyltransferase (HGPRT)*
2. inosine kinase
3. *de novo* biosynthetic pathway - last enzyme was inosinate cyclohydrolase
4. adenylosuccinate synthetase
5. adenylosuccinate lyase
6. adenylate deaminase
7. inosinate dehydrogenase
8. guanylate synthetase (xanthylate aminase
9. guanylate reductase**
10. adenine phosphoribosyl transferase (APRT)
11. adenosine kinase
12. hypoxanthine-guanine phosphoribosyltransferase (HGPRT)*
13. guanosine kinase

-nucleotide monophosphates are phosphorylated to form di- and triphosphates
-ribonucleotide diphosphates can be reduced to form deoxyribonucleotides

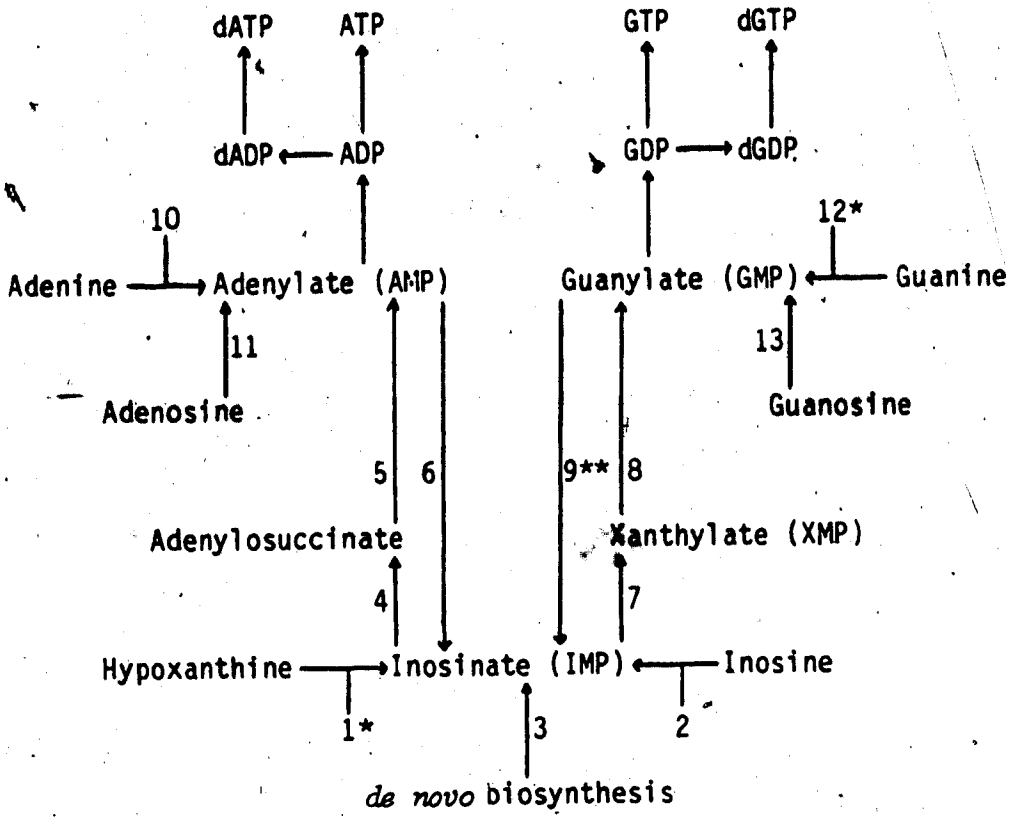


Figure 2. Salvage pathways and Interconversion of purine nucleotides (Henderson, 1972)

*this pathway has weak or reduced activity in *Drosophila*

**this pathway is probably absent in *Drosophila*

FIGURE 3. Catabolism of purine nucleotides

The names of the enzymes and/or the type of reaction involved at each step are as follows:

1. xanthine dehydrogenase (XDH) - oxidation reaction
2. inosine phosphorylase - phosphorolysis reaction
3. dephosphorylation reaction
4. adenylate deaminase - deamination reaction
5. guanylate deaminase (reductase)** - deamination reaction
6. dephosphorylation reaction
7. dephosphorylation reaction
8. xanthine phosphorylase - phosphorolysis reaction
9. adenosine deaminase - deamination reaction
10. adenosine phosphorylase - phosphorylsis reaction*
11. adenine deaminase - deamination reaction
12. guanosine phosphorylase - phosphorolysis reaction
13. guanine deaminase - deamination reaction

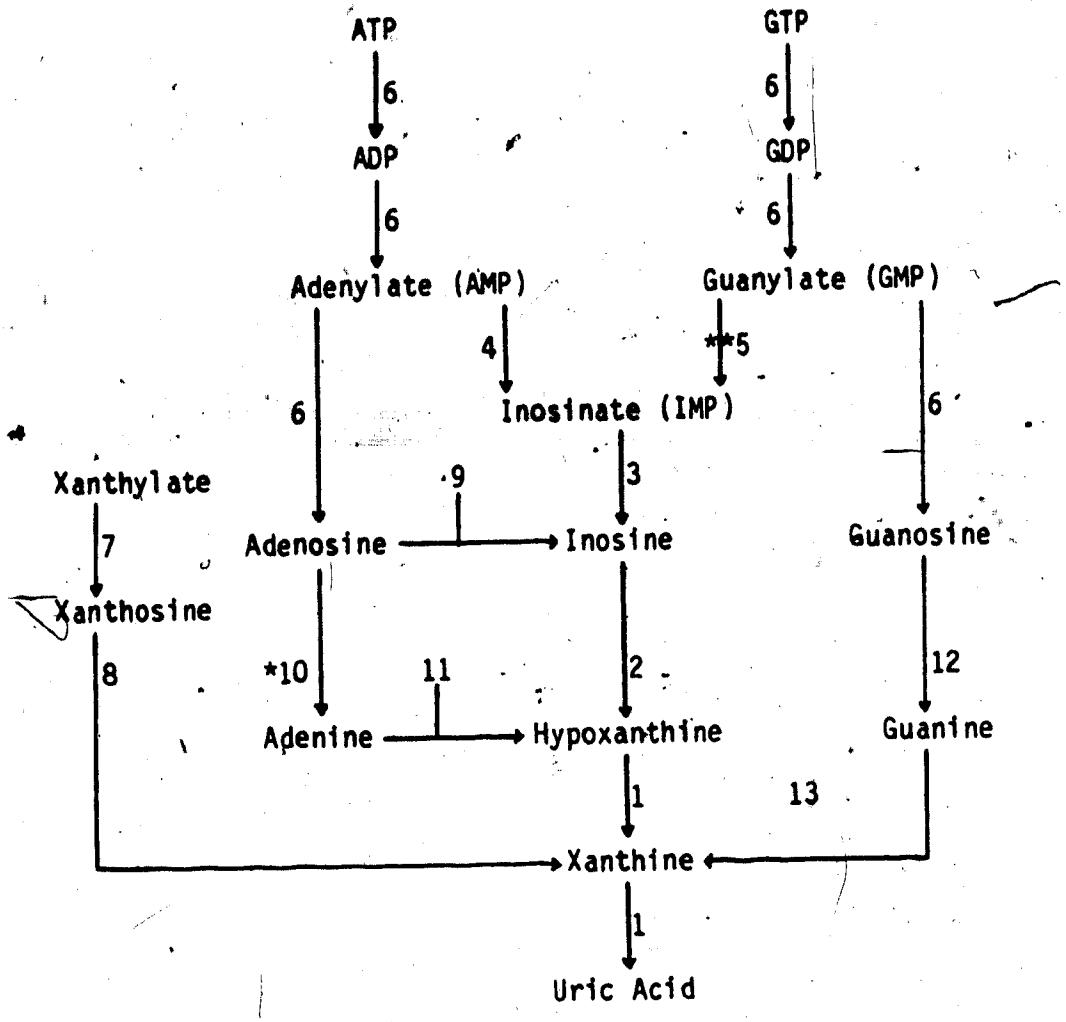


Figure 3. Catabolism of purine nucleotides

*this pathway has weak or reduced activity in *Drosophila*

**this pathway probably absent in *Drosophila*

excretory products. The catabolism of purine nucleotides involves four processes: dephosphorylation, deamination, phosphorolysis and oxidation as diagrammed in Figure 3.

C. Regulation of purine metabolism in prokaryotes

Fourteen biochemical steps are involved in *de novo* purine biosynthesis. Mutants in thirteen of the enzymes have been identified in E. coli and are associated with specific loci (see Figure 1). The genes are generally monocistronic operons scattered throughout the chromosome except for purJHD and guaAB which are two unlinked. multicistronic operons (Gots et al., 1977). The structural gene encoding the third enzyme in *de novo* purine biosynthesis has only recently been identified by Henikoff and coworkers (personal communication). An additional gene, purR encodes a repressor protein that represses seven of the enzymes involved in the synthesis of IMP (purE, purF, purI, purG and purJHD) (Benson and Gots, 1976). The guaAB operon does not respond to the purR repressor protein but is hypothesized to be regulated by a product of the guaR gene which may specify a trans acting regulatory element (Tiedemna and Smith, 1984). Transcription regulation of the other genes has not yet been demonstrated.

Feedback regulation plays an important role. The first enzyme PP-ribose-P amidotransferase (purF) is inhibited by both AMP and GMP derivatives to control the synthesis of IMP. The enzyme adenylosuccinate synthetase (purA) that converts IMP to AMP requires GTP to function. GMP and GDP compete with GTP for the site resulting in

competitive inhibition. Guanylate synthetase involved in the cnoversion of IMP to GMP requires ATP in a similar manner (Bagnara et al., 1974).

Feedback regulation may also play a role in the activity of the repressor protein purR. Studies indicate that the purE operator will only bind purR repressor protein in the presence of ATP whereas purF and purI require GTP. The differential action of the 2 nucleotide effectors suggests a model of regulation whereby gene expression is controlled jointly by the level of ATP and GTP in the cell (Koduri and Gots, 1980).

D. The enzymes of purine metabolism in *Drosophila melanogaster*

i. Studies of purine metabolism in *Drosophila*

The study of purine metabolism in *Drosophila melanogaster* has been an endeavor since 1942 when the purine base adenine was found to be toxic to fruit flies, reducing survival or slowing development (review in Henderson and Scott, 1980, Henderson et al, 1980). Other studies indicate that certain purines can stimulate growth or development (House, 1962, Sang 1956, 1978).

To date many of the enzymes associated with purine metabolism have not be identified in *Drosophila melanogaster* although many have been isolated and characterized in a wide variety of organisms and animal cell systems (Henderson, 1972, Patterson et al., 1974, Patterson, 1975, 1976) (Section 1, I, C.)

Those enzymes that have been identified in *Drosophila* have been detected through a series of different experiments.

Tracer studies utilizing radioactive precursors have been used to

detect the activity of purine metabolism enzymes (McMaster-Kaye and Taylor, 1959; Hodge and Glassman, 1967; Burridge et al., 1977; Johnson et al., 1980 a, b, c). The rates of enzyme activity can be estimated based on the amount of precursor converted to various substances in the organism.

Nutritional studies wherein purine metabolic substrates are used to supplement auxotrophic mutants, have helped discern which enzymes are active in *Drosophila*. (Villee and Bissell, 1945; Sang, 1956, 1957; Hinton et al., 1951, Hinton, 1956, 1959; Ellis, 1959; Burnett and Sang, 1963; Geer, 1964, Wyss, 1977).

Crude cell extracts from *Drosophila* embryos, imaginal disks and adult flies have been studied to detect the presence of purine metabolism enzymes (Seecof, 1961; Morita, 1964; Uda et al, 1969; Friedman, 1973; Becker 1974 a, b; 1978). Only one enzyme of purine metabolism in *Drosophila* has been successfully purified, XDH (Seybold, 1973; Andres, 1976).

The combined evidence from radioactive tracer studies, nutritional studies and crude cell extracts has helped to formulate an overall understanding of purine metabolism in *Drosophila melanogaster*.

ii. *de novo* purine nucleotide biosynthesis

The *de novo* purine nucleotide biosynthetic pathway operates in *Drosophila* because wildtype flies do not have an absolute requirement for purine bases or purine nucleosides in their diet (Sang, 1956; Geer, 1964). Growth of cultured *Drosophila* cells grown in purine free media also occurs; furthermore, such growth can be inhibited by drugs that block *de novo* purine biosynthesis (Wyss, 1977). Evidence that dietary

purines can stimulate development rate indicates that the reactions of *de novo* biosynthesis are limiting for growth. Tracer studies using radioactive formate and glycine showed radioactive material being incorporated into IMP, AMP and GMP and their metabolites, indicating *de novo* biosynthesis probably follows the same pattern as in other organisms (Johnson et al., 1980 a, b, c).

In the course of studies of the *Gart* locus of *Drosophila* (structural gene for GART) Henikoff and his collaborators (in press) have demonstrated *in vitro* activities equivalent to the first five steps of purine biosynthesis, three of which are encoded by *Gart*. *Gart* is a gene that encodes three *de novo* purine biosynthetic pathway activities, GARS, GART and AIRS (see Figure 1), the second, third and fifth steps of the pathway respectively. All three enzyme activities are encoded in a single gene transcript but alternative transcription termination produces two different RNA classes (Henikoff et al., 1983). The adenosine 3 locus (Sec 1, II, B.) is the *Gart* locus.

iii. Purine salvage pathways

Dietary purine bases stimulate growth in wildtype flies and auxotrophic mutants and have toxic effects at higher concentrations implying their conversion to nucleotides. This provides evidence of the existence of salvage pathways (Villee and Bissell, 1948; Hinton et al., 1951; Hinton, 1956, 1959; Sang, 1956, 1957; Ellis, 1959; Burnett and Sang, 1963).

In *Drosophila* adenine phosphoribosyl transferase (APRT) is active (McMaster-Kaye and Taylor, 1959; Wyss, 1977; Johnson et al., 1980 a, b, c) whereas HGPRT is absent (Becker, 1974 a, b) or detected only at

reduced levels (Wyss, 1977; Becker, 1978; Johnson, 1980 a, b, c). The apparent low HGPRT activity may be due to the high rates of XDH and guanine deaminase activity in *Drosophila* (Nash and Henderson, 1982). Johnson and Friedman (1981, 1983) have demonstrated that purine resistance, due to a deficiency of APRT results fromalterations at a single locus *Aprt* (3-3.03). *Aprt* appears to be the structural gene for APRT based on gene dosage dependence studies.

The nucleoside kinases (inosine, adenosine and guanosine) appear to all be active in *Drosophila*, unlike in mammalian cells (Becker, 1974 a, b, 1975; Wyss, 1977, Johnson et al., 1980 a, b, c).

No other salvage pathway enzymes have been studied to date.

iv. Interconversion of purines

Adenylate deaminase activity has been detected in *Drosophila* yet no guanylate reductase activity was found (Becker, 1974 b; Johnson et al., 1980 a, b, c). It is important to note however that a very small rate of guanylate reductase activity has been demonstrated by radioactive guanine and guanosine being converted to AMP and IMP in larvae (Burridge et al., 1977).

Guanylate reductase is at such a low level that its role in interconversion of purines in *Drosophila* is extremely limited. This is significant when trying to isolate auxotrophic mutants. Auxotrophic mutants blocked in *de novo* biosynthesis can be supplemented by adenylate or inosinate derivatives yet are not supplementable with guanylate derivatives inasmuch as GMP cannot be converted to IMP.

v. Catabolism of purines

None of the enzymes responsible for dephosphorylation have been characterized in *Drosophila* although their presence has been deduced from radioactive precursor studies (Hodge and Glassman, 1967; Becker, 1974 a,; Johnson et al., 1980 a, b, c).

Adenylate, adenosine, adenine and guanine deaminase activities have been detected in *Drosophila* , with adenosine deaminase and guanine deaminase displaying high activity rates (Seecof, 1961; Morita, 1964; Uda et al., 1969.; Becker 1974b, 1975; Johnson et al., 1980 a, b, c). Inosine, guanosine and adenosine phosphorylases are present in *Drosophila* with the latter enzyme demonstrating only very reduced activity. The reduced activity was detected by extremely low levels of adenosine being converted to adenine, as is seen in several cell systems (Becker, 1974 b, 1975; Hodge and Glassman, 1967; Snyder and Henderson, 1973; Henderson, 1979).

One enzyme that has been well characterized in *Drosophila* , XDH, has been found to show tissue specificity (Ursprung and Hadorn, 1961; Horikawa et al., 1967; Collins et al., 1970; Sayles et al., 1973; Seybold, 1973; Andres, 1976; Johnson et al., 1980 a, b, c). The catabolic enzyme urate oxidase (uricase) has been detected at elevated levels in XDH mutant flies. This enzyme oxidizes uric acid to allantoin which is excreted in *Drosophila* (Friedman, 1973; Johnson et al., 1980 a, b, c).

E. Pteridine metabolism in *Drosophila*

Pteridines are derived from purines and are found in most organisms, performing a variety of functions. In *Drosophila*, they are particularly evident because they form eye pigments that influence the eye color of the imago. *Drosophila* wildtype eye color is produced by the combination of brown (ommochromes) and red (drosopterin) pigments, with the yellow pigment (sepiapterin) playing a minor role (Ziegler, 1961; Ziegler and Harmsen, 1969). The red pigments in the eye, drosopterins, are dimeric pteridines (Rokos and Pfleiderer, 1975).

Pteridines are derived from GTP by the cleavage of the imidazole five membered ring of the guanine base. Removal of the carbon atom at position 8 and incorporation of 2 carbons from the ribose then generates a six-membered heterocyclic ring (Brown, 1971). The basic structure of pteridines is 2-amino-4-hydroxypteridine (Ziegler and Harmsen, 1969).

The exact pathway involved in the production of the drosopterins is as yet undefined. At least 30 different *Drosophila* eye color loci have been identified that have reduced or absent levels of drosopterins (Hadorn and Mitchell, 1951; Nolte, 1954 a, b, 1955, 1959; Forrest et al., 1956; Counce, 1957; Gregg and Smuckler, 1965; Baker, 1973). This large number of genes is in excess of the number of enzymes suspected of being involved in the production of drosopterins.

Many of these eye color mutants have been studied to determine if any could be associated with enzymes involved in pteridine metabolism (Fan et al., 1976; Krivi and Brown, 1979). Of particular interest to to this study are the results involving the raspberry mutants of the raspberry locus that produce flies with a purplish eyecolor. the raspberry and the prune loci were found to produce altered pteridine

metabolism. Mutants in both loci influence the regulation of GTP cyclohydrolase that converts GTP to dihydroneopterin triphosphate (Fan et al., 1976; Evans and Howells, 1978; Fan and Brown, 1979), however, neither was found to be the structural gene for GTP cyclohydrolase. Evans and Howells (1978) postulated that the wildtype gene products of *ras* and *prune* must regulate the activity of GTP cyclohydrolase. Mackay and O'Donnell (1983) have found the structural gene to be at the Punch locus (2-97.0).

II. PURINE MUTANTS IN *DROSOPHILA*

A number of purine auxotrophic mutants have been isolated in *Drosophila melanogaster* and are described in brief below.

A. Nucleoside requiring mutants

The nucleoside 1 gene located on the second chromosome near brown at position 104.5 has 3 alleles all displaying an auxotrophic requirement for a purine or a pyrimidine nucleoside (Naguib, 1976; Naguib and Nash, 1976). The auxotrophic mutant alleles are believed to be deficient in phosphoribosyl pyrophosphate (PP-ribose-P) which is involved in both the purine and pyrimidine biosynthetic pathways.

B. Adenosine requiring mutants

Three adenosine requiring auxotrophs have been isolated and studied (Falk, 1973; Falk and Nash, 1974 b; Naguib, 1976; Naguib and Nash, 1976; Johnson et al., 1976; Johnson, 1978; Henikoff et al., in press).

Adenosine 1 mapped to position 57.0 on the X chromosome and had one allele. This slow growing mutant was found to have purine metabolism similar to wildtype flies in radioactive tracer studies (Johnson et al., 1976). Unfortunately this mutant has been lost.

The adenosine 2 mutant *(ade2-1)* displays a dull red eye color resembling rosy, demonstrates a strong pteridine deficiency and has an absolute dependence on exogenous purines (Naguib, 1976, Johnstone et *al., 1985). The ade2-1* flies can be supplemented with exogenous RNA, adenine, adenosine or inosine. Guanosine derivatives fail to supplement these mutants due to the lack of guanylate deaminase activity in *Drosophila*. The *ade2-1* mutant appears to be defective in *de novo* purine biosynthesis. Johnson (1978), however, found no evidence of defective biosynthesis of purine nucleotides in this mutant. In radioactive tracer studies she noted accumulation of guanine and guanosine which seems to indicate these substrates are not being catabolized. Johnson (1978) postulated that a defect might lie in the catabolic enzyme guanine deaminase that converts guanine to xanthine which is subsequently oxidized to uric acid. This hypothesis was supported by Johnstone et al. (1985) who discovered a six-fold lower guanine deaminase activity in *ade2-1* flies in comparison to wildtype.

Recent enzyme studies by Henikoff et al. (in press) seem to indicate that (FGARAT) is absent in *ade2-1* mutant flies (see page 1). This enzyme is involved in the fourth step of *de novo* purine biosynthesis. Henikoff et al. assayed for the presence of the first five *de novo* purine biosynthetic pathway enzymes in both wildtype and *ade2-1* homozygous stocks and found FGARAT to be absent in *ade2-1* flies. However unpublished results (Keizer and Tiong,

personal communication) suggest that the mutant isolated by Naguib (1976) may not be at the same locus as that studied by Johnstone et al. (1985), and Henikoff et al. (in press). Further work is being carried out to elucidate the situation.

The adenosine 3 locus has 1 mutant allele, *ade3-1* , that displays wildtype eye color, poor survival and very slow growth on unsupplemented medium. Normal growth rate is seen when the mutant *ade3-1* flies are supplemented with RNA, adenine, adenosine or inosine. The locus maps to position 20.0 on the second chromosome (26F-27E). Henikoff et al. (1981) isolated the *Drosophila* DNA encoding the GART locus (Sec.1, I, B, i) and found it hybridized to a single site near 27C, the location of adenosine 3. Subsequent enzyme studies confirmed that the *ade3-1* mutation is a base substitution in the GART coding region leading to the substitution of a highly conserved glycine by a serine (Henikoff et al., in press). The two additional enzymes encoded at the GART locus (GARS, AIRS) are not defective in *ade3-1* flies.

C. Guanosine requiring mutants

Two mutants have been isolated as guanosine auxotrophs. The guanosine 1 mutant, *gua1-1*^{ts} (Falk, 1973; Falk and Nash, 1974b; Johnson et al., 1979; Nash et al., 1981) maps near to position 32.8 on the X chromosome at cytological bands 9E1-9E4 (Johnson et al., 1979). This mutant is a temperature sensitive auxotroph showing reduced viability at elevated temperatures (29°C) and 50% viability at 25°C in the absence of guanosine. Adenosine fails to supplement this mutant which seems to indicate the defect may lie in the conversion of IMP to GMP.

A second guanosine requiring auxotroph maps to the second chromosome

at position 55.7 (Johnstone and Nash, 1979). Originally designated *gua2-1* it displays an eye color defect and is allelic to the *bur* mutant (1-55.7) and is therefore designated burgundy-guanosine 2-1 (bur^{gua2-1}) (Johnstone et al, 1985). The mutant shows a low level of pteridine deficiency and an absolute requirement for guanosine supplementation. Guanine fails to act as an effective supplement for this auxotroph presumably due to low levels of guanosine phosphoribosyltransferase activity and/or high levels of guanine deaminase activity that are found in *Drosophila melanogaster*.

A mutant that is supplemented by guanosine alone is likely to be defective in the conversion of IMP to GMP. Two enzymes are found in the conversion, IMP dehydrogenase and GMP synthetase. Neither bur^{gua2-1} nor $gua1-1^{ts}$ has been proven defective in the structural loci encoding either enzyme. Johnstone (1985) studied IMP dehydrogenase in bur^{gua2-1}. Initial mutant isolates showed reduced levels of IMP dehydrogenase but over time, apparently as a result of natural selection, enzyme activity returned to normal. The auxotrophic phenotype and eye color defect showed no concomitant change and so appears not to be the primary cause of the auxotrophy.

D. Purine requiring mutants

The final class of purine auxotroph isolated in *Drosophila melanogaster* is one that can utilize either an adenosine or a guanosine source as supplement. (Falk, 1973; Falk and Nash, 1974 b; Johnson et al., 1976; Johnson, 1978; Johnson et al., 1979). The single locus identified, called purine 1, has two alleles (*pur1-1, pur1-2*). Purine 1 maps near position 32.8 on the X chromosome (Johnstone and Nash,

1979). The auxotrophic mutants *pur1-1* and *pur1-8* display a slight morphological wing defect, different from those associated with pyrimidine auxotrophy, and retains a wildtype eye color (Nash et al., 1981). The two known auxotrophic alleles of the purine 1 locus differ qualitatively (Falk and Nash, 1974 b). The allele *pur1-2* responds equally well to supplementation with adenosine or guanosine. Johnson et al. (1976) noted increased rates of purine catabolism which may deplete levels of IMP produced in the biosynthetic pathway. Purine 1 encodes an essential function (Johnson et al, 1979) as indicated by the lethal phenotype when the *pur 1* mutants are placed against a deficiency on supplemented medium; the *pur 1* mutations are, it appears, hypomorphic alleles (Johnson et al., 1979).

III. THE *gua-pur-ras* COMPLEX

A. Raspberry mutants in *Drosophila*

Raspberry *(ras)* mutants in *Drosophila melanogaster* display mutant eye color due to reduction in the orange-red drosopterin eye pigments (Nolte, 1959). Evans and Howells (1978) noted that two sex-linked mutants, prune (1-0.8) and raspberry (1-32.8) which display similar eye color defects cause a marked reduction in GTP cyclohydrolase activity in the heads of young adult flies. However, neither locus appeared to contain the structural gene for GTP cyclohydrolase for a variety of reasons (Fan et al., 1976; Evans and Howells, 1978). Subsequently, Mackay and O'Donnell (1983) established the Punch locus, (2-97.0) to be the structural gene for the enzyme. Evans and Howells (1978) speculated

that prune and raspberry play a role in the developmental regulation of GTP cyclohydrolase.

Numerous lethal alleles have been found to exist at the raspberry locus suggesting the raspberry locus to be essential for *Drosophila* (Johnson et al., 1979; Nash et al., 1981; Nash and Janca, 1983). Raspberry, guanosine 1 and purine 1 mutants were found to map cytologically at bands 9E1-9E4 to the left of vermillion and very near to each other on the X chromosome (Johnson et al., 1979)

B. *gua1-pur1-ras* interactions

Since purine 1 is expected to encode an essential function (Sec1, II, C.) lethal alleles of this gene would be expected. However, lethal alleles of purine 1 often prove to be allelic to the raspberry and guanosine loci as well (Johnson et al., 1979; Nash et al., 1981) indicating that the three loci are related in some way. These mutants form a single recessive lethal complementation group and because they were initially discovered by allelism with *ras* are called raspberry lethals *(ras-1)* (Janca et al., 1986).

In complementation studies the mutants *gua1-1*^{ts}, *pur1-1* and *pur1-2*, which all display auxotrophic phenotypes and wildtype eye color, were studied in double heterozygotes with ras^2 an allele, when homozygous, produced viable flies with the classical raspberry eyecolor phenotype. Double heterozygotes were competely wild type with respect to auxotrophic phenotype and eye color (Johnson et al., 1979, Nash et al., 1981). It will be recalled that *pur1-1* is a purine requiring mutant whose developmental rate is supplemented by guanosine as opposed to *pur1-2* which is supplemented by either adenosine or guanosine. This

difference was refected in complementation tests with *gua1-1^{ts}*. On unsupplemented medium, *pur1-1/gua1-1^{ts}* develops more slowly than *pur1-2/gua1-1^{ts}* (Falk, 1973). The special requirement of guanosine in *pur1-1* appears to lie in the same function that altered the *gua1-1^{ts}* mutant. This small lack of complementation implies that *pur1* and *gua1* may be functionally related as part of a single 'complex' locus. The slow growing phenotype of *pur1-1/gua1-1^{ts}* may then be explained as a weak polar effect (Falk, 1973).

Janca et al., (1986) selected a large number (24) of EMS induced recessive lethal mutations in the region 9E1-9F13 on the X chromosome, eight of which failed to complement two or more of the functions of the *gua1-pur1-ras* complex. These 8 mutations constituted a single lethal complementation group. Of the eight, six failed to complement *pur1-1* and *gua1-1*. Two recessive lethals failed to complement *pur1-1* and *gua1-1^{ts}* yet complemented ras^2 as evidenced by wild type eye color and an auxotrophic phenotype. Therefore, raspberry is not necessarily defective in an essential function. Rather, the lethality of *ras-1* mutants is probably due to their *pur1* defect. Janca et al., (1986) interpret their results as showing that various *ras-1* alleles are qualitatively different with regard to the component of the complex which is most strongly affected and therefore not a single series of hypomorphic alleles. This conclusion is compatible with the notion that there are three quasi-independent functions within the locus.

Further characterization of the complex locus using classical genetic techniques is difficult, thus we have chosen to continue studies of the raspberry locus at the molecular level.

IV. CLONING THE RASPBERRY LOCUS OF *DROSOPHILA MELANOGASTER*

A. Techniques for cloning genes of *Drosophila melanogaster*

Many routes are available for the cloning of genes in *Drosophila*.

i. Chromsome walking and jumping

Chromosome walking and jumping is carried out by obtaining a series of overlapping clones from a genomic library. With appropriate restriction endonuclease analysis, it is possible to ascertain the order of the clones along the chromosome. In this way, large regions can be cloned by slowly "walking" along the chromosome. Various parameters may be employed to determine which genes are present in the cloned region. (Example, Barber et al., 1983).

Jumping involves a similar procedure but incorporates genomic Southern analysis of rearranged chromosomes with breakpoints close to the initial probe and in or near a remote gene of interest. When the "walk" reaches the rearrangement breakpoint there will be a radical divergence between the restriction pattern of the wildtype and the rearranged stock.

Walking and jumping procedures are feasible only if one has a cloned piece of DNA that lies near the gene of interest or near a rearrangement breakpoint that interrupts or falls near the same gene.

ii. Utilizing a potentially homologous probe to isolate genes.

Several methods are employed to isolate genes in *Drosophila* with homologous probe sequences. These sequences may be synthesized based

upon a known protein amino acid sequence or alternatively, cloned genes from other organisms may be used to identify partially homologous sequences in *Drosophila*. Isolated message from other organisms may also prove to be homologous.

iii. Cloning by complementation

Segments of *Drosophila* DNA may be able to complement a mutant phenotype in another experimental organism.

Henikoff et al. (1981) used a transformation and complementation procedure to clone the *Drosophila* adenosine 3 gene (Sec.1, I, B, i.). Utilizing a yeast strain deficient for GART activity, he transformed the mutant cells with cloned genomic *Drosophila* DNA and screened for those segments that complemented the yeast deficiency. This procedure is limited to those genes that can produce a gene product that complements established mutants in other organisms. The Gart locus fulfills this requirement in that it contains a promoter 5' to the GART coding region that enabled the yeast DNA to transcribe the *Drosophila* DNA to produce functional GART. Most yeast auxotrophic mutants, which could conceivably be complemented by *Drosophila* genes, have been tested and are not complemented (pers. comm.).

iv. Microdissection

A novel approach that is becoming more common is the use of microdissection of polytene bands as devised by Scalenghe et al. (1981) Micromanipulation is used to dissect single polytene chromosome bands in an oil chamber by microscopic monitoring. The material is subsequently cloned into a vector in nanolitre quantities. This

technique is useful in that the gene, if mapped to a particular band, can most likely be isolated in its entirety in the sample of clones produced. A major drawback to the procedure is that it appears to be complicated, requiring special equipment and technical skill.

v. Transposon tagging

The most common procedure, at present, to isolate genes that are genetically and cytogenetically characterized involves the use of transposon tagging. The P transposable element in *Drosophila* is used in dysgenic crosses to induce transposition-mediated insertion events in the gene of interest. The cloned transposable element serves as a probe to identify those sequences that carry the transposable element. The unique *Drosophila* DNA that flanks the transposon can be identified through *in situ* hybridization. Limitations arise when the cytogenetic location of the gene has not been determined (Bingham et al., 1981).

B. Procedure for cloning the raspberry locus of *Drosophila melanogaster*

Of the techniques described above transposon tagging appeared to be the most useful technique available for the raspberry locus. Chromosome walking and jumping was not possible because a cloned piece of DNA near the region or near a rearrangement that breaks the 9E region is not available. Homologous material, in the form of a protein, an mRNA or DNA from another organism, has not yet been identified since the function of the region is unknown. Transformation/complementation experiments are not possible for the same reason. Microdissection

represents a realistic, perhaps even optimal approach to cloning the raspberry locus, inasmuch as the cytological location has been established. However, this technique has only recently been perfected and may be difficult to master technically. Given that raspberry lethal mutations are easy to select phenotypically and had previously been demonstrated as products of hybrid dysgenesis, transposon tagging, using the P transposable element, was selected as the means to isolate the *gua-pur-ras* complex in *Drosophila melanogaster*.

Hybrid dysgenesis in *Drosophila melanogaster* has been described as a "syndrome of correlated genetic traits that is spontaneously induced in hybrids between certain mutually interacting strains, usually in one direction only" (Kidwell and Kidwell, 1976). In the PM system it has been found that this syndrome is due to the transposition of the P transposable element (Rubin et al. 1982; Bingham et al., 1982). A P strain is one that contains several genomic P elements and a "P cytotype" which suppresses P transposition. An M strain is one that lacks P elements and possesses the non-suppressive "M cytotype". In the progeny of the cross between a P strain male with an M strain female P elements from the paternal complement, activated by the M cytotype, transpose actively from one genomic location to another. A brief review of the molecular mechanism of P transposition is presented by Engels, (1983). The P element inserts at "random" locations throughout the genome sporadically producing visible or lethal insertion mutations. For example, Rubin et al. (1982) and Bingham et al. (1982) were able to clone the P factor from P insertion mutations at the white locus using DNA clones produced from previous work (Bingham et al., 1981).

Bingham et al.(1981) proposed use of P element mutagenesis as a general method for the cloning of DNA from loci that have been identified by genetic and cytogenetic analysis, but where no RNA products have been found. Through hybrid dysgenesis, P transposable element insertions are produced at the locus under consideration and are selected on the basis of altered phenotype. The P element can then be used to probe a genomic library from the mutant strain. Cloned sequences flanking the P element can be identified by *in situ* hybridization of the cloned DNA to polytene chromosomes from an M strain (P element free). Appropriate subcloning and "walking" procedures can then be used to develop a series of clones covering the entire wildtype gene.

2. MATERIALS AND METHODS

I. STOCKS

A. *Drosophila melanogaster* Stocks

Drosophila melanogaster stocks used for the isolation and maintenance of the P element induced raspberry lethal mutations are described in Table 1.

B. Bacteria and Bacteriophage Stocks

All bacteria stocks were stored in 15% glycerol at -20°C. Bacteriophage were stored at 4°C over chloroform.

i. HB101 containing plasmid pπ25.1

Escherichia coli strain HB101 has the genotype F^-hsdS20 (r^-B, m^-B, $recA_13$, ara-14, proA2, $lacY_1$, galK2, rpsL20(Sm^r), xyl-5, mtl-1, supE44, λ^-) (Boyer and Roulland-Dussoix, 1969; Bolivar and Backman, 1979). HB101 was transformed with the plasmid pπ25.1 that is a derivative of pBR322 that carries a complete P element and unique Drosophila DNA from the X chromosome 17C region inserted into the tetracycline resistance gene (O'Hare and Rubin, 1983). An intact ampicillin resistance gene enables the plasmid to be selected by growth on ampicillin supplemented LB plates (to 100 µg/ml). The plasmid DNA of pπ25.1 was used as a P element probe in subsequent hybridizations. The restriction map of the plasmid is diagrammed in Figure 4. HindIII, EcoRI and BamHI restrictions shown in Figure 20 were used to confirm the presence of pπ25.1.

TABLE 1. DESCRIPTION OF *DROSOPHILA MELANOGASTER* STOCKS

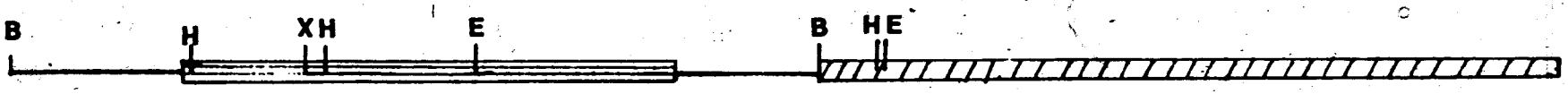
STOCK	DESCRIPTION	M OR P STRAIN	SOURCE
Amherst, Am^+	Inbred derivative of OregonR, Amherst College, Drosophila Information Service (1968); wildtype.	M	Amherst College
ycvvf	y = yellow body (1-0.0) cv= crossveinless wing (1-13.7) v = vermillion (1-33.0) f = forked bristles (1-56.7)	M	Origin unknown
ycvras^{2}vf	y = yellow body (1-0.0) cv= crossveinless wing (1-13.7) ras^2=raspberry, nonlethal eye color phenotype (1-32.8) v = vermillion (1-33.0) f = forked bristles (1-56.7)	M	Nash, unpublished
π2P	Phenotypically wildtype stock contains approximately 20-30 P elements dispersed throughout the genome	P	Engels, (1979); Engels and Preston, (1979)
v(I)	vermillion strain with "I" activity in IR dysgenic system Shown to be P strain as a preliminary to this study.	P	Alain Pellison
H6/FM6	EMS induced raspberry lethal balanced against the first chromosome balancer FM6. This stock was used for the FM6 balancer chromosome.	M	Nash and Janca, (1983)
In(1)123/FM6	Balanced inversion of the X chromosome. Breakpoints within the centromere and region 9E. Raspberry lethal phenotype.	M	Nash, unpublished

FIGURE 4. Restriction map of plasmid pπ25.1

Diagrammed is a restriction map of the plasmid pπ25.1, the plasmid that was used as a P element probe in this study. Restriction sites of restriction enzymes employed in this study are shown.

FIGURE 5. Restriction map of bacteriophage EMBL4

Diagrammed is a restriction map of the bacteriophage EMBL4 used as the cloning vector in this study. The internal fragment is flanked by the 3 restriction sites SalI, BamHI and EcoRI.

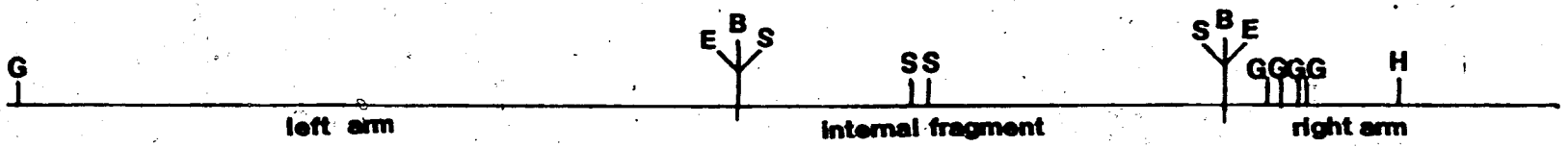


P ELEMENT DNA

DROSOPHILA 17C DNA

pBR322 PLASMID DNA

FIGURE 4. RESTRICTION MAP OF PLASMID Pπ25.1 (O'Hare and Rubin, 1983)



B=BamHI E=EcoRI G=BglII H=HindIII S=SalI X=XhoI

FIGURE 5. RESTRICTION MAP OF BACTERIOPHAGE EMBL4 (Frischauf et al., 1983)

ii. Q358

An *E.coli* strain that is permissive for growth of EMBL4 (hsdR$^-$K, hsdM$^+$K, supE, ϕ80^r) (Karn et al., 1980).

iii. Q359

An *E.coli* strain restricts growth of EMBL4. This strain is a P2 lysogen which prevents the growth of intact EMBL4 possessing a *spi*$^+$ phenotype (sensitive to P2 interference). Replacement of the red and gam genes in EMBL4 result in a *spi*$^-$ phenotype and ability to grow on Q359 (hsdR$^-$K, hsdM$^+$K, supE, ϕ 80^r, P2) (Karn et al., 1980).

iv. BHB2688

An *E.coli* strain used in the preparation of freeze-thaw lysate packaging extracts (N205 recA$^-$ (λimm^{434}, cIts, b2, red$^-$, Eam, Sam)/λ) (Hohn, 1979).

v. BHB2690

An *E.coli* strain used in the preparation of sonicated packaging extracts (N205 recA$^-$ (λimm^{434}, cIts, b2, red$^-$, Dam, Sam)/λ) (Hohn, 1979).

vi. Bacteriophage EMBL4

EMBL4 is a BamHI substitution vector derived from λ1059 (Karn et al., 1980; Frischauf et al., 1983). EMBL4 is useful as a cloning vector for making genomic DNA libraries. An internal BamHI fragment carrying λred and gam genes conferring a *spi*$^+$ phenotype,

can be replaced by a 19-23 kb fragment of insert DNA. The BamHI sites that remove the internal fragment are flanked by SalI and EcoRI restriction sites. SalI digestion of EMBL4 ensures the internal fragment will not religate to the BamHI cohesive ends of the vector arms. EcoRI digestion may be used to discern the size and restriction pattern of inserted material. When the internal BamHI fragment is replaced with *Drosophila* DNA the λ red and gam genes are absent, conferring a *spi*⁻ phenotype. Thus, EMBL4 phage that contain a Drosophila DNA insert instead of the BamHI insert can be isolated selectively on E.coli Q359. The restriction map of EMBL4 is illustrated in Figure 5.BamHI, SalI and EcoRI digestions, shown in Figure 22, were used to confirm the presence of EMBL4 DNA.

II. MATING SCHEME

The protocols used to obtain and confirm P element induced raspberry lethal mutations are shown in Figures 6, 7 and 8. In generation 2, flies were selected that displayed a raspberry eyecolor. Raspberry mutants are deficient in the orange-red drosopterins (Nolte, 1959). Vermillion mutants are deficient in the brown ommochrome pigments (Lewin, 1983) of the eye. Consequently, double mutants for raspberry and vermillion display light orange eyes, which are readily detected. Vermillion maps to position 33.0 on the X chromosome, 0.2 map units proximal to the raspberry locus.

FIGURE 6. Protocol used for obtaining P element induced raspberry lethal mutations.

In generation 1, 10 M strain females were mated to 10 P strain males. In generation 2, 25 bottles of 10 hybrid dysgenic females were mated to 10 *ycvras*^{2}*vf* males and were set over a 10 day period.

Hybrid dysgenic females in Generation 2 were created by mating *ycvvf* (M) strain females to P strain males in Generation 1. Dysgenic females were mated to *ycvras*^{2}*vf* males to reveal those progeny carrying a raspberry mutation.

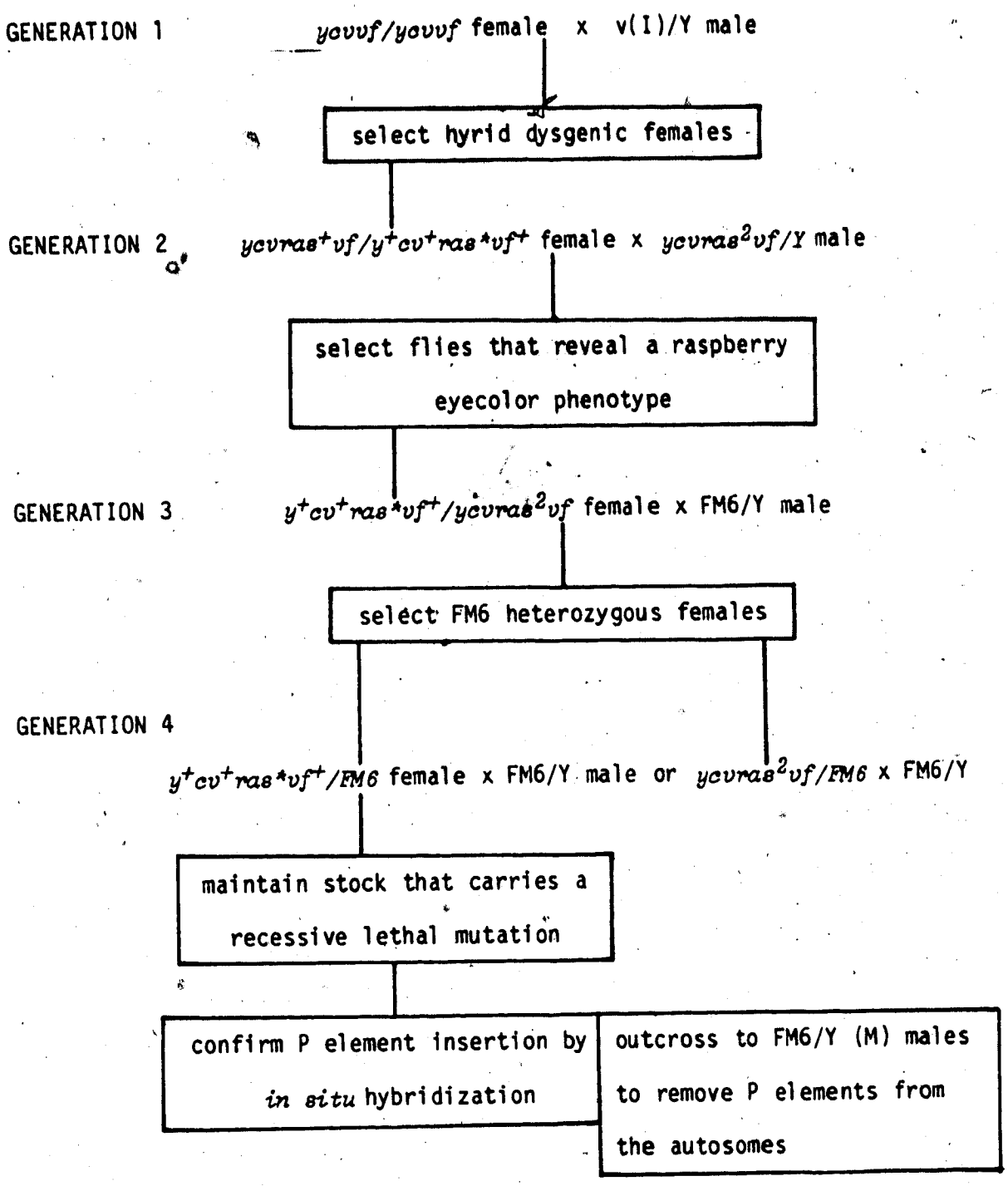
In generation 3, the mutant female was mated singly to 3-5 FM6 males.

In generation 4, FM6 heterozygous females were mated singly to FM6 males to confirm if the *ras* bearing chromosome carried a lethal mutation.

The suspected P induced raspberry mutations were maintained by mating flies to a first chromosome balancer stock (FM6).

All stocks were outcrossed at least 4 times to M strain (FM6) males to remove P elements from the autosomes.

FIGURE 6. MATING SCHEME FOR ISOLATION OF NJ-5 STRAIN



* = lethal mutation

FIGURE 7. Confirmation of a sex-linked recessive lethal

This mating scheme illustrates how a sex-linked recessive lethal phenotype was confirmed for each mutant.

FIGURE 8. Confirmation of a raspberry eye color mutant

This mating scheme illustrates how a raspberry eye color mutant was confirmed for each mutant.

Note that the above two mating schemes only indicate that a recessive lethal is present on the X chromosome and that a mutation is present at the raspberry locus on the X chromosome. It does not determine whether or not the lethal is present at the raspberry locus.

FIGURE 7. CONFIRMATION OF A SEX-LINKED RECESSIVE LETHAL

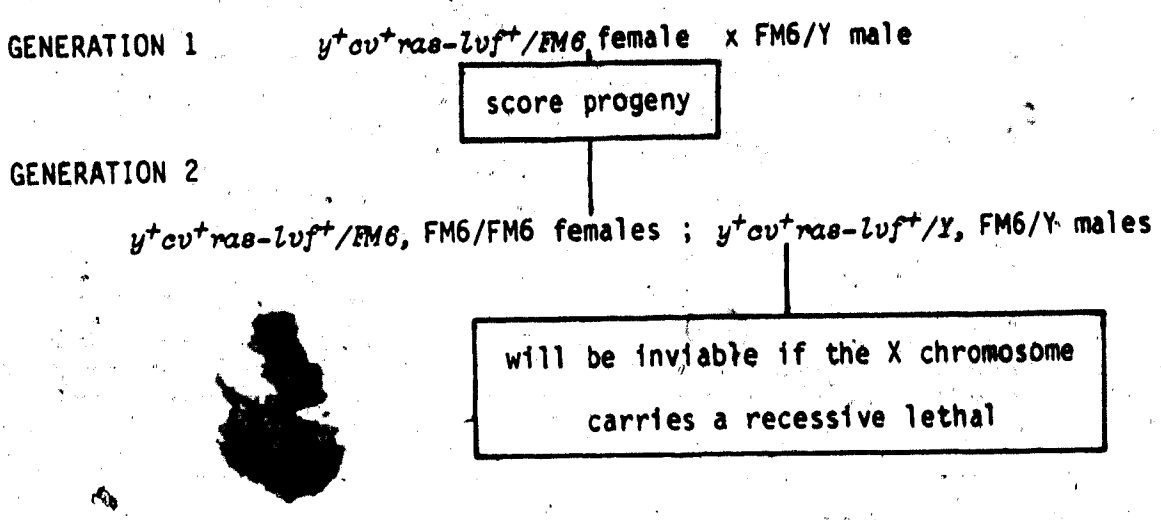
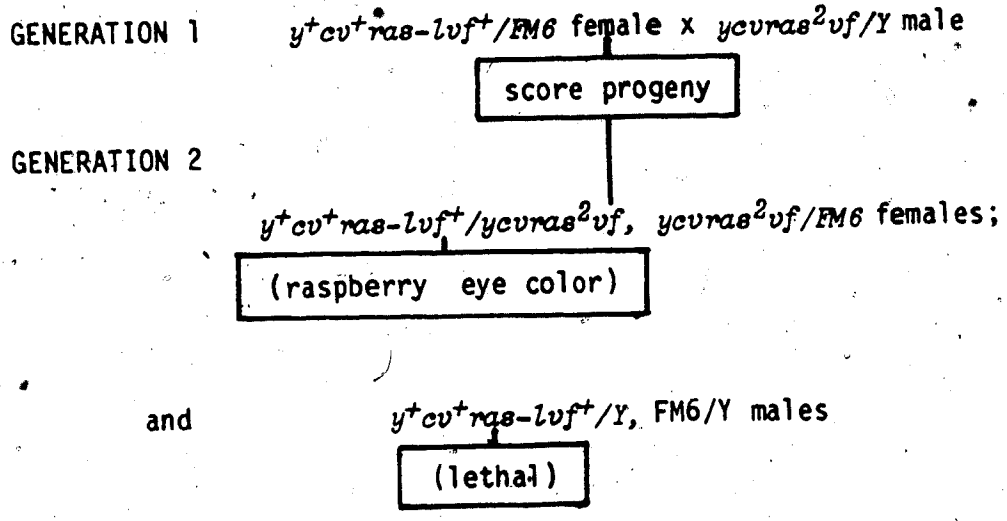


FIGURE 8. CONFIRMATION OF MUTATION AT RASPBERRY LOCUS



III. MEDIA

All media and solutions were prepared using chemicals from Sigma or Fisher-Scientific unless otherwise stated.

A. Yeast-Sucrose Medium:

All *Drosophila* stocks were grown on yeast-sucrose medium composed of 12.5 g brewer's yeast (Difco), 10.0 g sucrose, 2.0 g granulated agar (Bacto), 25.0 mg streptomycin, 25,000 I.U. penicillin, 1.0 ml propionic acid to a final volume of 100.0 ml of water.

B. Luria-Bertani (LB) Broth

All *E. coli* strains were grown in LB broth which consists of 10.0 g tryptone (Difco), 5.0 g yeast extract (Difco) and 10.0 g NaCl per litre water. The pH was adjusted to 7.5 using NaOH. For growth of plasmid containing strains, 100 µg/ml ampicillin was added to the medium after autoclaving.

LB plates were prepared by adding 15.0 g/litre of Agar.

LB medium plus 10mM $MgSO_4$ or LB medium plus 10mM $MgSO_4$ and 0.2% maltose were used in the preparation of bacteriophage.

C. Tryptone (T) Medium

Tryptone medium was used in the preparation of some overnight bacteria cultures and bacteriophage plates. This medium contained 10.0 g tryptone and 5.0 g NaCl per litre water. The pH was adjusted to 7.5 using NaOH.

TM plates were prepared by adding 15.0 g/litre of agar, 10mM $MgSO_4$.

TM Top Agar or TM Top Agarose were prepared using 7.0 g/litre of agar or agarose.

IV. HANDLING OF DNA

A. Phenol-chloroform extraction

In order to remove contaminating proteins or carbohydrates a DNA solution was extracted in a series of steps using phenol and chloroform. Phenol was prepared by equilibrating with an equal volume of 1.0M Tris(pH8.0). The phenol was then reequilibrated with an equal volume of 10mM Tris(pH8.0). One-tenth% 8-hydroxyquinoline was added to the phenol to provide a color indicator for the organic phase and to act as an anti-oxidant, a partial inhibitor of RNase and a weak chelator of metal ions (Kirby, 1956). Chloroform was prepared with isoamyl alcohol in a 24:1 ratio.

The steps involved in a phenol-chloroform extraction were as follows. The DNA solution was extracted with an equal volume of the prepared phenol by gentle inversion, ensuring thorough mixing. Organic and aqueous phases were separated by centrifugation and the aqueous layer was then extracted with an equal volume of a 1:1 prepared phenol-chloroform mixture. The resultant aqueous phase was extracted with an equal volume of the prepared chloroform. DNA was precipitated from the final aqueous phase by ethanol precipitation.

B. Ethanol precipitation

Salt concentrations of DNA samples were adjusted to a final

concentration of 0.25M sodium acetate, 0.1M NaCl or 2.0M ammonium acetate. Two volumes of 95% ethanol (-20°C) were added to the DNA-salt solution which was mixed by gentle inversion and chilled (-20°C overnight or -70°C for 30 minutes). DNA was pelleted by centrifugation (SS-34, 12K, 10', 0°C) washed with 70% ethanol, dessicated and resuspended in TE buffer (10mM Tris(pH8.0),1mM EDTA).

C. Dialysis of DNA

Dialysis tubing (Spectapor Membrane Tubing MW 6,000-8,000) was prepared by boiling 10 minutes in 2.0% sodium bicarbonate, 1mM EDTA and then rinsed with distilled water before use (Maniatis et al., 1982). The tubing was sealed with clips and left to dialyze in the appropriate buffer (three - 1 litre changes) for a minimum of 3 hours.

D. Restriction endonuclease digests of DNA

Table 2 shows the buffer conditions used for each of the restriction enzymes used in this study. The special conditions for use of Sau3A are described below (Sec.2, VII, B.). Digestions involving two restriction enzymes were performed sequentially. Buffer conditions were altered on addition of the second restriction enzyme. In general 0.5 - 1.0 μg of DNA were digested with 3 - 10 units of enzyme in a 20 μl reaction mixture and incubated 1 hour at 37°C. The reaction was stopped by heating to 68°C for 10 minutes. All restriction enzymes were from Bethseda Research Laboratories (BRL).

E. Ligation reaction

TABLE 2. RESTRICTION ENDONUCLEASE BUFFER CONSTITUENTS

ENZYME	BUFFER
BamHI, SalI, XhoI	100mM NaCl 50mM Tris(pH7.5) 10mM $MgCl_2$ 1mM DTT
BglII	10mM Tris(pH7.5) 10mM $MgCl_2$ 1mM DTT
HindIII	75mM NaCl 50mM Tris(pH7.5) 10mM $MgCl_2$ 1mM DTT
EcoRI	100mM NaCl 150mM Tris(pH7.5) 10mM $MgCl_2$ 1mM DTT

Up to 1 μg of DNA was ligated in 80mM Tris(pH8.0), 20mM $MgCl_2$, 15mM DTT, and 1mM ATP with 1 unit of T4 DNA Ligase (BRL) in a final volume of 50 μl. The reaction mixture was incubated at 15°C for at least 18 hours.

F. Nick translation of DNA

DNA was nick translated by the method of Maniatis et al. (1975) and Rigby et al. (1977). In all nick translations, 1 μg of DNA was treated in 50 μl reaction mixtures. When the probe was to be used for *in situ* hybridizations to polytene chromosomes approximately 156 picomole aliquots of tritiated thymidine triphosphate (Amersham - specific activity approximately 100Ci/mmol) were prepared by lyophilizing the ethanol solution the nucleotides were packaged in. When filter hybridizations were to be carried out, 10 μl of fresh ^{32}P-dCTP was used (Amersham - 10mCi/ml, 0.000314 mol/0.1ml).

Two methods were used for nick translation. The first utilized a nick translation kit as provided by BRL. 1 μg of DNA was nick translated as described in material provided with the kit.

The second method involved the following procedure (Maniatis et al.,1982). In a final 50 μl reaction mixture, 1 μg of DNA was mixed in 50mM Tris(pH7.2), 10mM $MgSO_4$, 0.1M DTT, 50 μg/ml BSA (Pentax Fraction V) and 20 μM of the appropriate cold nucleotides on ice. 5 units of polymerase I (PolI) (BRL) and 4 x 10^{-5} g of deoxyribonuclease I (DNaseI) were added to complete the reaction. DNaseI was prepared in 0.15M NaCl and 50%glycerol. Optimal DNaseI concentrations were determined from preliminary tests.

Nick translation reaction mixtures were incubated for 1 hour at

16°C. The reaction was stopped by adding 50 µl of 10mM Tris, 5mM EDTA, 0.1% SDS and 100 µg/ml denatured, sonicated salmon sperm DNA. Salmon sperm DNA was prepared by shearing the DNA through an 18 gauge hypodermic needle several times and sonicating until the mixture appeared less viscous. The DNA was boiled for 10 minutes and then stored at -20°C. DNA was denatured by boiling for 5 minutes prior to use.

Unincorporated nucleotides were removed by two subsequent ethanol precipitations using *E.coli* tRNA to a final concentration of 0.4mg/ml as a co-precipitate, by a NACS prepac column (BRL-1525) or by a mini-Sephadex G-50 column (Maniatis et al., 1982). A Sephadex G-50(med) column was formed in a 1 ml disposable syringe via centrifugation in a clinical benchtop centrifuge. The column was equilibrated twice with 0.1 ml of TE(pH8.0). Ten µl of bromophenol blue in water was added to the nick translated material which was subsequently loaded onto the Sephadex column. The material was centrifuged for 2 minutes. The nick translated DNA was collected in a 100 µl aliquot in an Eppendorf (1.5ml) at the bottom of a clinical centrifuge tube. Unincorporated nucleotides remained in the Sephadex column at the same level as the bromophenol blue.

Incorporation was estimated by scintillation counting.

G. Agarose gel electrophoresis and lambda markers

Appropriate amounts of agarose were dissolved in a solution of 100mM Tris borate (pH8.3), 2mM EDTA (1 x TBE). Ethidium bromide (EtBr) was added to a final concentration of 2 µg/ml. EtBr intercalates into nucleic acids and fluoresces with ultraviolet irradiation. DNA

samples were loaded into gel wells with the gel loading buffer BSU (0.5 g sucrose, 0.42 g urea and 5 mg bromophenol blue/ml). BSU stops the reaction and aids in loading of DNA samples due to its high density. The bromophenol blue helps monitor electrophoresis in that it moves at about the same velocity as a 400 bp DNA fragment. Lambda DNA (BRL) was used to quantitate the amount of DNA present in a sample and to act as molecular weight standards. HindIII digested lambda DNA produced fragments of size 23.13, 9.42, 6.68, 4.36, 2.32, 2.03, 0.564 and 0.125 kb. HindIII/EcoRI digested lambda DNA produced fragments of size 21.7, 5.25, 5.05, 4.21, 3.41, 2.03, 1.90, 1.67, 1.32, 0.93, 0.84, 0.56 and 0.13 kb. Bacteriophage λDNA and restriction endonuclease digests were heated at 68°C to denature the 12 bp cohesive ends.

V. PREPARATION OF BACTERIOPHAGE

A. Plating of bacteriophage EMBL4

Bacterial cultures were grown overnight with shaking at 37°C in LB medium, 10mM $MgSO_4$ and 0.2% maltose. Magnesium cations aid growth and adsorption of bacteriophage by stabilizing the head whereas maltose enhances attachment of the phage to bacterial cell membranes.

All bacteriophage were stored and diluted in TM buffer (10mM Tris(pH7.5), 10mM $MgSO_4$).

Host bacteria, 0.1 ml of overnight cells for 100mm petri dishes (0.3ml for the large 150mm petri dishes) were mixed with 0.1 ml of the appropriate dilution of the bacteriophage. Cells and phage were left at room temperature for 15 minutes to allow adsorption of the phage to the

cell membrane. Three ml of warm (50-55°C) TM Top agar or agarose (7-10 ml for 150mm petri dishes) were added to the cell/phage mixture, vortexed and then plated onto TM plates. Plates were inverted and incubated at 37°C.

B. Patching of bacteriophage

An innoculum of 0.1 ml of bacterial cells (0.3 ml for 150mm plates) was made by plating the cells in TM Top agar or agarose onto a TM plate. The narrow tip of a sterile toothpick was used to stab a single plaque which was then "patched" by lightly touching an area on the lawn of cells.

Single plaques were purified by taking an agar plug of the plaque in the tip of a pasteur pipette and placing in 1.0 ml of TM buffer containing a drop of chloroform. The plug/buffer mixture was vortexed gently and left to stand for 1-2 hours to allow the bacteriophage to diffuse out of the agar. The concentration of bacteriophage in this mixture was approximately 10^6-10^7 pfu (plaque forming units)/ml. The mixture was stored at 4°C.

C. Preparation of plate lysate stocks

One-tenth ml of bacterial cells was mixed with 10^5 pfu of the bacteriophage and then plated onto TM plates. The plates were incubated upright for 9 - 12 hours until lysis was nearly confluent. Five ml of TM buffer was added to the plate and stored with gentle shaking at 4°C overnight. The TM buffer was then removed into a tube by pouring or using a pasteur pipette. Another 1.0 ml of TM buffer was then added to the plate and the procedure repeated. The 1.0 ml lysate was then

combined with the 5.0 ml. One-tenth ml of chloroform was added to the lysate, the mixture vortexed and centrifuged (SS-34, 4K, 10', 4°C) to remove any contaminating debris. The supernatant was recovered and stored over chloroform at 4°C.

D. Preparation of mini-lysates

Host bacteria, 50 µl of fresh overnight cells grown in LB plus 10mM $MgSO_4$, were mixed with approximately 10^7 pfu of virus obtained from a single plaque in an agar plug or from 10 - 20 µl of a lysate. The cells and phage were incubated for 15 minutes at room temperature. Five ml of LB with 10mM $MgSO_4$ were added and the culture incubated at 37°C with vigorous shaking (250-300rpm). Lysis was evident within 5 - 8 hours by clearing of the culture. Chloroform was added to 0.3% with a further 15 minute incubation at 37°C. The lysate was centrifuged (SS-34, 8K, 10', 4°C) to remove bacterial debris and unlysed cells.

VI. PREPARATION OF DNA

A. Large scale isolation of pT25.1 DNA

i) Preparation of DNA

DNA was prepared by the method of Holmes and Quigley (1981). *E.coli* strain HB101 containing the plasmid pT25.1 was grown overnight at 37°C in LB broth plus 100 µg/ml ampicillin (LB-amp). Overnight cells were used to innoculate 500ml LB-amp prewarmed in a 2 litre flask. The cells were incubated at 37°C with moderate shaking until the culture was in log phase ($O.D._{600}$=0.45). Chloramphenicol was added to 170 µg/ml.

Under these circumstances replication of bacterial, but not plasmid, DNA is inhibited with consequent relative enhancement of plasmid DNA content. The culture was incubated for an additional 12 - 16 hours and harvested by centrifugation (GSA, 4K, 10', 4°C). The bacterial pellet was resuspended in 10 ml of TE buffer and transferred to a 50 ml Erlenmeyer flask to which was added 1.0 ml of a solution of 20 mg lysozyme/ml in 10mM Tris(pH8.0). The mixture was heated over a Bunsen burner, with constant shaking, until the liquid just started to boil. The flask was submerged in boiling water for 40 seconds and subsequently submerged in ice-cold water for 5 minutes. At this stage the cells should all be lysed and the DNA released into the solution making it viscous. The mixture was centrifuged (SS-34, 19K, 45', 4°C) to remove debris.

ii. Cesium chloride density gradient.

The preparation was readied for cesium chloride (CsCl) density gradient centrifugation by adding 1.0 g CsCl to each 1.0 ml of supernatant and 0.8 ml of EtBr solution (10 mg/ml in water) to each 10.0 ml of the resulting CsCl solution. The centrifugation was carried out in a Beckman VTi50 rotor (50K, 16 hours, 20°C). The plasmid DNA, monitored by EtBr fluorescence, was found midway up the tube below bacterial DNA and above the RNA, which was found at the bottom of the tube. The plasmid band was is removed with a hypodermic needle.

iii. Removal of ethidium bromide

EtBr was removed by extracting with an equal volume of isopropanol which has been saturated with NaCl (100.0 g NaCl,250 ml water, 500 ml

2-propanol, 10.0 ml 1.0M Tris (pH7.5). The extraction was repeated until all the pink color of the EtBr disappears from the aqueous phase. Themixture was dialyzed against several changes of TE buffer to remove CsCl. The quantity of DNA isolated was estimated by electrophoresing DNA against a series of uncut lambda standards.

B. Extraction of *Drosophila* genomic DNA

i. Embryo collection and dechorionation

Two to three thousand flies were placed in a population cage with 150mm petri dishes filled with yeast-sucrose medium coated with yeast paste. Plates were left a maximum of 18 hours to ensure females oviposit on the medium and yeast plate. The embryos were washed from the petri dishes onto a fine mesh size screen with the aid of gentle brushing. Embryos were rinsed with cold running water for approximately 10 minutes to remove any contaminating yeast or medium. The embryos were then placed in a Buchner funnel with filter paper, incubated at room temperature with a 1:1 mixture of commercial bleach (6.0% sodium hypochlorite):water for 2 minutes then rinsed with 1 - 2 litres of water.

ii. Extraction of *Drosophila* embryo DNA

The method of Henikoff (1981) was employed. Not more than 2.5 g of dechorionated embryos were homogenized in 10.0 ml of 2.0% SDS, 7M Urea, 0.35M NaCl, 10mM Tris(pH8.0) and 1mM EDTA. The embryos were homogenized in a Dounce homogeniser using 15 strokes with a loose-fitting pestle

followed by 15 strokes with a tight-fitting pestle. The homogenate was placed in a shaking bottle with 2.0 ml of 10.0% SDS. The mixture was extracted three times with gentle shaking for 15 - 30 minutes with a 1:1 phenol-chloroform mixture (Sec.2, IV, A). The organic and inorganic phases were separated by centrifugation (SS-34, 5K, 10', 4°C). DNA was precipitated from the final aqueous phase by ethanol precipitation. The final mass of DNA was pelleted by centrifugation (SS-34, 5K, 5', 4°C), dessicated and redissolved in TE buffer. RNA and protein contaminants were removed on a CsCl density gradient (Sec.2, VI, A, ii). EtBr was removed from the solution with isopropanol and the remaining DNA was dialyzed against TE buffer overnight (4°C). Quantity and size of DNA was estimated by electrophoresing the DNA on a 0.4% agarose gel with various amounts of lambda standard DNA.

C. Large scale preparation of EMBL4 DNA

i. Isolation of large scale liquid lysate

EMBL4 bacteriophage was added to 10.0 ml of fresh overnight cells grown in LB broth with 10mM $MgSO_4$ at a multiplicity of infection of 0.1. Ten ml of a fresh overnight should contain approximately 1.25 x 10^{10} cells. The cells and bacteriophage were incubated for 10 minutes at room temperature then transferred to 500 ml of prewarmed LB broth at 10mM $MgSO_4$ in a 2 litre flask and incubated at 37°C with vigorous shaking. Lysis was usually evident 5-9 hours after infection. Chloroform (to 0.3%) was added to complete lysis of remaining bacterial cells. The lysate was then titered. A concentration of 10^{10} pfu/ml was considered sufficient for continued extraction of the EMBL4 DNA. When

large scale liquid lysates were difficult to obtain due to poor growth of the bacteriophage, numerous mini-lysate preparations were combined to provide enough virus particles.

ii. Preparation of lambda phage.

The method of Yamamoto et al. (1970) was used. Ribonuclease A (RNaseA) and DNaseI were added to the liquid lysate at room temperature to a final concentration of 1 µg/ml. After digesting RNA and DNA for 30 minutes at room temperature, NaCl was added to 0.7M by gentle swirling. The lysate was left on ice for one hour. This step allows Mg^{++} stabilization of the bacteriophage. The mixture was centrifuged (GSA, 11K, 10', 4°C) to remove bacterial debris. The supernatant was transferred to a 1 litre erlyenmeyer flask. Solid polyethylene glycol (PEG-8000) was added to a final concentration of 10% w/v which was gently swirled on ice until all the PEG had dissolved. The lysate was stored on ice for at least 1 hour or overnight at 4°C, then swirled gently to resuspend any precipitated phage and transferred to centrifuge bottles. Centrifugation (GSA, 11K, 10', 4°C) precipitated the bacteriophage and PEG. The supernatant was discarded and any excess moisture was removed with a pasteur pipette. The pellet from a 500 ml lysate was resuspended in 8.0 ml of TM buffer with gentle swirling to prevent breakage of the bacteriophage. PEG was removed by successive 30 second extractions with chloroform (Sec.2, IV, A). 0.75 g CsCl was then added for each 1.0 ml of phage suspension and dissolved gently by inversion. After centrifugation (SW50.1, 35K, 24 hours), a single thin white band of phage was seen after centrifugation. The CsCl was removed by dialyzing against 10mM NaCl, 50mM Tris(pH8.0) and 10mM $MgSO_4$.

iii. Extraction of EMBL4 DNA

The bacteriophage suspension was transferred to a centrifuge tube and brought to 20mM EDTA, 50 µg/ml Proteinase K and 0.5% SDS. The tube was inverted several times to ensure thorough mixing and then incubated at 65°C for 1 hour. After deproteinization with phenol/chloroform and overnight dialysis at 4°C against TE, the DNA was ethanol precipitated.

D. Mini-preparation of bacteriophage DNA

Mini-preparations were prepared by the method of E.F. Fritsch (Maniatis et al., 1982). Mini-lysates of EMBL4 or EMBL4 derivatives were prepared (Sec.2, V, D.). The lysate was brought to 1 µg/ml with RNaseA and DNaseI and incubated for 30 minutes at 37°C. An equal volume of a solution of 20% (w/v) PEG, 2M NaCl, 10mM Tris(pH7.5) and 10mM MgSO was added to the lysate. After gentle mixing by inversion, the solution was stored in an ice bath for 1 hour. The precipitated bacteriophage are recovered by centrifugation (SS-34, 10K, 20', 4°C). The pellet of phage particles was resuspended in 0.5 ml of TM buffer. The suspension was centrifuged 2 minutes in a microfuge to remove debris. The supernatant was transferred to a new microcentrifuge tube and incubated at 68°C for 15 minutes with 0.1% SDS and 5mM EDTA (pH8.0). A subsequent phenol/chloroform extraction removed the proteins. DNA was precipitated by adding to the final aqueous phase an equal volume of isopropanol and storing at -70°C for 20 minutes. The DNA was precipitated by centrifugation in a microcentrifuge for 15 minutes at 4°C. The pellet was washed with 70% ethanol, dessicated and resuspended in 50 µl TE buffer.

VII. MAKING A GENOMIC LIBRARY

A. Preparation of arms

The bacteriophage vector EMBL4 is useful in making a genomic library (Sec.2, I, B, vi.). EMBL4 was initially digested with BamHI restriction enzyme which digests the vector into a left arm, a right arm and an internal fragment that can be replaced with genomic DNA. A sample of the DNA run on a minigel confirmed complete digestion. Digestion with SalI ensured the internal fragment was completely removed and destroyed by four internal restriction sites. An additional sample of DNA was run on a minigel to confirm complete digestion.

B. Large scale preparation of partially digested Sau3A *Drosophila* DNA

Genomic DNA digested with the 4 bp restriction enzyme Sau3A produces staggered cohesive ends homologous to the BamHI cohesive ends found on the prepared EMBL4 vector arms (Sec.2, VII, A.). A commercial preparation of Sau3A restriction enzyme was diluted in Sau3A storage buffer (10mM Tris(pH7.4), 50mM KCl, 0.1mM EDTA, 1mM DTT, 500 µg/ml BSA and 50%v/v glycerol). Prior dilution allowed repeatable production of very low enzyme concentrations of 0.01U/µl. Initially, 1 µg aliquots of genomic *Drosophila* DNA were digested at 37°C with Sau3A at 5 - 10 minute intervals from 0 - 80 minutes. The reaction mixture consisted of 1 µg of genomic *Drosophila* DNA, 0.02 units of Sau3A restriction enzyme in 2 µl in the appropriate digestion buffer (6mM Tris(pH7.5), 6mM $MgCl_2$, 50mM NaCl, 100 µg/ml BSA). Samples were heated to 68°C for 10 minutes to stop the reaction and electrophoresed on a 0.4% agarose gel. The optimal conditions for a full scale digestion were determined from these

results. Larger quantities of genomic DNA were then digested using these conditions and a sample analyzed by electrophoresis to check the digestion.

The Sau3A partial digestion products were then separated by size on a gradient, (5 to 29% NaCl in TE (pH8.0)). DNA in a volume up to 0.3 ml was loaded on the gradient which was centrifuged in an SW40 rotor (35K, 6 hours, 22°C). One-half ml fractions were collected and samples electrophoresed on a 0.4% agarose gel to ascertain which fractions contained appropriately sized DNA fragments. Salt concentrations of lambda standards were adjusted accordingly. The chosen fractions were purified by running them over an ion-exchange resin (NACS prepac, BRL) followed by ethanol precipitation.

C. Ligation reaction

Test ligations were set between EMBL4 arms and Sau3A partial digests of *Drosophila* DNA in ratios of 4:1, 3:1, 2:1, 1:1 and 0.5:1 for arms:inserts (Sec.2, IV, E.). Each ligation reaction was packaged *in vitro* to form viable plaque forming units.

D. Preparation of packaging extracts

Packaging extracts were prepared by the method of Hohn and Murray (1977). Two bacterial strains BHB2688 and BHB2690 (Sec.2, I, B, iv. and v.) each carry a different prophage that possess amber mutations in the bacteriophage genes encoding protein E and protein D respectively. Protein E encodes a major component of the bacteriophage head, whereas protein D is involved in the stabilization and maturation of the head after assisting the insertion of the lambda DNA. Both prophages carry a

temperature sensitive lambda repressor molecule (cIts) which maintains the lambda DNA in a lysogenic state at 32°C yet allows induction at 45°C due to the inactivation of the cI repressor. An amber mutation in the lambda S gene prevents cells from lysing upon induction of the endogenous lambda prophage. A b region deletion reduces the chance of packaging the endogenous lambda DNA. A red3 mutation in the prophage combined with a recA⁻ host reduces recombination between the induced prophage and the exogenous DNA to be packaged.

i. Preparation of preheads from λ lysogen BHB2690: Sonicated extract

The strain BHB2690 was streaked on LB plates which were incubated at 32°C and 42°C. Lack of growth at 42°C confirmed the presence of the lysogen. A 500 ml aliquot of NZ medium (10.0 g NZ-amine(Type A hydrolysate of casein, Humko, Sheffield Chem.Div.), 5.0 g NaCl, 2.0 g $MgCl_2$/litre H_2O) was innoculated with BHB2690 bacteria to an O.D.600 of 0.08-0.1 and incubated at 32°C with moderate shaking until O.D.600 of 0.3 was reached. The prophage were induced by slowly shaking the flask in a 45°C waterbath for 15 minutes followed by subsequent incubation at 37°C for 3 hours with vigorous shaking. Chloroform was added to a sample to check for induction. If induction was successful; the solution cleared. The cells were harvested by centrifugation (GSA, 5K, 10', 4°C). The pellet was resuspended in 2.5ml of cold sonication buffer (20mM Tris(pH8.0), 0.1mM EDTA, 3mM $MgCl_2$ and 5mM β-mercaptoethanol. The suspension was sonicated on ice with 5 - 10 second pulses and 30 second breaks to ensure that the temperature remained below 4°C. Sonication was complete when the mixture was no longer viscous. Debris was removed by centrifugation (SS-34, 10K, 10',

4°C). An equal volume of sonication buffer was added to the supernatant followed by one-sixth volume of packaging buffer (6mM Tris(pH8.0), 50mM spermidine, 50mM putrescine, 20mM $MgCl_2$, 30mM ATP and 30mM β-mercaptoethanol). After complete mixing, the preparation was distributed to microcentrifuge tubes in 15-30 µl aliquots and frozen in liquid nitrogen. The tubes were then stoppered and stored at -70°C.

ii. Preparation of packaging protein from λlysogen BHB2688: Freeze Thaw Lysate

BHB2688 cells were grown in the same way as the BHB2690 cells. Three 500 ml aliquots of NZ medium were innoculated with BHB2688 bacteria in the same manner as described for BHB2690. After harvesting the cells by centrifugation (GSA, 10K, 10', 4°C) the cells were resuspended in 2.0 ml of 10% sucrose solution in 50mM Tris(pH7.5) at 4°C. The suspension was transferred to a new tube and 100 µl of fresh lysozyme (2mg/ml, 0.25M Tris(pH7.5) was added. After gentle mixing, the suspension was frozen in liquid nitrogen and thawed slowly on ice. 100 µl of packaging buffer was added and mixed thoroughly by inversion. Following centrifugation (SW50.1, 35K, 60', 4°C) 10 µl aliquots of supernatant were frozen in microcentrifuge tubes using liquid nitrogen, capped and stored at -70°C.

E. *In vitro* packaging of bacteriophage DNA utilizing prepared packaging extracts

Tubes containing prehead (sonicated extract) and packaging protein (freeze-thaw lysate) were removed from the -70°C freezer and thawed on ice. The DNA to be packaged was added to 10 µl of the freeze-thaw

lysate in volumes of 5 - 10 μl. After mixing gently with the micropipet tip, 15 μl of the sonicated extract was added. The extracts were again mixed gently and then incubated for 1 hour at room temperature. One-half ml of TM buffer was then added to the mixture. The extract was titered on Q358 and Q359. The expected rates of packaging were 10^4 - 10^6 pfu/ug DNA (Hohn and Murray, 1977).

F. Plating of the packaged library

Having achieved a satisfactory packaging, the packaged material was plated on the P2 lysogen at a density of approximately 10^4 plaques on 150mm plates at which point they do not quite reach confluence overnight (9 - 12 hours). These unamplified libraries were usually probed with nick translated ^{32}P-pπ25.1 after transfer to nitrocellulose paper.

Alternatively, amplified libraries were prepared by making plate lysates (Sec. 2, V., C.) and stored at 4°C over chloroform.

G. Postulated library size

In preparing a library of genomic DNA one must calculate how many plaques are needed to carry all fragments of the genomic DNA under study. A formula described by Maniatis et al. (1982) is used to calculate the number of plaques needed to ensure with 99% probability that all segments of the genomic DNA are inserted into the vector. The formula is based on an average size of insert and on the size of the genomic DNA.

formula is based on an average size of insert and on the size of the genomic DNA.

$$N = \frac{\ln (1 - 0.99)}{\ln (1 - (x/y))}$$

where:

N = the necessary number of recombinant plaques needed in the library

0.99 = the 99% probability of having a given DNA sequence represented in a library of 20kb fragments of a genome

x = the size in base pairs of the average inserted DNA fragment

y = the size in base pairs of the entire genomic DNA of the organism

Assuming the average size of insert in EMBL4 is 20kb and the genome size of *Drosophila melanogaster* is 1.65×10^8bp, the number of plaques needed in the library is calculated to be 37,995.05 with 99% probability.

$$N = \frac{\ln (1 - 0.99)}{\ln (1 - (2.0 \times 10^4/1.65 \times 10^8)}$$

$$N = 37,995.05$$

Approximately 38,000 plaques are needed to form a complete *Drosophila melanogaster* library of 20kb size fragments.

III FILTER TRANSFERS

A. Plaque lifts of EMBL4 libraries

150mm plates with approximately 10^4 plaques were prepared directly from packaging extracts or from an amplified library. The plates were chilled at 4°C to harden the top agarose. Filters (150mm diameter Biodyne nitrocellulose) were placed carefully on the agarose surface and plates were marked asymmetrically with a syringe and India ink. After one minute on the agarose surface the filter was lifted carefully and placed plaque side up on Whatman #3 filter paper saturated with denaturing solution (1.5M NaCl, 0.5M NaOH) for 5 minutes. It was then transferred to a neutralizing solution (3M sodium acetate(pH5.5)) for an additional 5 minutes, air dried for 30 minutes, at room temperature, and baked at 80°C for 1 hour (Benton and Davis, 1977).

B. Southern transfers

electrophoretic separation of DNA on agarose gels was followed by denaturion with 1.5M NaCl and 0.5M NaOH for 1 hour, at room temperature, with gentle shaking. After neutralizing with 1M Tris(pH8.0), 1.5M NaCl for 1 hour, at room temperature, the gel was placed on Whatman#1 filter paper arranged on a glass support leaving wicks in a reservoir of 10x SSC (1.5M NaCl, 0.15M sodium citrate). Nitrocellulose cut to dimensions 1-2mm larger than the gel (the nitrocellulose is handled with gloves at all times) is floated on a solution of 2x SSC (0.3M NaCl, [illegible] citrate) and then submerged for 2 - 3 minutes. The wet [illegible] was placed on the gel, taking care to prevent its making contact more than once and avoiding air bubbles. Two pieces of Whatman #1 filter

paper soaked in 2x SSC were then placed on the nitrocellulose paper followed by a 3" stack of dry paper towels and weights. The flow of buffer from reservoir to the dry towels transfers the DNA from gel to nitrocellulose in 12 - 24 hours. The nitrocellulose was then washed in 6x SSC for 5 minutes and the paper air dried at room temperature. After drying, the filter is baked at 80°C for 2 hours in a vacum oven.

IX. HYBRIDIZATIONS

A. Prehybridization

i. Plaque lifts

Plaque lift filters were pre-hybridized to nonhomologous, nonradioactive DNA to prevent non-specific binding of probe. Plaque lift filters were placed in 4 ml of hybridization buffer (5x Denhardt's composed of 0.5 g Ficoll Type 400, 0.5 g polyvinylpyrrolidone, 0.5 g BSA - Pentax Fraction V in 50ml H_2O, 5x SSPE composed of 0.9M NaCl, 50mM NaH_2PO_4(pH8.3) and 5mM EDTA, 0.2% SDS) in a heat sealable plastic food storage bag (Phillips). Denatured, sonicated salmon sperm DNA was added to the filters at 250 µg/ml (denatured by boiling 10 minutes at 100°C and placed on ice immediately). Bags were sealed and incubated at 65°C for at least 2 hours.

ii. Southern transfers

Southern transfer filters were treated in a similar fashion. Baked

filters were submerged in 6x SSC (0.9M NaCl, 0.09M Na citrate) for 2 minutes, placed in a heat sealable bag with prehybridization fluid (6x SSC, 0.5% SDS, 5x Denhardts, 100 μg/ml of denatured salmon DNA, 4ml of solution/100cm^2), sealed and incubated 2 - 4 hours at 65°C.

B. Hybridization

i. Plaque lifts

A corner was cut from the sealed bag and as much prehybridization solution as possible removed. The ^{32}P-labelled probe was denatured (100°C, 10') and added to provide 10^6 - 10^7 CPM/filter which usually required 0.01 - 0.1 μg of DNA. Two 2 ml of additional hybridization buffer was added, the bag resealed and then placed in a second sealed bag containing water. Hybridization was carried out at 65°C for at least 18 hours.

ii. Southern transfers

Excess solution was removed and ^{32}P-labelled probe was denatured (100°C, 10') and added. For hybridization to cloned DNA fragments, 0.01 - 0.1 μg of probe DNA of minimum specific activity of 10^7CPM/μg was used. When *Drosophila* genomic DNA was to be probed, a minimum of 10^7CPM in DNA of 10^8CPM/μg specific activity (or more) was used. Hybridization buffer was added (6x SSC, 0.5% SDS, 5x Denhardt's, 0.01M EDTA, 100 μg/ml of denatured salmon DNA, 2 ml of solution/100cm^2), the bag resealed and then placed in a second sealed bag containing water. Hybridization was carried out at 65°C for 3 - 8 hours for cloned DNA fragments and at least 24 hours for genomic DNA.

C. Washing of unhybridized probe

i. Plaque lifts

Biodyne filters were washed in 3 - 1 litre changes of wash buffer (5mM Na phosphate(pH7.0), 1mM EDTA and 0.2% SDS) at room temperature with constant shaking over a period of 1 1/2 hours. Washed filters were left to dry at room temperature.

ii. Southern transfers

Southern filters were washed in 3 - 1 litre changes of wash buffer (2x SSC, 0.5% SDS) at room temperature with constant shaking over a period of 1 1/2 hours. A stringent temperature of 60°C was sometimes used for the second wash. Washed filters were left to air dry at room temperature.

D. Autoradiography of nitrocellulose filters

All filters were wrapped in Saran Wrap to prevent unwanted dispersal of radioactivity. X-ray film (Kodak, X-Omat AR5) was placed against the filters. Markings on the filters were matched on the film utilizing a sharp instrument. Film and filters were placed in a film cassette containing an intensifying screen (Lightning plus) to minimize the time of exposure.

Film was left to expose at -70°C for a period of hours or days, developed under Kodak #13 safe lights, in Kodak Microdol developer (2 minutes), stopped in 2% acetic acid (5 seconds), fixed in Kodak fixer (5 - 10 minutes), washed in running water (5 minutes) and air dried.

X. PLAQUE PURIFICATION OF P-POSITIVE CLONES

Positive areas, detected on a plaque lift of genomic libraries using the P element probe pπ25.1, were resuspended in TM buffer and replated to produce dispersed, distinct plaques on 100mm plates. When subsequent plaque lifts and rehybridization with the P element probe revealed positive plaques, they were patched individually onto a lawn of bacterial cells. A series of 10 - 15 plaques in an area, suspected of carrying a positive plaque, were patched to ensure the single positive plaque would be isolated. Patched viruses were transferred to filter and hybridized. Mini-lysates were prepared from confirmed positive clones from which the DNA was isolated and digested with EcoRI to characterize the insert DNA. Nick translation of the DNA and subsequent *in situ* hybridization to polytene salivary chromosomes (Amherst - M strain) confirmed the cytological location of the *Drosophila* DNA present in the plaque.

XI. *IN SITU* HYBRIDIZATION TO *DROSOPHILA* POLYTENE CHROMOSOMES

The method of Pardue and Gall (1975) was used.

A. Preparation of *Drosophila* polytene salivary chromosomes

Salivary glands were dissected from a third instar female larva under 45% acetic acid and transferred to a drop of fresh 45% acetic acid on a microscope slide previously coated with a 0.5% gelatin solution. The gland was covered with a presiliconized 22mm square #2 coverglass. The tissue was macerated by tapping gently on the coverglass with the eraser end of a pencil or by applying pressure to it with a dissecting

needle. The coverglass was covered with blotting paper and pressure was applied with the thumb to spread and flatten the tissue. The coverglass was removed with a razor blade after freezing the slide for 10 - 20 seconds in liquid nitrogen. The slide was washed once in 3:1 95% ethanol:glacial acetic acid (1 minute) and twice in 95% ethanol (5 minutes each) then air dried.

B. Pretreatment of polytene chromosome slides

To improve chromosome morphology, slides were incubated in 2x SSC for 30 minutes at 65°C (Bonner and Pardue, 1976), rinsed twice in 70% ethanol (5'), once in 95% ethanol (5') and then air dried. Endogenous RNA was removed with 0.1 µg/ml RNase A in 2x SSC (heated to 100°C, 30' to destroy any contaminating DNase) for 1 hour at 37°C. After treatment the slides were washed three times in 2x SSC, dehydrated in ethanol and air dried. As final preparation, just prior to the *in situ* hybridization, slides were treated wtih 0.07N NaOH (3', room temperature) to denature DNA, washed three times in 70% ethanol, twice in 95% ethanol and air dried.

C. Hybridization and Washes

At least 2 - 4 x 10^5CPM of tritiated probe was added to each slide which required approximately 0.02 - 0.04 µg of DNA in 20 µl of hybridization buffer (1x Denhardt's, 5% dextran sulphate, 0.33M NaCl, 10mM $MgCl_2$, 10mM Na phosphate buffer (pH7.0), and 0.1mg/ml denatured, sonicated *E.coli* DNA) were added per slide. Probe DNA and carrier DNA were denatured by boiling for 5 - 10 minutes and placed on ice immediately, prior to addition of hybridization buffer. Twenty µl of

the reaction mixture was placed over the tissue on a prepared slide, covered with a prewashed coverglass (boiled in 1N HCl, 5'), and the slide placed in a moist chamber (2x SSC) which was sealed and submerged in a 65°C waterbath for a minimum of 4 hours.

The hybridization was terminated by washing the slides in 2x SSC three times at 65°C (15 minutes each) and twice at room temperature (15 minutes each). The slides were washed once in 70% ethanol, twice in 95% ethanol and air-dried.

D. Autoradiography and staining of *in situ* hybridization slides

Kodak Ilford NTB-2 emulsion was mixed 1:1 with warmed distilled water (52°C). Emulsion is handled and applied using Kodak #2 safelights only. Slides were dipped and allowed to drain and dry for 30 - 60 minutes before sealing in a light tight slide box and storing at 4°C. The autoradiographic images were developed 1 - 4 weeks after application of emulsion. The slides were submerged 1 minute in water, 2 minutes in a 1:1 mixture of Kodak Dektol developer and water, rinsed in water and fixed for 4 minutes in Kodak fixer. The fixed slides were washed for 5 - 15 minutes in running cold tap water.

If staining was required, 2.5 ml of Geimsa stock solution (Gurr, BDH Chemicals) was brought to 50 ml with 0.01M phosphate buffer (pH7.0). Slides were dipped in stain for 5 minutes and rinsed with running water.

Bridge's cytological map was used to determine regions on the polytene chromosomes (Bridges, 1938, Lindsley and Grell, 1968).

3. RESULTS AND DISCUSSION

I. PM STATUS OF PARENTAL STOCKS

In situ hybridizations of various *Drosophila melanogaster* stocks with the P element containing probe pπ25.1 established their PM status. The presence of unique *Drosophila* DNA from cytological region 17C (X chromosome) provided an internal control to ensure that the hybridization was successful. Autoradiographic images varied considerably between preparations and even within preparations; therefore the repeated appearance of site specific grain on numerous (but not all) nuclei was taken to indicate homology. Several slide preparations were inspected to confirm site specificity.

The *ycvvf* strain as seen in Figure 9 is an M strain that contains no P elements in its genome. Likewise, the *ycvras*^{2}*vf*, H6/FM6, Amherst and In(1)123/FM6 strains were confirmed to be "M" strains. The v(I) and π2P strains displayed in Figures 10, 11 and 12 are P strains with approximately 20 and 30 P elements dispersed throughout their genomes, respectively.

PM dysgenic hybrids of *Drosophila* often display reduced fertility at 29°C, in comparison to 22°C, due to increased levels of gonadial dysgenesis (Bregliano and Kidwell, 1983; Engels, 1983). Table 3 illustrates the results of a fertility test wherein the average number of progeny, from 10 hybrid dysgenic females (from each cross) and M strain males incubated at 22°C and 29°C for a period of 10 days, are scored. The M strain used, as parents to the hybrid dysgenic females,

FIGURE 9. *In situ* hybridization of *ycvvf* nucleus with pπ25.1 probe

ycvvf is an M strain possessing no P elements in the genome. Note the single site of hybridization at position 17C homologous to the unique *Drosophila* DNA in the pπ25.1 probe.
Tissue: *ycvvf/ycvvf* female, two week exposure
Magnification: 880 X

FIGURE 10. *In situ* hybridization of π2P nucleus with pπ25.1 probe

π2P is a P strain possessing approximately 30 P elements in the genome. π2P displays approximately 5 P elements on the X chromosome. The cytological region 9E (*ras*) is P element free. The 17C region displays grain.
Tissue: π2P/π2P female, two week exposure
Magnification: 590 X

FIGURE 11 and 12. *In situ* hybridization of v(I) nuclei with pπ25.1 probe

v(I) is a P strain possessing approximately 20 P elements in the genome. v(I) displays approximately 3 P elements on the X chromosome. The nuclei display a P element-free 9E region and 17C hybridization.
Tissue: v(I)/v(I) female, two week exposure
Magnification: 690 X

FIG.9. *y cv v f*

FIG.10. TT2P

FIG.11. v(1)

FIG.12. v(1)

was *ycvvf* in all cases. Only a slight decrease in fertility, in comparison with the reciprocal non-dysgenic cross, was seen in the v(I) P strain whereas the π2P strain showed a marked reduction. The results indicate that π2P is a "classical" P strain whereas v(I) may be a Q strain which possesses many properties associated with P strains, excluding sterility (Bregliano and Kidwell, 1983). In subsequent experiments designed to generate P element induced *raspberry* mutants, both v(I) and π2P were used as the male parents. The M strain female parents were *ycvvf*.

II. ISOLATION OF P ELEMENT INDUCED RASPBERRY LETHALS

Three cycles of matings as described in materials and methods (Sec.2, II) were carried out using *ycvvf* females as the M strain parent and π2P or v(I) males as the P strain parent. Hybrid dysgenesis is seen in the offspring (the hybrid) between an M strain female and a P strain male. No hybrid dysgenic events were detected in the reciprocal cross.

After the initial mating of 10 *ycvvf* females to 10 P strain males, of 10 hybrid dysgenic females were mated with 10 *ycvras²vf* males at 22°C in 25 bottles. Female progeny displaying the raspberry eyecolor phenotype were expected to carry a mutation in the *raspberry* locus. The first two cycles employed v(I) males as the paternal P strain with π2P males being used in the final cycle. In each cycle it was estimated, based on fertility studies, that each female produced around 200 progeny over a 10 day period for v(I) matings and approximately 155

TABLE 3. FERTILITY TEST OF PM DYSGENIC CROSSES

AVERAGE NUMBER OF PROGENY/HYBRID DYSGENIC FEMALE OF 10 HYBRID DYSGENIC FEMALES SET FOR 10 DAYS

INCUBATION TEMPERATURE	ORIGINAL CROSS: v(I) STRAIN, M female x P male	v(I) STRAIN, P female x M male	π2P STRAIN, M female x P male	π2P STRAIN, P female x M male
22°C	201	222	156	271
°C	119	242	0	212

TABLE 4. RESULTS OF HYBRID DYSGENIC MATINGS

	ORIGINAL CROSS: ycvvf (M) female x v(I) (P) male	ycvvf (M) female x π2P (P) male	ycvvf (M) male x (P) female
Total No. of: Flies screened	50,000	19,375	24,600
Flies apparently mutant at *ras*	5	7	0
Sterile	2	5	0
Confirmed as recessive lethals	3	2	0
Rate of dysgenic mutation at *ras* locus	6.0 - 10.0 / 10^5 chromosomes	10.0 - 36.0 / 10^5 chromosomes	

for π2P matings. Therefore, 25 bottles with 10 hybrid dysgenic females set over a 10 day period produced approximately 50,000 flies for v(I) matings and 38,750 flies for π2P matings. Five bottles of the reciprocal cross of an M strain male with a P strain female produced approximately 11,100 flies for v(I) and 13,500 for π2P matings. The total number of flies screened in all three cycles are summarized in Table 4.

Green (1977) found the frequency of P element insertions at the raspberry locus to be in a range of 13 - 34 mutations per 10^5 chromosomes using the male recombination (MR) chromosome in *Drosophila melanogaster*. These mutations were due to the insertion of P elements in the raspberry gene (Bregliano and Kidwell, 1983). Eeken (unpublished) also reported MR induced raspberry mutations which Eeken notes involves the transposition of P elements. Assuming sterile mutants were true positives, the v(I) strain produced a rate of insertion of 10 mutants per 10^5 chromosomes in comparison to 36 mutants per 10^5 chromosomes for the π2P strain. These rates are comparable to those already calculated for the raspberry locus as cited above (Green, 1977). The v(I) strain shows a slightly lower rate. This may either reflect the observed difference in the strains found in the fertility tests or be a statistical fluctuation. The reciprocal mating produced no raspberry mutants, a finding consistnet with the low rate of spontaneous mutations at the raspberry locus of 0.04 mutations per 10^5 chromosomes as established by Green (1977). No additional eye color or morphological mutants were detected.

Approximately 1 *ycvrasvf* male per 10^3 chromosomes was detected in the dysgenic matings versus none in the nondysgenic matings. They are

presumed to be non-disjunction products. Chromosomal nondisjunction is a trait common to hybrid dysgenic matings in the PM system (Bregliano and Kidwell, 1983). Nondisjunction events arising from primary or secondary nondisjunction in the female would produce nullo-X eggs. These eggs would generate XO male offspring which observed abberant phenotype if fertilized by a *yc* [illegible]. As predicted for XO males, all *ycvras*^{ts}*f* males were found to be sterile.

III. CHARACTERIZATION OF THE *ras*$_{1}$ MUTANTS

A. Genetic status

Of the 12 females isolated with raspberry eyecolor, only 5 were fertile. The phenotypes of the original females isolated are listed in Table 5. Recombinants which arose in these females were sometimes preserved in the stocks established from them, resulting in a mixture of *ras* mutant bearing chromosomes in some stocks. All 5 mutants showed lethality when tested by mating to FM6 males as diagrammed in Figure 7. Confirmation of a raspberry phenotype associated with the mutated chromosomes was performed as illustrated in Figure 8. The relatively extensive analysis required to confirm that the lethal mutation is at the raspberry locus was not considered necessary. All stocks were outcrossed four times to M strain males to reduce the number of P elements present in the autosomes.

Reversion to wildtype of the lethal phenotype has been detected on a recurrent basis in stocks of NJ-5 and NJ-8. The remaining stocks have not displayed reversion to date. Selection for the lethal phenotype is

TABLE 5. PHENOTYPE OF P ELEMENT INDUCED RECESSIVE LETHALS DISPLAYING RASPBERRY EYE COLOR PHENOTYPE AT INITIAL ISOLATION

MUTANT STOCK	PATERNAL PARENT	ORIGINAL PHENOTYPE	P ELEMENT AT 9E
NJ-1	v(I)	y cv ras v	no
NJ-4	v(I)	y^+cv^+ras v	yes
NJ-5	v(I)	y^+cv^+ras v	yes
NJ-6	π2P	y cv ras v f	yes
NJ-8	π2P	y^+cv^+ras v^+f^+	yes

maintained within stocks. The occurence of reversion supports the view that these mutations are due to the insertion of P elements into the gene with a resultant lethal phenotype. Precise excision of P elements, with concomitant reversion, is common (Rubin et al., 1982).

B. Confirmation of P element insertion at the raspberry locus by *in situ* hybridization.

In situ hybridizations with a tritiated P element probe, p125.1 were carried out on each of the outcrossed stocks NJ-1, NJ-4, NJ-5, NJ-6 and NJ-8 to determine whether the observed mutant phenotypes were associated with a P element insertion in the raspberry gene. The raspberry gene is located at map position 32.8, which cytologically is in the region 9E1-9E4 as identified by Johnson et al. (1979). The P element probe was hybridized to tissue prepared from larvae heterozygous for the raspberry, lethal-bearing X chromosome and a wildtype M strain (Amherst) X chromosome. Such female flies were produced by crossing *rsb-1*/FM6 females to wildtype males: individual FM6-free (inversion free) preparations were selected directly after squashing, before removal of coverglass in preparation for hybridization.

All strains except NJ-1 were found to possess P elements at the raspberry locus (9E1-E4). Although NJ-1 carries a recessive lethal and displays a raspberry eye color phenotype it does not have a P element present at the raspberry locus. A P element may have inserted into the gene and transposed to a new site with imprecise excision, either deleting or inserting a small amount of material at the locus. Alternatively the PM dysgenic cross may have prompted the transposition

of another transposable element such as copia (Engels, 1983). Furthermore, v(I) is known to possess active I factor, the transposable element responsible for another dysgenic system (IR) (Bregliano and Kidwell, 1983). The IR status of the *y cv v f* stock is unknown, but it is possible that the cross producing NJ-1 (and NJ-4, NJ-5) could have been exhibiting both PM and IR dysgenesis simultaneously. NJ-1 could, therefore be a product of the later system. Finally, a spontaneous mutation may have produced the lethal phenotype.

Strains NJ-4, NJ-5, NJ-6 and NJ-8 all displayed grain over the region 9E1-E3 on the X chromosome, indicating that the *ras-1* mutation may be associated with a P element insertion event.

Figures 13, 14 and 15 are photographs of an *in situ* hybridization with P element probe to NJ-5/Am^{+} tissue. Approximately 7 P elements may be found on the X chromosome, including the site at 9E1-E4, and 3 P elements on the autosomes. The NJ-5 strain was selected for cloning the raspbery locus. The NJ-5 strain has a strong signal at the 9E band indicating extensive homology to the P element probe and contains only 10 genomic P elements, a low number compared to alternate strains. A final factor was that the NJ-5 *Drosophila* stocks grew well in comparison to the other stocks which were hard to expand. Such problems can be due to bacterial infection or to genetic factors that reduced the viability or fertility of the stocks.

IV. PREPARATION OF NJ-5 LIBRARY

A library of NJ-5 genomic DNA was made by ligating the arms of the

FIGURE 13, 14 and 15. *In situ* hybridizations of NJ-5 strain tissue with pπ25.1 probe

NJ-5 possesses at least 10 P elements in its genome. Three autosomal P elements are seen at positions 93 and 95 (3R) and 56F (2R). The *in situ* shown in figure 14 displays an NJ-5/Am^+ X chromosome possessing 8 P elements at positions 3C, 3F, 5B, 7C, 7E, 8C, 9E and 10C. No other P elements were seen on this chromosome. Figure 15 displays an NJ-5/Am^+ X chromosome possessing 6 P element sites at position 5B, 7C, 7E, 8C, 9E and 10C. The P elements in the tip are absent due to recombination with an M strain chromosome. Grain is present at 17C in all preparations.
Tissue: NJ-5/Am^+ female, two week exposure

Fig.13 Magnification: 760 X
Fig.14 720 X
Fig.15 730 X

FIGURES 16, 17 and 18. *In situ* hybridizations to M strain polytene chromosomes with 3 different P positive bacteriophage.

The three figures illustrate typical *in situ* hybridizations of 3 different clones obtained. Figure 16 demonstrates an *in situ* hybridization of the clone R-1a (pattern a, Fig.25) that hybridizes only to 3C on the X chromosome. Figure 17 utilizes the probe H-1 (pattern x, Fig.25) and hybridizes to region 56F whereas the probe Q-1 (pattern p, Fig.25) in Figure 18 carries a multicopy sequence of DNA that hybridizes extensively to the centromere. There is no grain seen at cytological region 9E in all cases.
Tissue: Am^+/Am^+ female, two week exposure

Fig.16 Magnification: 870 X
Fig.17 1200 X
Fig.18 630 X

FIG.13. NJ-5 NUCLEUS

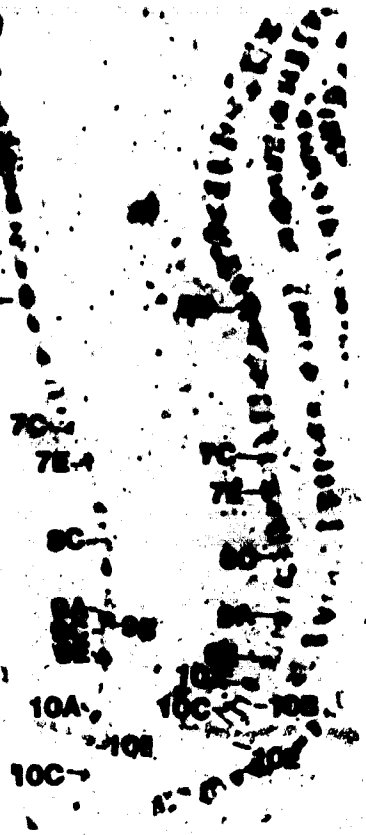


FIG.14. NJ-5

FIG.15. NJ-5

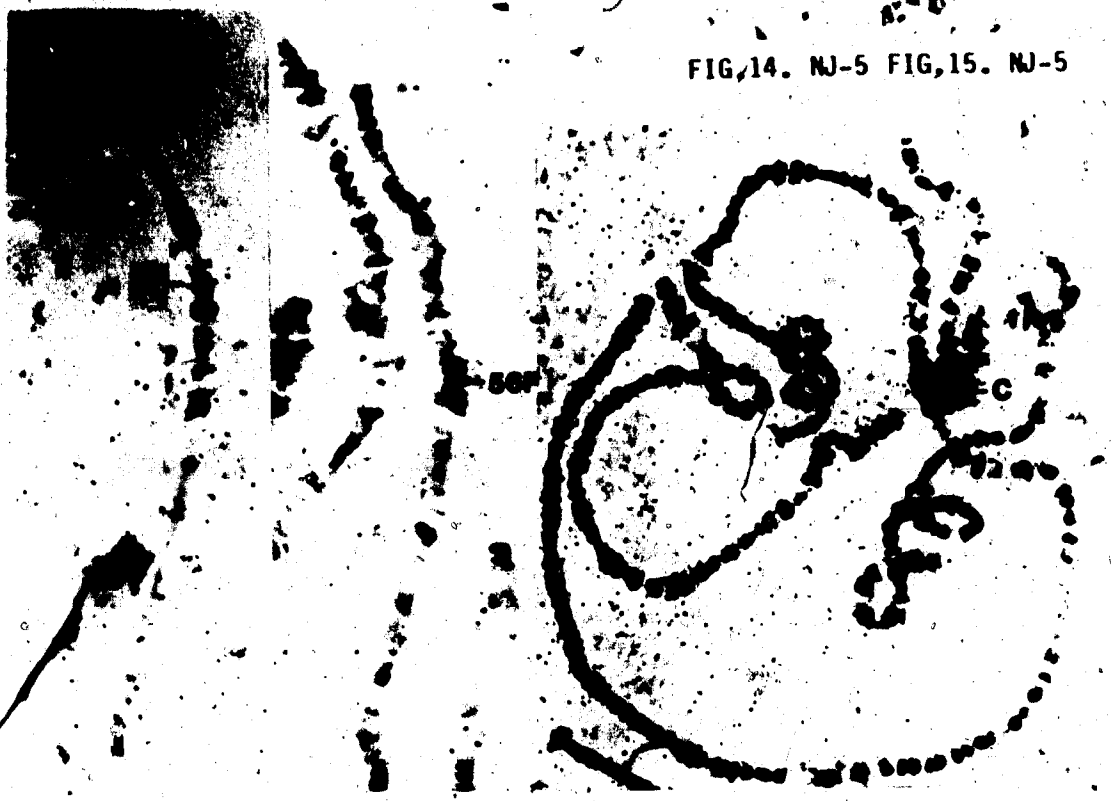


FIG.16. 3C PROBE

FIG.17. 56F PROBE

FIG.18. MULTICOPY PROBE

bacteriophage vector EMBL4 to 18-23kb sized Sau3A fragments of NJ-5 genomic DNA. The results of one such preparation are illustrated in Figures 19 and 21. Sau3A digestion of NJ-5 *Drosophila* DNA required 0.02U of enzyme/μg of DNA with an optimal incubation time of approximately 25 minutes at 37°C (Figure 19). Sau3A digested NJ-5 DNA was run on an NaCl gradient to separate the various fragment sizes. Samples of fractions collected were run on a 0.4% agarose gel to determine which fractions contained 18-23kb sized fragments (Figure 21). EMBL4 bacteriophage arms were prepared (Sec.2, VII, A.). To confirm that both the EMBL4 DNA and the Sau3A partial digests were capable of ligating, 0.25 μg of small MW partials of Sau3A digested DNA were ligated. EMBL4 was also ligated (Figure 23). The ligated combination of Sau3A partials with EMBL4 DNA was not analyzed by gel electrophoresis due to limited amounts of DNA.

The ligated EMBL4 arms and Sau3A (18-23kb) insert DNA was packaged (Sec. 2, VII, E.) and plated on the bacterial strain Q359 to detect insertion of the genomic DNA (spi⁻ phenotype). Results of one such packaging experiment are diagrammed in Table 6. The DNA was packaged with an efficiency of 7.2×10^4pfu/μg of insert DNA with a background religation rate of approximately 1.8×10^3pfu/μg EMBL4 DNA on Q359 (P2 lysogen). Control DNA (EMBL4 intact) was packaged with an efficiency of 1×10^7pfu/μg DNA as estimated on Q358 cells. Fedoroff [illegible] testing the efficiency of the EMBL4 vector in various host strains had optimal rates as high as 1.0×10^5pfu/μg of insert DNA with a background religation rate of 1.9×10^3pfu/μg EMBL4 DNA. The rates I observed are comparable although the packaging efficiency of insert DNA is somewhat low. Hohn and Murray (1977) predict packaging efficiencies of 10^4 - 10^6

FIGURE 19. Sau3A Digestion of NJ-5 DNA

This is a photograph of a preliminary digestion of NJ-5 DNA. 0.02Units of Sau3A/ g of NJ-5 DNA. Lanes a, b, and s represent marker DNA lanes. Lanes c - r represent 1 μg of NJ-5 DNA digested with 0.02U of Sau3A at various time intervals.

Lane a : uncut EMBL4 DNA (48kb)
Lanes b, s: HindIII digested lambda DNA
Lanes: c. 0 minute digestion of NJ-5
d. 5 min.
e. 10 min.
f. 15 min.
g. 20 min.
h. 25 min.
i. 30 min.
j. 35 min.
k. 40 min.
l. 45 min.
m. 50 min.
n. 60 min.
o. 70 min.
p. 80 min.
q. 80 min.-no enzyme
r. 1.0 g of uncut NJ-5 DNA.

0.6% agarose gel

FIGURE 20. Restriciton Endonuclease digests of Plasmid pπ25.1

This figure displays single restriction digests of the plasmid pπ25.1. Correlating this information with the published restriction map (Figure 4) confirms that plasmid pπ25.1 DNA has been isolated. Fragment sizes (in kb) are listed after each restriction. Lane a and f represent HindIII digested lambda DNA (0.25μg).

Lanes: b. HindIII digested pπ25.1 DNA (0.125μg) (5.06, 3.25, 0.81)
c. BamHI " " (4.68, 4.44)
d. XhoI " " (9.0)
e. EcoRI " " (6.63, 2.37)

0.9% agarose gel

FIGURE 21. NaCl gradient of sized fragments.

Ten μl samples of 500 μl fractions were separated on a 0.4% agarose gel. Lanes a and s represent HindIII digested lambda DNA. Lanes b - r represent DNA in NaCl fractions (approximately 0.04 μg).

Lanes: b. Tube #1 (largest fragments)
c. #4
d. #7
e. #10
f. #13*
g. #16*
h. #19*
i. #22
j. #25
k. #28
l. #31
m. #34
n. #37
o. #40
p. #50
q. #60

* tubes carrying appropriate sized fragments

0.4% agarose gel

FIGURE 22. Restriction Endonuclease digests of EMBL4

Single digests of EMBL4 DNA confirms that the correct bacteriophage DNA has been isolated (Figure 5). Fragment size (in kb) is listed for each digestion.

Lanes a and e: HindIII digested lambda DNA (0.25μg)
Lanes: b. BamHI digested EMBL4 DNA (0.25μg) (20.7, 14.0, 9.2)
c. EcoRI " " (20.7, 14.0, 9.2)
d. SalI " " (20.7, 9.2, 8.5, 5.5)

0.6% agarose gel

FIGURE 23. Ligation reaction of EMBL4 and NJ-5 DNA

This figure represents preliminary ligation tests of Sau3A digested NJ-5 DNA and BamHI digested EMBL4 DNA. Small molecular weight fractions of NJ-5 DNA and SalI/BamHI digested EMBL4 DNA were religated separately to ensure that both samples were capable of ligating.

Lanes a and f: HindIII digested lambda DNA
Lanes: b. ligated sample of NJ-5 small MW fragments (0.25 μg)
c. unligated sample of NJ-5 small MW fragments (<0.25 μg)
d. unligated sample of SalI/BamHI digested EMBL4 (0.25 μg)
e. ligated sample of SalI/BamHI digested EMBL4 (0.25 μg)
0.4% agarose gel

FIGURE 24. EcoRI restriction patterns of 10 P Positive Clones

The DNA of 10 different P positive bacteriophage was digested with EcoRI to determine the restriction pattern of the internal fragment. Lane c displays the pattern of LF1 bacteriophage. The site of *in situ* hybridization for each clone is diagrammed in brackets followed by the label pattern (Figure 25).

Lanes: a. Hind III digested lambda DNA
b. BamHI digested EMBL4 DNA
c. EcoRI digested clone LF1 (9E, pat. y)
d. " " GG-4b (3C, pat. a)
e. " " GG-4a (3C, pat. a)
f. " " MM-1a (3C, pat. 1)
g. " " QQ-5b (3C, pat. d)
h. " " QQ-5a (3C, pat. d)
i. " " 48-3b (3C, pat. c)
j. " " 48-3a (3C, pat. c)
k. " " 48-5a (3C, pat. a)
l. " " R-1 a (3C, pat. a)
0.7% agarose gel

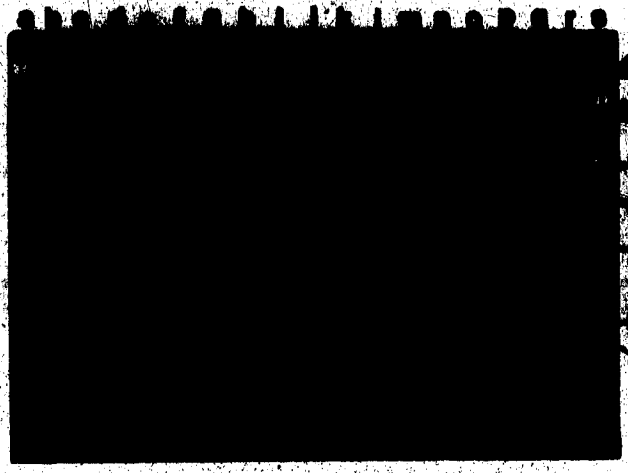


FIG.19. SAU3A RESTRICTION OF NJ-5 DNA

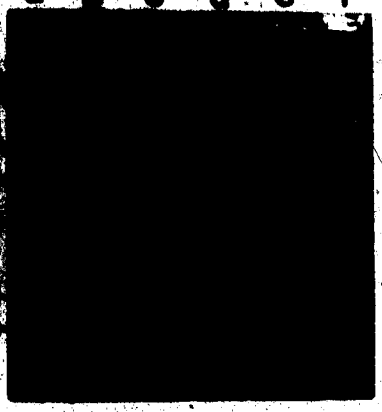


FIG.20. PLASMID PW25.1

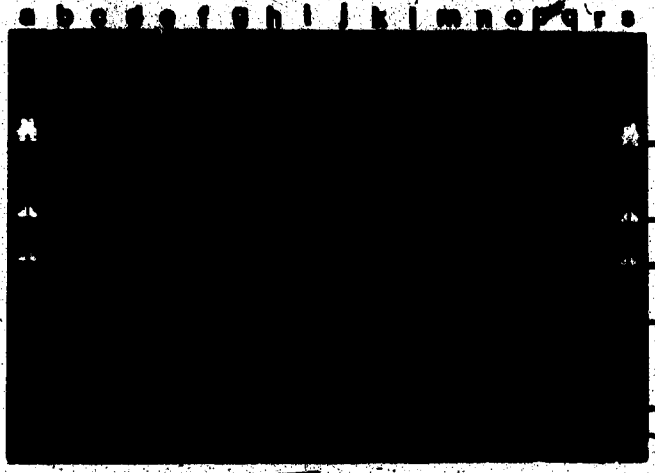


FIG.21. NaCl GRADIENT OF SIZED FRAGMENTS

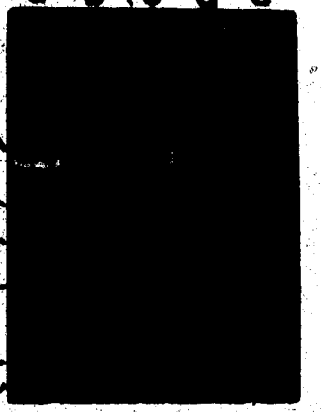


FIG.22. EMBL4

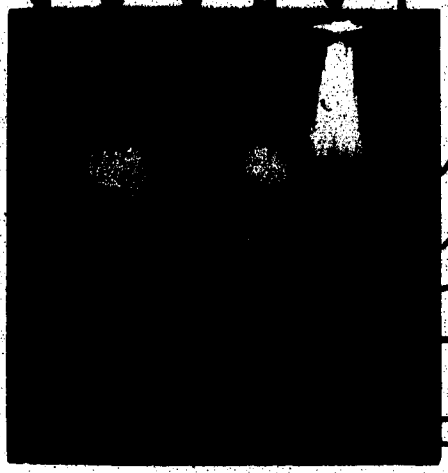


FIG.23. LIGATION REACTION

FIG.24. 10 P POSITIVE CLONES

TABLE 6. *IN VITRO* PACKAGING OF RELIGATED DNA

LIGATION MIXTURES Arms:Inserts(0.125μg)	Number of plaques detected in 0.1ml of the appropriate dilution of *in vitro* packaged DNA mixture on Q359 cells: 10^0	10^{-1}	10^{-2}
4 : 1	259	25	2
	252	23	3
3 : 1	291	32	2
	309	20	2
2 : 1	TMC	134	12
	TMC	129	13
1 : 1	TMC	183	16
	TMC	171	18
0.5 : 1	TMC	124	11
	TMC	101	13
CONTROL MIXTURES			
EMBL4 Arms only (0.5 μg)	TMC	48	4
	TMC	46	5
No DNA packaged	0	0	0
	0	0	0
Q359 cells only	0	0	0
	0	0	0
Intact EMBL4 DNA (0.5 μg)	TMC	99	1
	TMC	103	0

	on Q358 cells: 10^{-4}	10^{-5}	10^{-6}
Intact EMBL4 DNA (0.5 μg)	99	11	2
	140	8	0

TMC = Too many to count

pfu/μg insert DNA. All *in vitro* packaging rates, excluding the rates for intact EMBL4 (representing nonligated DNA) were at the lower end of this range, at 10^4 pfu/μg insert DNA.

To ensure with 99% probability that a copy of all segments of the *Drosophila* genomic DNA are present in the library approximately 38,000 plaques are needed (Sec. 2, VII, G.). Three libraries were prepared with approximately 12,000, 30,000 and 50,000 plaques respectively. All were hybridized to the P element probe pπ25.1. The first library, composed of 12,000 plaques was unamplified. The second and third libraries were prepared as plate lysates and combined to provide an estimated 80,000 clones in an amplified library.

To ensure the isolated clones carried inserted *Drosophila* DNA, the DNA from 16 plaques was EcoRI digested and analyzed. Eleven of the 16 samples displayed unique EcoRI patterns indicating the insertion of *Drosophila* DNA had been successful. This eliminated the possibility of having vector religation in a way that might produce a spi⁻ phenotype and a single repeated pattern.

Material plated directly from the amplified library was transferred to Biodyne filters (plaque lift, Sec.2, VII, A, i.). Plaques were hybridized to the P element probe, pπ25.1 as described earlier (Sec.2, IX, B, i.) revealing a number of positive signals.

V. P-POSITIVE CLONES

A number of P positive plaques were detected upon hybridization of the unamplified library. On the second cycle of retesting, none were

confirmed to be positive. This could be explained by inexperience in differentiating a true positive signal from background.

245 putative positive clones were detected in the amplified library of which 141 were reconfirmed as positive, sharing P element homology. Out of these 141, 54 were tested for specifice hybridization to band 9E The remaining 87 either failed to produce enough DNA, failed to digest with EcoRI, were unsuccessfully nick translated or did not show clear *in situ* hybridization. The 54 positive clones displayed at least 25 different EcoRI restriction patterns as diagrammed in Figure 25which also shows the results of the *in situ* hybridizations.

Various EcoRI digests of 10 positive clones are shown in Figure 24, including the raspberry clone LF1 (Lane 3). It is interesting to note that the remaining clones displayed in this particular photograph hybridized to the 3C region on the X chromosome. After numerous *in situ* hybridizations confirming a correlation between various EcoRI patterns and hybridization to the cytological region 3C, clones with a suspected 3C EcoRI restriction pattern were pooled together into a single *in situ* hybridization reaction mixture.

In all, 38/54 positive clones were homologous to the 3C cytological band. This fact initiated an investigation of the 3C region. At the time when a 9E positive band was isolated, an attempt was underway to produce a probe which would help eliminate 3C positive clones in future screens. This was abandoned as no longer relevant.

The abundance of 3C clones may be explained if there are few Sau3A restriction sites that would lead to an enrichment of 3C DNA in fragments of 18-23 kb in size. The 4 base pair recognition sequence (GATC) would be expected to occur approximately once per 256 bp.

FIGURE 25. EcoRI restriction and *in situ* hybridization results of P positive bacteriophage.

This figure represents a summary of the various EcoRI restriction patterns that were isolated for the 54 clones studied. The cytological site of hybridization and the number of bacteriophage displaying the representative pattern are listed for each unique restriction pattern detected.

The left and right arms of the bacteriophage are not diagrammed for purposes of simplicity. It should be noted that many of the restriction patterns displayed may represent partial restriction digests. Since one could not be assured, at this early mini-lysate preparation stage, that they were not complete digestions, all restriction patterns were noted.

MC '= multicopy DNA that hybridized to many genomic locations, including the centromere.

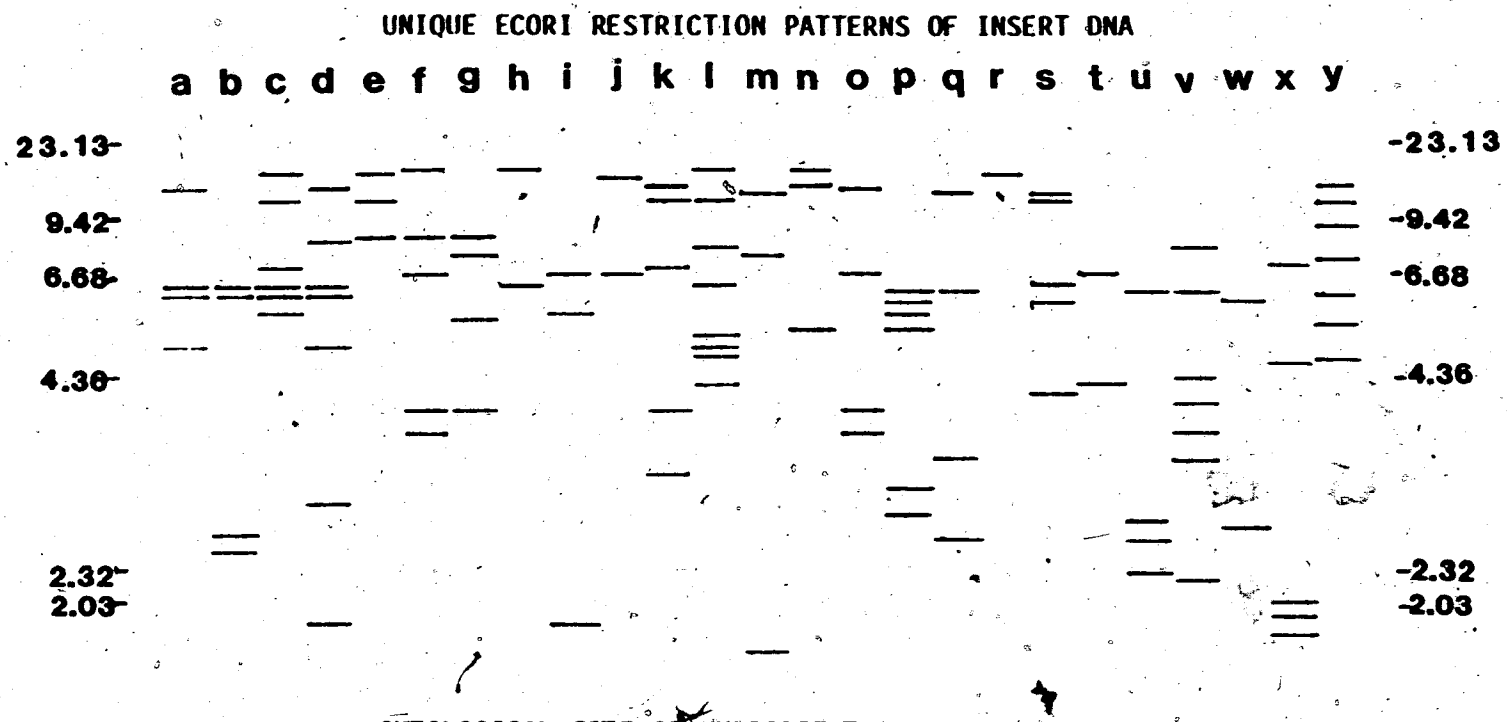


FIGURE 25. ECORI RESTRICTION AND *IN SITU* HYBRIDIZATION RESULTS OF P POSITIVE BACTERIOPHAGE

A more likely alternative is that a bacteriophage carrying 3C DNA is able to grow particularly successfully and is therefore detected at a higher frequency in an amplified library. For example, the 3C DNA may contain numerous Chi sequences that stimulate recombination to produce dimeric circles necessary for effective DNA packaging, in the absence of gam^+ mediated rolling-circle mechanism. Bacteriophage possessing Chi sequences are known to be at a selective advantage, although EMBL4 does possess a Chi sequence in its right arm. Conceivably the 3C DNA contains some other sequences that stimulate lambda growth. Conversely, the abundance of 3C clones in relation to all other P positive clones, may be due to some factor in the non-3C P elements rendering then unable to be cloned.

In figures 16, 17 and 18, *in situ* hybridizations of 3 different probes to *Drosophila* M strain polytene chromosomes (Amherst) are shown. Figure 16 displays the common 3C probe that was seen hybridizing solely to 3C. Figures 17 and 18 represent probes containing 56F and multicopy (centromeric) DNA respectively.

VI. RASPBERRY CLONE LF1

The mutant *Drosophila* strain NJ-5, after repeated outcrosses to M strain males, possessed 7 P elements on the X chromosome including the site at 9E and 3 P elements on the autosomes. Theoretically, if all P element sites are inserted into the EMBL4 vectors with equal frequency, 1/10 (10%) of the P positive clones isolated should be from the raspberry locus. Nonetheless, only 1/54 (1.9%) of P positive clones

This low rate may be due to the high number of 3C clones isolated. Excluding all 3C clones, 1/16 (6.25%) of the P positive clones were from the raspberry locus, a rate approaching the expected value based on the *in situ* hybridization results.

The sole clone that hybridized to 9E was called LF1. LF1 hybridized solely to the 9E cytological region of the X chromosome. (Figures 26 - 29 and 31). An accompanying photograph displays the region in question (Figure 30).

LF1 was found to be a very slow growing bacteriophage. Originally the LF1 clone was isolated as a single plaque on plates that displayed distinct individual plaques. The plaque was patched to a new innoculum of bacterial cells (Sec. 2, V, B.), which was used to make a liquid lysate preparation from which DNA was isolated, EcoRI digested and used for *in situ* hybridizations. Originally, LF1 showed only hybridization to 9E on the X chromosome (2 week exposure) with no hint of any other site displaying homology to the probe. Unfortunately, after the bacteriophage had been grown up on a large scale, to isolate large quantities of DNA, two new sites (autosomal) were detected in *in situ* hybridizations (3 - 4 week exposure). The large scale lysate was found to possess two contaminating clones that displayed different EcoRI restriction patterns and which in turn were found to hybridize to each of the autosomal hybridization sites. These contaminating phage displayed large, well growing plaques whereas the LF1 bacteriophage produced very small plaques that were difficult to grow on purification. The contaminating clones may have been found at a very low frequency in the original plaque, due to diffusion of bacteriophage within the plate. As lysates were prepared for large scale isolation of

FIGURES 26, 27, 28, 29 and 31. *In situ* hybridizations to M strain polytene chromsomes with LF1 bacteriophage

M strain (Amherst) salivary gland chromosomes are hybridized with the LF1 probe. Grain is seen localized solely over the cytological region 9E on the X chromosome. No other sites display grain indicating the probe contains DNA homologous only to the 9E region (raspberry).
Tissue: Am^+/Am^+ female, four week exposure

Fig.26 Magnification: 640 X
Fig.27 800 X
Fig.28 800 X
Fig.29 800 X
Fig.31 590 X

FIGURE 30. M Strain X Chromosome
Cytological bands surrounding the 9E region are shown on the aceto-orcein stained polytene chromosome.
Tissue: Am^+/Am^+ female, aceto-orcein stained

Fig.30 Magnification: 590 X

▼ [illegible]. HYBRIDIZATION OF LF1 PROBE TO M STRAIN POLYTENE CHROMOSOMES

FIG.27. LF1 PROBE▼

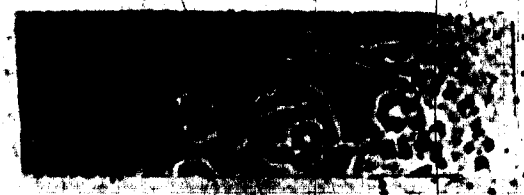


FIG.28. LF1 PROBE▼

FIG.29. LF1 PROBE ▲

FIG.30. M STRAIN X CHROMOSOME ▼

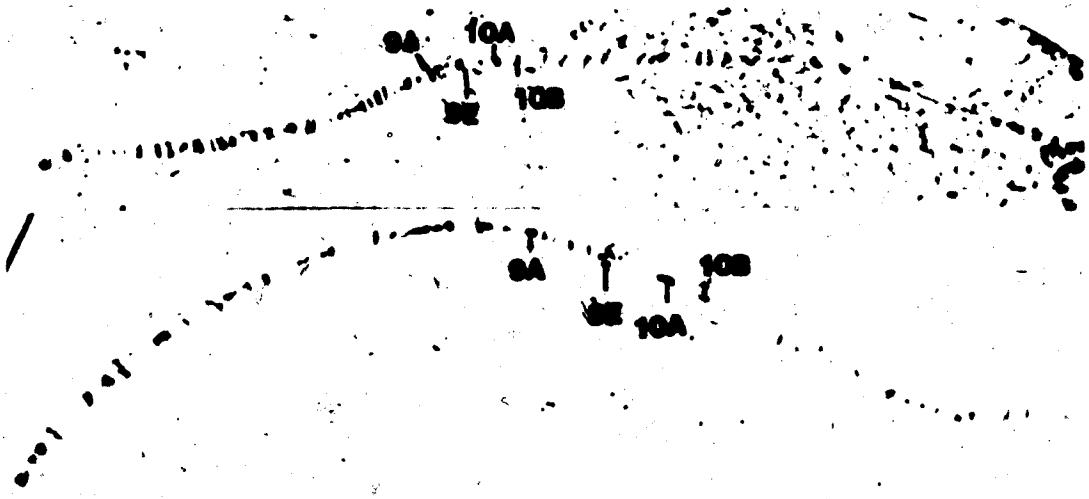


FIG.31. HYBRIDIZATION OF LF1 PROBE TO M STRAIN POLYTENE CHROMOSOMES

DNA, these phage had a selective advantage over LF1 and soon became predominant in the lysate. Subsequent repurification and *in situ* hybridization (4 weeks exposure) has confirmed LF1 hybridizes solely to region 9E on the X chromosome.

To confirm the location of LF1 cytologically, an *in situ* hybridization was performed with the LF1 probe to the inversion stock In(1)123/FM6 which bears inversion breakpoints in the raspberry locus and centromere and demonstrates a raspberry lethal phenotype. The DNA homologous to LF1 appears to be situated across the inversion breakpoint at band 9E. Figures 32, 33, 34 and 35 demonstrate cytology of the inversion In(1)123/Am$^+$ and the *in situ* hybridization of LF1 to In(1)123/Am$^+$

VII. CHARACTERIZATION OF LF1 BACTERIOPHAGE

A. Single and double restriction digests of LF1 Bacteriophage

Bacteriophage LF1 single and double restriction digests are shown in Figure 36. The restriction endonucleases BglII, XhoI, EcoRI were used. A tentative restriction map of the LF1 bacteriophage is illustrated in Figure 42 (See Sec. 3, VIII, D. for discussion).

B. Subcloning of LF1 bacteriophage

LF1 is a weak growing bacteriophage characterized by small placques. To ensure the inserted DNA is not lost and to place it in a non-EMBL4 vector, Dr. S. Tiong has prepared subclones of 6 EcoRI

FIGURE 32. Inversion heterozygote: In(1)123/Am$^+$ X chromosome

Aceto-orcein prepared chromosomes with the inversion breakpoint regions in 9E and the centromere. An inversion loop is associated with the centromere in this photograph. The bands surrounding the region 9E are indicated.
Tissue: In(1)123/Am$^+$, aceto-orcein stained
Fig.32 Magnification: 990 X

FIGURES 33, 34 and 35. *In situ* hybridizations of In(1)123/Am$^+$ X chromosome with LF1 (GG1) probe

In situ hybridization of the probe LF1 to the inversion heterozygote reveals that the DNA in LF1 hybridizes across the inversion breakpoint indicating that the DNA homologous to the raspberry gene has been cloned.
Tissue: In(1)123/Am$^+$, four week exposure
Fig.33 Magnification: 990 X
Fig.34 2130 X
Fig.35 1280 X

Line diagrams of all four figures (minus grain) are inset with each photograph.

FIG.32. IN(1)123/AM$^+$ X CHROMOSOME

FIG.33. HYBRIDIZATION OF GG1 TO IN(1)123/AM$^+$

FIG.34. HYBRIDIZATION OF LF1 TO IN(1)123/AM$^+$

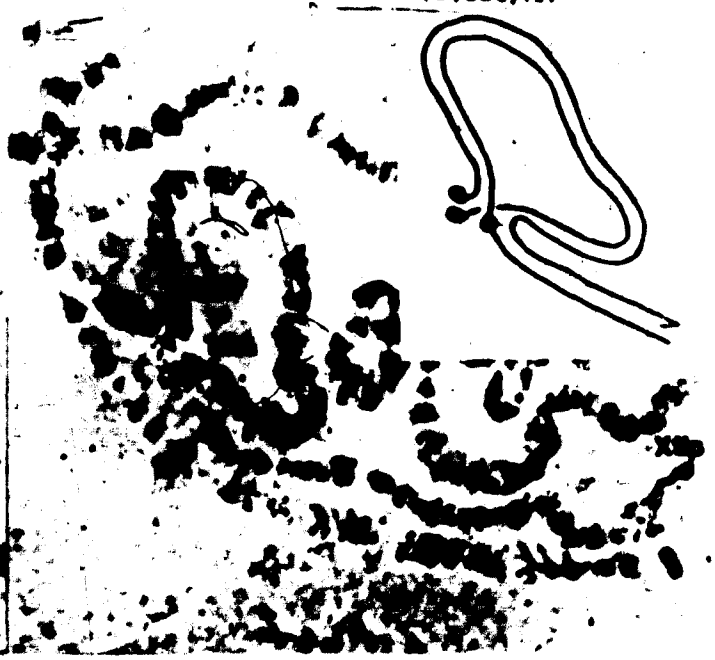


FIG.35. HYBRIDIZATION OF GG1 TO IN(1)123/AM$^+$

FIGURE 36. Single and double restriction digests of LF1

Displayed are single and double restriction digests of the bacteriophage LF1 with appropriate markers. Fragment sizes are only approximate estimates of molecular weights. The lanes and approximate fragment sizes in each restriction are as follows:

Lane a. HindIII/EcoRI digested lambda DNA (fragment sizes diagrammed)
b. BamHI digested EMBL4 (21, 14, 9.2)
c. HindIII digested lambda DNA (fragment sizes diagrammed)
d. BglII digested LF1 DNA (40?, 7.2, 0.79, 0.75)
e. XhoI " " (40?, 13.3, 5.8, 1.1, 1.0, 0.8)
f. EcoRI " " (21.4, 9.1, 8.4, 5.0, 2.3, 1.6, 1.07, 0.79)
g. HindIII " " (26.3, 8.4, 7.6, 5.0, 4.3)
h. HindIII digested lambda DNA
i. BglII/XhoI digested LF1 DNA (40?, 7.2, 5.8, 3.6, 1.15, 1.04 0.89)
j. BglII/EcoRI " " (21.4, 8.4, 7.2, 5.0, 3.5, 2.3, 1.6, 1.3, 0.79)
k. BglII/HindIII" " (26.3, 8.4, 4.8, 4.3, 3.8, 2.5, 0.75)
l. HindIII digested lambda DNA
m. XhoI/EcoRI digested LF1 DNA (21.4, 9.1, 5.8, 4.8, 2.3, 1.6, 1.2, 1.04, 0.89)
n. XhoI/HindIII " " (26.3, 7.6, 5.0, 4.3, 3.4, 2.3, 1.15 0.89, 0.79)
o. EcoRI/HindIII" " (21.4, 5.0, 4.8, 4.3, 3.4, 3.4, 2.1, 1.48, 1.47, 1.07, 0.79)
p. HindIII digested lambda DNA
q. BamHI digested EMBL4 DNA
r. HindIII/EcoRI digested lambda DNA
0.7% agarose gel

FIGURE 37. Single and Double restriction digests of LF1 subclones

The LF1 subclones are EcoRI fragments of LF1 ligated into the plasmid pUC19 (2.7kb). The subclone is labelled by the size of the EcoRI fragment inserted. Approximate fragment sizes of each restriction are listed in brackets and are only estimates based on calculations utilizing mw markers. Each lane lists the particular restriction enzymes used, the DNA digested and the fragment sizes. The symbol "p" represents those fragments that are partial digests. Those samples that are not digested by a particular enzyme are designated "uncut".

Lane:

a	HindIII/EcoRI, lambda DNA (fragment sizes diagrammed)		
b	undigested	Subclone LF1-8.4	(lmw, 20, 6)
c	EcoRI	" "	(8.4, 2.7)
d	HindIII	" "	(lmw(p), 8.4(p), 4.8, 3.3, 3.0)
e	HindIII/EcoRI	" "	(8.4(p), 5.3(p), 4.8, 3.25, 2.7, 0.4)
f	XhoI	" "	(5.4, 2.9, 1.1, 1.0, 0.9)
g	XhoI/EcoRI	" "	(5.4, 2.7, 1.1, 1.0, 0.9)
h	undigested	Subclone LF1-5.0	(3-lmw, 9, 3.6)
i	EcoRI	" "	(4.9, 2.7)
j	HindIII	" "	(8.0(p), 6.0, 1.5)
k	HindIII/EcoRI	" "	(4.9(p), 3.5, 2.7, 1.5)
l	XhoI	" "	(3-lmw, 9, 3.6) uncut
m	XhoI/EcoRI	" "	(4.9, 2.7)
n	HindIII, plasmid DNA p 25.1		(5.1, 3.3, 0.81)
o	HindIII/EcoRI, lambda DNA		
o	HindIII/EcoRI, lambda DNA - same lane as adjoining photograph		
p	undigested	Subclone LF1-2.3	(lmw, 5.0, 2.5)
q	EcoRI	" "	(2.7, 2.2)
r	HindIII	" "	(4.9)
s	HindIII/EcoRI	" "	(2.7, 2.2)
t	XhoI	" "	(lmw, 5.0, 2.5) uncut
u	XhoI/EcoRI	" "	(2.7, 2.2)
v	HindIII, plasmid DNA pUC19		(2.7)

0.8% agarose gel
lmw = large molecular weight

FIGURE 38. Single and Double restriction digests of LF1 subclones

The subclone is labelled by the size of the EcoRI fragment inserted. The approximate size of each restriction fragment are listed in brackets and are only estimates based on calculations utilizing the mw markers. Each lane lists the particular restriction enzymes used, the DNA digested and the fragment sizes. The symbol "p" represents those fragments that are partial digests. Those samples that are not digested by a particular enzyme are designated "uncut".

Lane:

a	HindIII/EcoRI, lambda DNA (fragment sizes diagrammed)			
b	undigested	Subclone LF1-1.6	(2-lmw, 10, 3.8)	
c	EcoRI	"	"	(2.7, 1.5)
d	HindIII	"	"	(2-lmw, 7.6, 3.8) uncut
e	HindIII/EcoRI	"	"	(2.7, 1.5)
f	XhoI	"	"	(2-lmw, 10, 3.8) uncut
g	XhoI/EcoRI	"	"	(2.7, 1.5)
h	undigested	Subclone LF1-1.1	(lmw, 3.7, 2.0)	
i	EcoRI	"	"	(2.7, 1.0)
j	HindIII	"	"	(3.7)
k	HindIII/EcoRI	"	"	(2.7, 1.0)
l	XhoI	"	"	(lmw, 3.7, 2.0) uncut
m	XhoI/EcoRI	"	"	(2.7, 1.0)
n	HindIII/EcoRI lambda DNA			

n	HindIII/EcoRI, lambda DNA - same lane as adjoining photograph			
o	undigested	Subclone LF1-0.8	(lmw, 3.4, 1.8)	
p	EcoRI	"	"	(2.7, 0.7)
q	HindIII	"	"	(3.4)
r	HindIII/EcoRI	"	"	(2.7, 0.7)
s	XhoI	"	"	(lmw, 3.4, 1.8) uncut
t	XhoI/EcoRI	"	"	(2.7, 0.7)
u	HindIII, plasmid DNA pUC19			(2.7)
v	XhoI, plasmid DNA pUC19			(7.0, 2.7, 1.5) uncut

0.8% agarose gel
lmw = large molecular weight

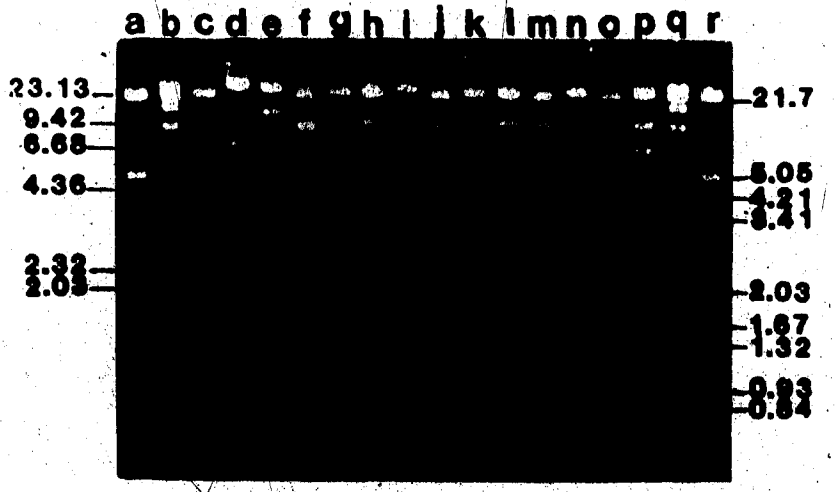


FIG.36. SINGLE/DOUBLE RESTRICTION DIGESTS OF LF1

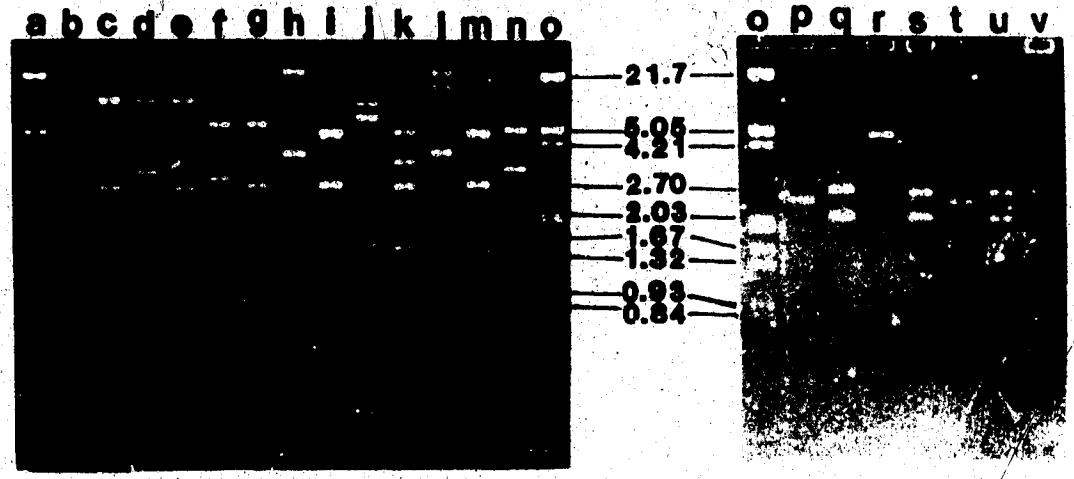


FIG.37. SINGLE/DOUBLE RESTRICTION DIGESTS OF LF1 SUBCLONES

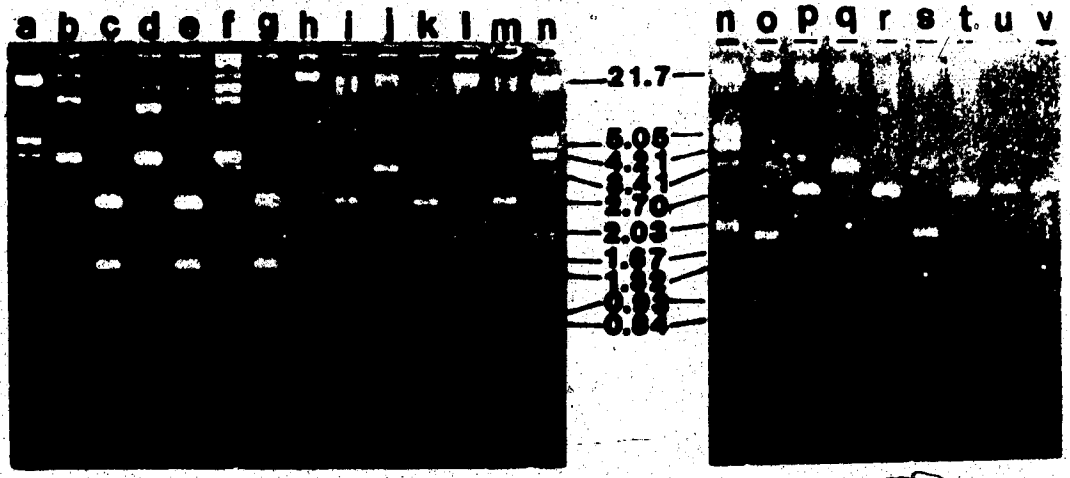


FIG.38. SINGLE/DOUBLE RESTRICTION DIGESTS OF LF1 SUBCLONES

fragments of the LF1 bacteriophage; LF1-8.4, LF1-5.0, LF1-2.3, LF1-1.6, LF1-1.1, LF1-0.8 in pUC19. Figures 37 and 38 demonstrate EcoRI, HindIII/EcoRI, XhoI, and XhoI/EcoRI digests of subclones spanning the entire LF1 insert DNA. The fragments detected support the restriction map established (Figure 42). If additional small molecular weight EcoRI fragments are present in LF1 bacteriophage, they have not been detected to date. Sequencing of the region may reveal any missing fragments.

C. Southern hybridization of pπ25.1 to single and double digests of LF1 bacteriophage and LF1 subclones

Southern hybridization of pπ25.1 to single and double digests of LF1 bacteriophage and LF1 subclones (Figures 36, 37 and 38) was carried out. Photographs of the autoradiograms are displayed in Figures 39, 40 and 41. The single and double digests of the LF1 bacteriophage seems to indicate that the P element resides in the 8.4kb EcoRI fragment, the large BglII fragment, in two or all three of the small XhoI fragments and the 5.0kb HindIII fragment. This places the P element approximately 2-4 kb from the right arm of the EMBL4 vector. The exact size of the inserted P element is not known. Further investigation of which XhoI pieces share P element homology will help to clarify this situation.

The Southern hybridization of the single/double digests of the LF1 subclones supports the view that the P element is present only in the 8.4kb EcoRI fragment. This autoradiogram is complicated by the fact that the plasmid pπ25.1 contains pBR322 sequences that hybridize to pUC19. Information on pUC19 can be found in Yanisch-Perron et al., (1985). For this study, it is important to know that pUC19 is 2.7kb in

FIGURE 39. Hybridization of pπ25.1 probe to single and double restriction digests of LF1 bacteriophage

Single and double restriction digests of LF1 were probed with labeled pπ25.1 DNA using the Southern methodology. The lanes correspond to those listed in Figure 36. Listed in each lane are the restriction enzyme(s) used to digest the LF1 bacteriophage and the size of fragments displaying hybridization to the P element probe (in brackets). These fragments are suspected of carrying the P element.

Lane:

d. BglII digested LF1 DNA,(large molecular weight fragment)
e. XhoI " " (1.15, 1.01, 0.89)
f. EcoRI " " (8.4)
g. HindIII " " (5.0)
h. HindIII digested lambda DNA- blank
i. BglII/XhoI digested LF1 DNA (1.15, 1.04, 0.89)
j. BglII/EcoRI " " (8.4)
k. BglII/HindIII" " (4.8)
l. HindIII digested lambda DNA- blank
m. XhoI/EcoRI digested LF1 DNA (1.2, 1.04, 0.69)
n. XhoI/HindIII " " (1.15, 0.89, 0.79)
o. EcoRI/HindIII" " (5.0)

0.7% agarose gel

FIG.39. HYBRIDIZATION OF Pir25.1 PROBE TO LF1 SINGLE/DOUBLE DIGESTS

FIGURE 40. Hybridization of p 25.1 probe to single and double digests of LF1 Subclones

The lanes correspond to those listed in Figure 37. Listed in each lane are the restriction enzymes used to digest each subclone. The EcoRI restriction patterns (*) in lanes c, i and q reveal that only the 8.4 kb EcoRI fragment shares P element homology.

Lane:

b	undigested	Subclone LF1-8.4	
c*	EcoRI	"	"
d	HindIII	"	"
e	HindIII/EcoRI	"	"
f	XhoI	"	"
g	XhoI/EcoRI	"	"
h	undigested	Subclone LF1-5.0	
i*	EcoRI	"	"
j	HindIII	"	"
k	HindIII/EcoRI	"	"
l	XhoI	"	"
m	XhoI/EcoRI	"	"
n	HindIII, plasmid DNA p 25.1		
o	HindIII/EcoRI, lambda DNA - blank		
p	undigested	Subclone LF1-2.3	
q*	EcoRI	"	"
r	HindIII	"	"
s	HindIII/EcoRI	"	"
t	XhoI	"	"
u	XhoI/EcoRI	"	"
v	HindIII, plasmid DNA pUC19		

0.8% agarose gel
Probe: p 25.1
Exposure time: 3 hours

FIGURE 41 Hybridization of p25.1 probe to single and double digests of LF1 Subclones

The lanes correspond to those listed in Figure 38. Listed in each lane are the restriction enzymes used to digest each subclone. The EcoRI restriction patterns (*) in lanes c, i and p reveal that none of the subclones LF1-1.6, LF1-1.1 or LF1-0.8 share P element homology. All dark bands correspond to pBR322 sequences

Lane:

b	undigested	Subclone LF1-1.6
c*	EcoRI	" "
d	HindIII	" "
e	HindIII/EcoRI	" "
f	XhoI	" "
g	XhoI/EcoRI	" "
h	undigested	Subclone LF1-1.1
i*	EcoRI	" "
j	HindIII	" "
k	HindIII/EcoRI	" "
l	XhoI	" "
m	XhoI/EcoRI	" "
n	HindIII/EcoRI, lambda DNA - blank	
o	undigested	Subclone LF1-0.8
p*	EcoRI	" "
q	HindIII	" "
r	HindIII/EcoRI	" "
s	XhoI	" "
t	XhoI/EcoRI	" "
u	HindIII, plasmid DNA pUC19	
v	XhoI, plasmid DNA pUC19	

0.8% agarose gel
Probe: p 25.1
Exposure time: 3 hours

FIG.40 b c d e f g h i j k l m n o p q r s t u v

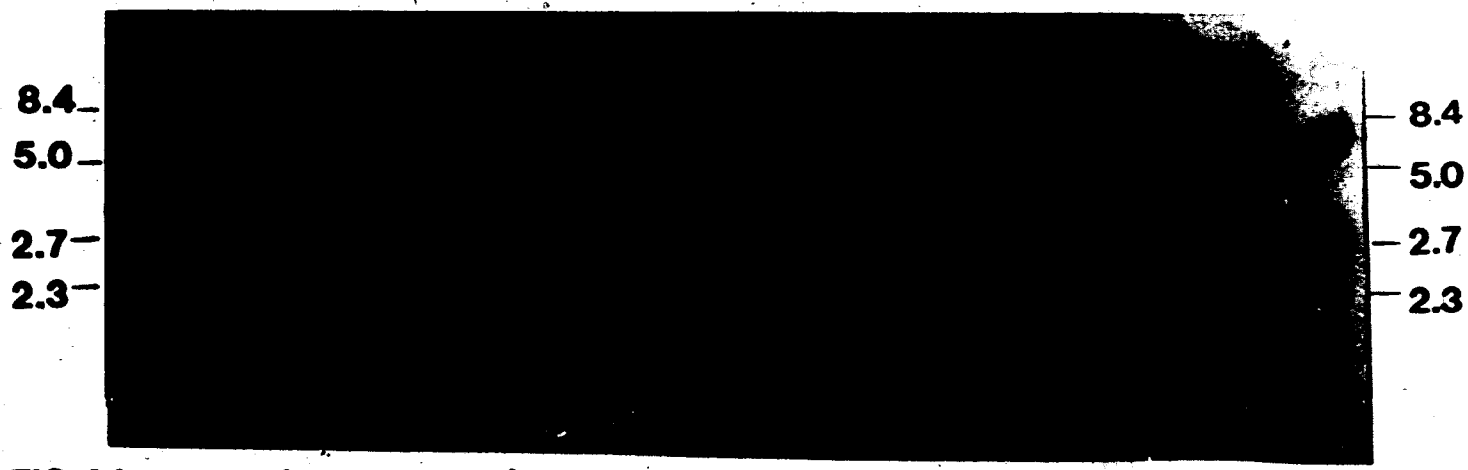
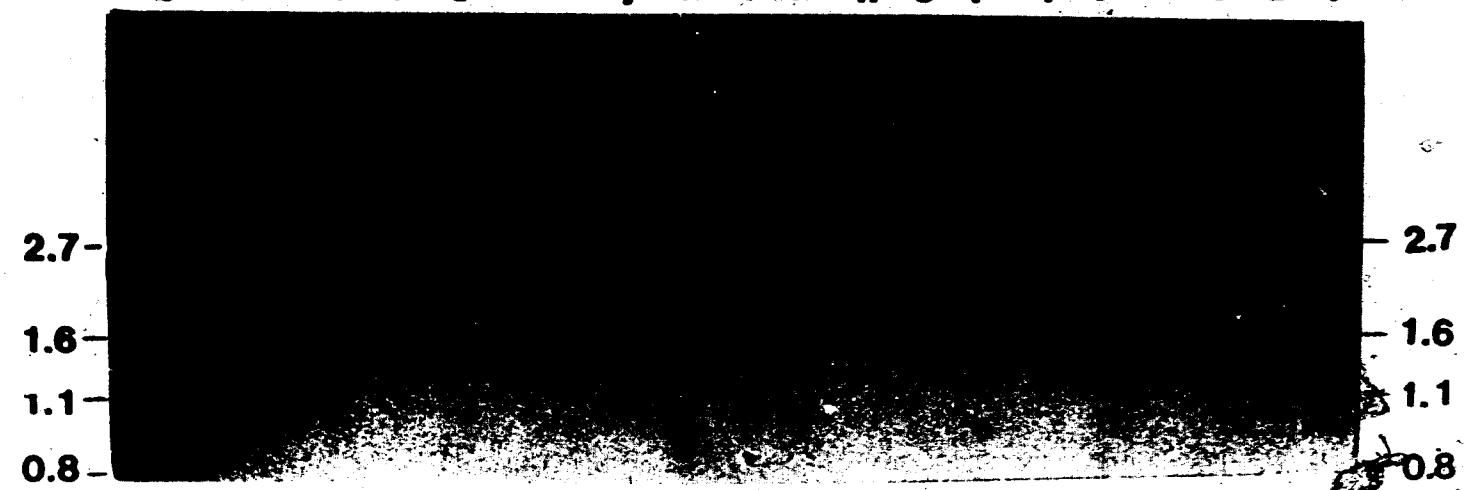


FIG.41 b c d e f g h i j k l m n o p q r s t u v



HYBRIDIZATION OF P~25.1 PROBE TO SINGLE/DOUBLE DIGESTS OF LF1 SUBCLONES

size and contains single EcoRI and HindIII restriction sites very near each other (20bp). In addition, uncut plasmid in the form of covalently closed circles and supercoiled DNA were readily transferred to the filter. These are seen in most of the samples and should not be mistaken for cut DNA.

D. Restriction map of LF1 bacteriophage

The insert DNA of LF1 bacteriophage is approximately 19 kb in size.

The tentative restriction map of LF1 is diagrammed in Figure 42. In establishing this map, the information obtained from the single and double digests of LF1 and LF1 subclones and hybridizations with the P element probe p25.1 were used.

The first fragment to be placed unambiguously was the HindIII 4.3kb fragment. The EMBL4 restriction map (Figure 5) has a single HindIII site that creates a 4.3kb fragment. The 4.3kb HindIII fragment is located at the right end of the bacteriophage leaving 3 possible internal HindIII fragments. BglII has four restriction sites in the EMBL4 right arm. The 7.6kb HindIII fragment is the only fragment that is digested by BglII. This places the 7.6kb fragment adjacent to the 4.3kb fragment. XhoI produces a large fragment approximately 11.4-13.3kb in size. This fragment carries a 9.1kb EcoRI piece (right arm) indicating it overlaps the right end of the bacteriophage. All the remaining internal XhoI fragments add up to a total of 8.8kb. The 5.8kb XhoI fragment is restricted into 2 smaller fragments (3.4, 2.3) by HindIII. This restriction pattern suggests that the 5.0kb HindIII fragment is adjacent to the 7.6kb HindIII piece since the 8.4kb fragment would produce 1 large and 1 very small fragment. The remaining small

FIGURE 42. Restriction map of bacteriophage LF1 and LF1 Subclones

A preliminary restriction map of LF1 and the LF1 subclones has been made with the four restriction enzymes BglII, XhoI, EcoRI and HindIII. The fragment sizes of the XhoI, HindIII and EcoRI restricted fragments are seen on line diagrams below the map.

The subclones LF1-8.4 and LF1-5.0 are the only subclones to reveal XhoI and HindIII restriction sites on the EcoRI inserts. Fragment sizes are illustrated directly on the subclone maps.

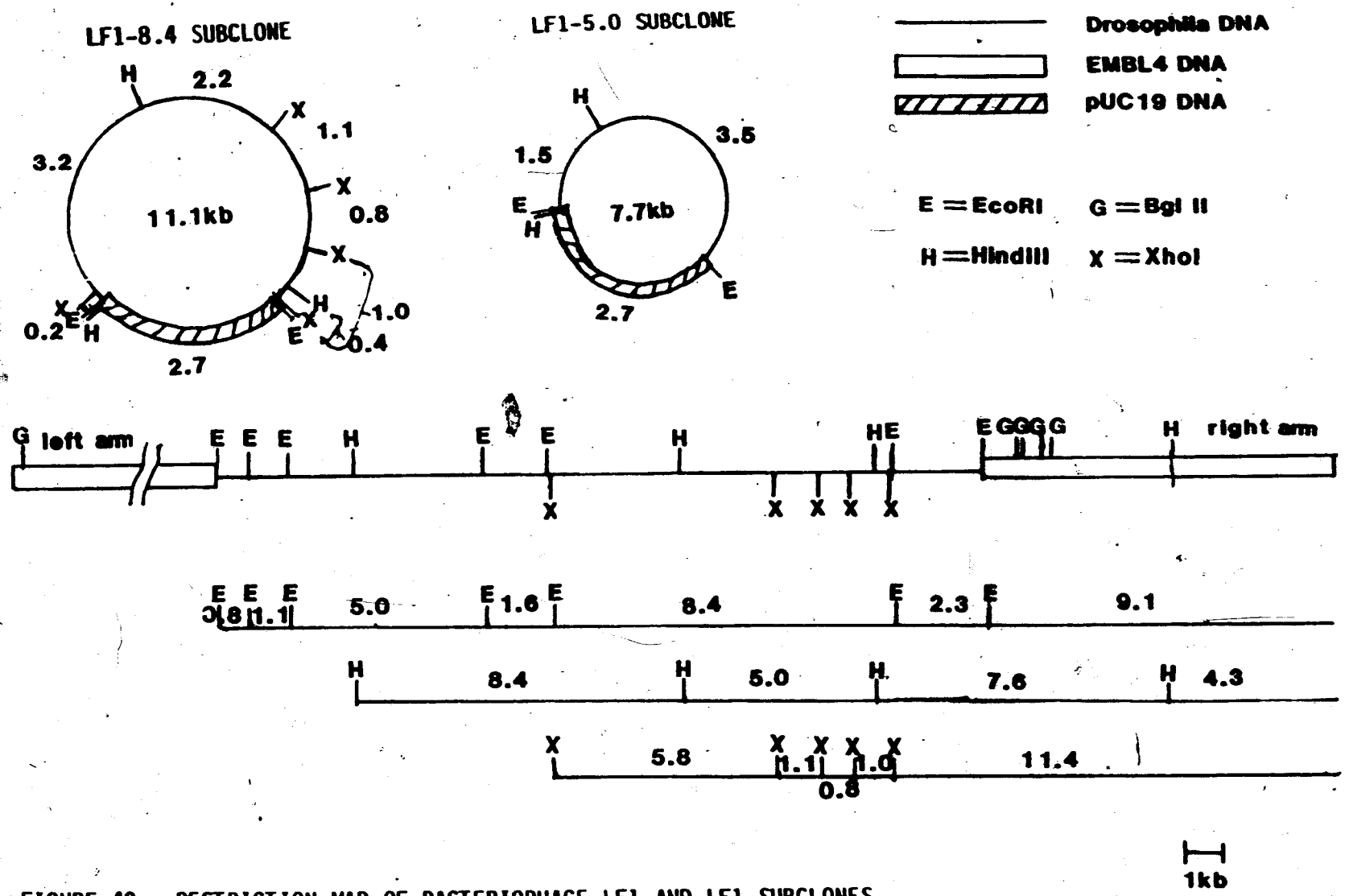


FIGURE 42. RESTRICTION MAP OF BACTERIOPHAGE LF1 AND LF1 SUBCLONES

XhoI sites share homology to the P element, indicating the three are located very near each other. Whether all three fragments share P element homology is yet to be determined. Their order in relation to each other is also unknown.

Southern hybridization reveals the small XhoI fragments, the 5.0kb HindIII fragment and the 8.4kb EcoRI fragments share P element homology. The 1.0 kb XhoI fragment appears to be restricted by HindIII placing it at the rightmost end. The large 8.4kb EcoRI fragment must include the 5.0kb HindIII fragment. The 5.0kb EcoRI fragment is to the left of the 8.4kb EcoRI fragment. This EcoRI fragment is restricted by HindIII to produce 3.5 and 1.5kb fragments. An additional 1.6 kb EcoRI fragment must be present betweeen the 8.4 and 5.0 kb EcoRI fragments in order to provide the proper length of the 8.4 kb HindIII fragment. The remaining EcoRI fragments have been arbitrarily placed. The precise positioning of the four small EcoRI fragments is not yet established.

The single and double digests and Southern hybridizations of the LF1 subclones confirm the map. The EcoRI and small XhoI fragment(s) share P element homology. It is difficult to discern, in this digest, if the 5.0kb HindIII fragment shares P element homology in that the HindIII restriction of LF1-8.4 subclone was incomplete.

The map of subclones LF1-8.4 and LF1-5.0 are illustrated in Figure 42. The orientation of pUC19 in subclone LF1-5.0 cannot be determined and is arbitrarily shown. All other subclones did not contain XhoI or HindIII restriction sites as was to be expected. The restriction map of the LF1-8.4 subclone suggests that the XhoI site that exists at the left end of the 8.4kb EcoRI fragment is approximately 0.2 kb from the EcoRI site. This is seen as a 2.9kb XhoI fragment carrying pUC19 in

comparison to the 2.7kb EcoRI fragment.

Sequencing will help to discern the final order of the restriction sites and may reveal small fragments that were not detected in this study.

4. CONCLUSIONS

I. ISOLATION OF DNA HOMOLOGOUS TO THE RASPBERRY LOCUS

A. *In situ* hybridization to M strain with LF1 probe

DNA from the LF1 clone hybridizes specifically to the 9E region on the X chromosome (Figures 26-29, 31). This region has been shown to carry the raspberry locus (Johnson et al., 1979). The original mutant strain, NJ-5, from which the LF1 clone was isolated has a raspberry lethal phenotype, due to a P element insertion event at the raspberry locus. Thus the LF1 clone contains DNA from the raspberry locus

B. *In situ* hybridization to In(1)123/Am^{+} with LF1 probe

In situ hybridization of the LF1 clone to the inversion stock heterozygote ~~In(1)123/Am^{+}~~ supports the above evidence. The In(1)123/FM6 *Drosophila* strain carries an inversion that has breakpoints in the raspberry gene region 9E and in the centromere. This strain displays a raspberry lethal phenotype suggesting that the inversion breakpoint lies in the raspberry gene. The LF1 probe appears to hybridize across the inversion breakpoint. Additional experiments intended to confirm this finding are underway.

C. LF1 Bacteriophage

DNA homologous to the 9E region of the *Drosophila* X chromosome is present as a 19 kb insert in the bacteriophage vector EMBL4. A tentative restriction map has been made (Figure 42), placing the P element approximately 2-4 kb from the right arm of the EMBL4 vector. Subclones of the insert DNA have helped establish the restriction map Additional investigation is required to clarify and confirm the restriction order.

II. FUTURE EXPERIMENTS

At present, work is being carried out by S. Tiong to isolate an M strain wildtype genomic library that may be probed with the subcloned LF1 material to identify wildtype sequences homologous to the Drosophila DNA cloned in LF1. To ensure that the entire gene has been cloned, various deletions that surround the locus may be studied in combination with cloned material. Analyzing the restriction maps of the P element induced strains NJ-1, NJ-4, NJ-6 and NJ-8 may reveal the location of the P elements in the raspberry gene and help establish its boundaries.

Messenger RNA studies will reveal if one or more transcripts are produced from this locus and if alternative processing is involved. Subsequent cDNA studies should reveal information about the number and size of introns and exons that may exist in the gene complex.

The molecular analysis of the *gua-pur-ras* locus is underway.

III. MODELS OF ORGANIZATION

The molecular analysis of the *gua-pur-ras* locus will help to determine how the three functions of the guanosine 1, purine 1 and raspberry loci are related. The three appear to be involved in a gene complex.

The classical view of a gene complex, as described in prokaryotes, is the operon (Jacob and Monod, 1961; Miller and Reznikoff, 1978). For example, the lac operon is a group of three linked, coordinately expressed genes that are related in function. The three genes encode the three different enzymes, β-galactosidase, permease and transacetylase that are involved in the uptake and metabolism of lactose within the cell. The three are transcribed in a single mRNA controlled by a promoter and operator site that responds to an inducible repressor. Translation of the mRNA results in 3 distinct enzyme products being produced.

In eukaryotes, polycistronic messages of this kind have not been identified to date. Nonetheless, the three gene products of the *gua1*, *pur1* and *ras* loci may be coordinately regulated and transcribed as a separate messages which are subsequently translated into three distinct gene products.

Another alternative is that the *gua-pur-ras* complex may display a similar genetic organization to the rudimentary or Gart loci in *Drosophila*. Rudimentary is a single locus in *Drosophila* whose alleles fall into a number of different complementing groups, yet display a complex pattern of interallelic interaction in the same way as

the *gua-pur-ras* complex (Falk and Nash, 1974a; Carlson, 1971). A single protein appears to encode the three functions; carbamyl phosphate synthetase (CPSase), aspartate transcarbamylase (ATCase) and dihydro-orotase; the first, second and third enzymes in the *de novo* pyrimidine biosynthetic pathways (Coleman et al., 1977; Jarry and Falk, 1974). The multifunctional properties of the polypeptide appear to display common developmental regulation (Mehl and Jarry, 1978). All three enzymes are encoded by a single mRNA transcript which directs synthesis of a multifunctional polypeptide with three active sites (Padgett et al., 1979). The Gart locus appears to involve alternative processing to produce a multifunctional polypeptide with three enzymatic functions (GARS, AIRS and GART) or a polypeptide encoding a single function (GARS) (Henikoff et al, in press).

Unlike rudimentary, the components of the *gua-pur-ras* locus appear to function in quite different ways. Each of the functions appears to act at different times in different tissues. Raspberry appears to regulate GTP cyclohydrolase activity in the head of young adults whereas the auxotrophs of guanosine 1 and purine 1 are believed to function in larvae fat bodies (Fan et al., 1976; Evans and Howells, 1978). It is unlikely that all three functions have a closely allied biochemical origin in contrast to the rudimentary multifunctional polypeptide which encodes three subsequent enzymatic steps in *de novo* pyrimidine biosynthesis.

As an alternative to a multifunctional polypeptide, the *gua-pur-ras* gene complex may produce a monofunctional gene product that is regulated differently in different tissues or at different times. The appearance of several complementing classes of mutations may stem from mutants in

several different cis-acting regulatory components of each gene. A gene product of this locus may function to alter transcription or translation of genes involved in different purine metabolic steps. A monofunctional gene product may also function by regulating gene products produced by several different genes, in a temporal or tissue specific manner.

Another route may involve the production of a primary transcript that may be alternatively processed through splicing mechanisms to produce several monocistronic messages to be translated. Alternative processing may involve alternate initiation or termintion sites, producing different sizes of message. Alternative splicing may result in a segment of DNA functioning as an intron or exon in various messenger RNAs. Alternative processing or splicing may involve temporal or tissue specificity. Introns of a transcript may encode a protein independent of exons (Lewin, 1983; Henikoff et al., 1986). One might also find that the coordinate regulation of the *gua-pur-ras* complex may involve the heterochromatization of adjacent genes.

Nonetheless, to distinguish between the many alternative models for the genetic organization of the *gua-pur-ras* complex, further molecular analysis of the wildtype gene and its mRNA and gene products is essential. The isolation of DNA homologous to the raspberry gene is a first step towards this goal.

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