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THE UNIVERSITY OF ALBERTA

THE FUNCTIONAL CHARACTERIZATION OF A 5' SEGMENT OF A VARIANT *DOPA DECARBOXYLASE* GENE IN DROSOPHILA MELANOGASTER

BY

ALBERTO MAZZA

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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OF MASTER OF SCIENCE

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled THE FUNCTIONAL CHARACTERIZATION OF A 5' SEGMENT OF A VARIANT *DOPA DECARBOXYLASE* GENE IN *DROSOPHILA MELANOGASTER* submitted by ALBERTO MAZZA in partial fulfilment of the requirements for the degree of Master of Science

Dr. R.B. Hodgetts (Supervisor)

Dr. J.B. Bell

Dr. R.H. Gooding

Date: _____

This thesis is dedicated to Vittorio and Therezinha Mazza for their love and unwavering support and to April for helping and encouraging me through difficult moments.

ABSTRACT

In order to determine the relevant regulatory sequences required for the tight temporal control of the *Dopa Decarboxylase* (*Ddc*) gene of *Drosophila melanogaster* the following strategy was employed. Two plasmids bearing hybrid *Ddc* genes that were composed of both wild type and Ddc^{+4} (a naturally occurring regulatory variant) sequences in various combinations were introduced back into live organisms via P-element mediated germ line transformation. The two constructs differed in a 1.0 kb AatII-ApaI fragment extending from -640 to +360 which includes: several 5' regulatory elements, the transcriptional start site, the entire first exon (non-translated leader), and part of the first intron. Quite unexpectedly only one transformant (TL-1) line was recovered and upon analysis was found to be most unusual. The putative location of the P-element insert was at 37C1-2 on 2L which is the location of the endogenous *Ddc* gene. Furthermore, *in situ* analysis revealed the presence of a non-autonomous P-element near the tip of the X-chromosome (5 EF). The source of this P-element was found to be one of the balancer stocks used in establishing transformed lines. Analysis of this P-element indicates that it is conferring repressor activity to the balancer stock and the transformed line. Preliminary evidence suggests that the repressor may be interfering with *Ddc* expression and this may account for the low transformation efficiency observed. The TL-1 line was transformed with plasmid pLuz which was comprised of Ddc^{+4} sequences throughout except for the -640 to +360 region, which consisted of wild type sequence. The developmental profile of DDC activity was nearly identical to that

of the wild type strain. This result indicates that the polymorphisms responsible for the Ddc^{+4} variant phenotype are located within the -640 to +360 fragment. This sets the stage for the construction of more hybrid genes which will allow a further refinement of the region in Ddc^{+4} responsible for the altered enzyme phenotype.

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INTRODUCTION

A The *Ddc* System

The *Dopa Decarboxylase (Ddc)* gene in *Drosophila melanogaster* is an ideal model system for the elucidation of the molecular events associated with the hormonal regulation of gene expression. Furthermore, *Drosophila* is of sufficient complexity to make such findings relevant with respect to other hormone gene systems in higher eukaryotes less amenable to molecular genetic studies. The *Ddc* gene itself is particularly useful in this regard because it is well characterized and is one of a variety of genes with known responses to the moulting hormone 20-OH-ecdysone (31,41). Understanding the structure of the *Ddc* locus and of its regulation is important in that current views on the mode of action of steroid hormones center on hormone-receptor and/or secondary messenger interactions with the 5' regulatory elements of target genes (43).

The DDC enzyme is active as a homodimer (54 kd per subunit) and catalyses the conversion of 3, 4 dihydroxyphenylalanine (DOPA) to Dopamine (39,59). In epidermis this reaction is part of a series of steps leading to the formation of N-Acetyl Dopamine-a compound required for the hardening and pigmentation of newly deposited cuticle (sclerotization) (59,60). In the central nervous system (CNS), the biological function of DDC is in the synthesis of the neurotransmittors dopamine and serotonin (10,11,38). The single copy *Ddc* gene which is located at 37C1-2 on 2L (26) encodes a polypeptide existing in two isoforms which differ by approximately 33 amino acid residues (16)and are believed to arise through tissue specific differential splicing of mRNA (16). The larger isoform (510 A.A.)

predominates in the neural tissue of the C.N.S (16) and is responsible for approximately 5% of the total DDC activity (59) whereas the smaller isoform (477 A.A.) is found mostly in the epidermis (16) and is responsible for greater than 90% of the total DDC activity (59).

In addition to tissue specific regulation, *Ddc* is subject to strict temporal control throughout ontogeny. During development, epidermal levels of DDC activity closely parallel the demand for N-acetyl dopamine; that is, DDC activity peaks are observed at each of the five stages where cuticle deposition occurs (34,41). These stages are: embryonic hatching, 1-2 and 2-3 larval moults, pupariation, and adult eclosion. The regulation of *Ddc*, then, is tied to the developmental process which is, in part, tied to the levels of the moulting hormone 20-OH-ecdysone. Interestingly, five major peaks of ecdysone also are observed during ontogeny (34). This suggests a causal relationship between hormone and DDC levels. The relationship is, nevertheless, inconsistent as ecdysone peaks preceed DDC peaks by variable amounts of time (10 to 60 hours) except at pupariation where the peaks are concurrent (34).

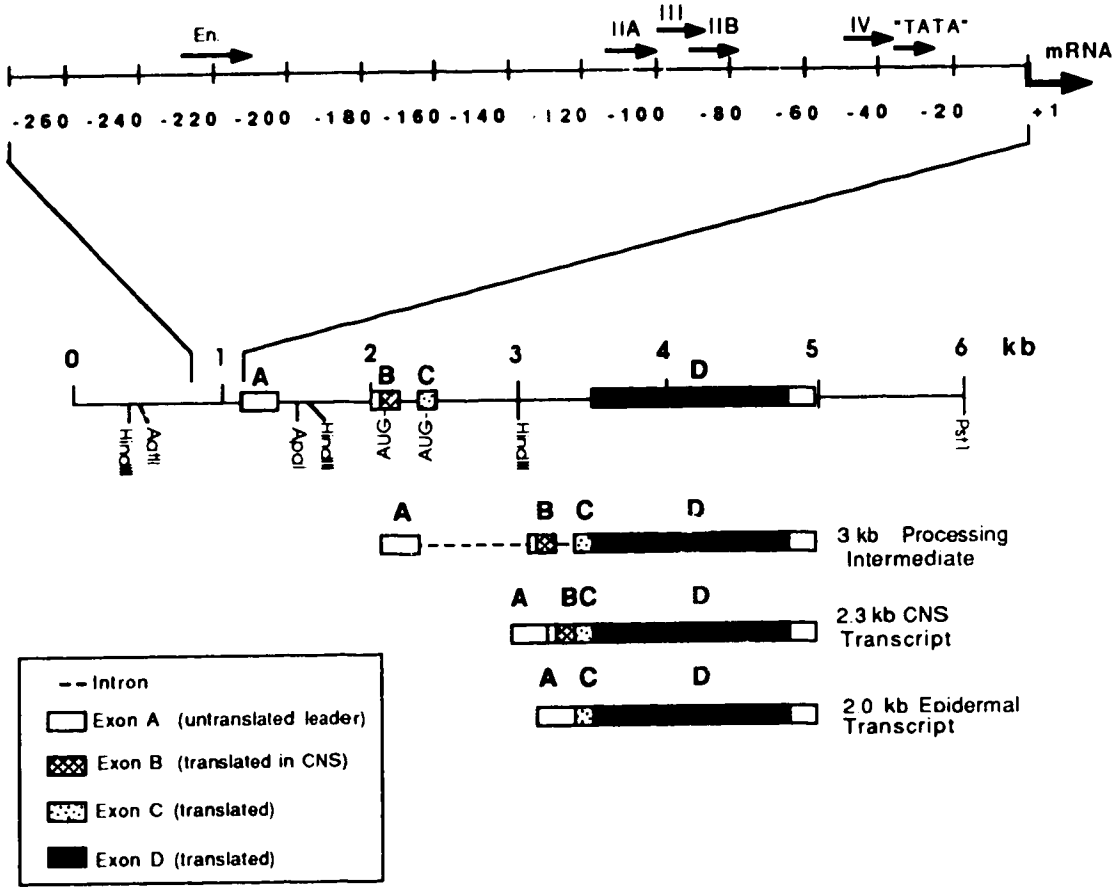
Several lines of evidence show that a direct relationship between hormone levels and expression of the *Ddc* gene exists at pupariation. Work with the temperature sensitive ecdysoneless mutant *ecd*^{1} provided some evidence as to the level at which ecdysone regulates *Ddc* expression (34). Experiments indicated that exogenous feeding of ecdysone to 3rd instar larvae maintained at the restrictive temperature significantly increased the levels of translatable mRNA. This was true in the absence of protein

synthesis as well (8). Taken together, these experiments suggest that elevated hormone levels increase transcription of *Ddc* and that this is a primary effect involving direct interaction of the hormone-receptor complex with the gene. Thus, the relationship between ecdysone levels and *Ddc* expression varies from seemingly disengaged (adult eclosion - 60 hr lag), to partially disengaged (embryogenesis, hatch, 1-2 moult, 2-3 moult - ~10 hr lag), to that of positive transcriptional regulator (pupariation) to possibly that of negative regulator in imaginal discs where a fall in hormone titer seems to be a prerequisite of DDC activity peaks (5,8,20,21). All this suggests the regulation of *Ddc* is complex, and that the effect of ecdysone on *Ddc* likely depends on what other regulatory signals are present at the time. The situation in the CNS contrasts sharply as DDC levels are relatively constant and seem to be independent of ecdysone levels.

B <u>The organization of the *Ddc* gene and its control region</u>

Sequence analysis of cDNAs made from a variety of *Ddc* transcripts (4 kb primary transcript, 3 kb processing intermediate transcript and two mature polysomal transcripts 2.3 kb and 2.0 kb)(1,16), as well as sequences from genomic clones reveal the following about the organization of the *Ddc* gene (see Figure 1). The transcribed region is approximately 4 kb in size and the gene itself is split into four exons (see Figure 1), three smaller 5' exons - A (211 bp), B (166 bp), C (110 bp) and one larger 3' exon - D (1500 bp)(20). Exon A is an untranslated leader present in both epidermal and CNS transcripts. Exon B is present in the larger CNS transcript (2.3 kb) and absent from the smaller (2.0 kb) epidermal

Figure 1. Restriction map of a 6.0 kb portion of the Ddc region including the approximate location of the coding regions within the Ddc transcriptional unit (indicated by boxes). Also indicated are the two mature transcripts and the splicing intermediates produced from this region as well as the relative positions of the translational start sites. Shown above the restriction map is a 280 bp sequence upstream of the transcriptional start site depicting the approximate positions of the TATA homology, regulatory elements IIA, IIB, III, and IV (52) and the enhancer homology (54).


En.
IIA
III
IIB
IV
"TATA"
mRNA
-260 -240 -220 -200 -180 -160 -140 -120 -100 -80 -60 -40 -20 +1
0
1
2
3
4
5
6
kb
A
B
C
D
HindIII
AbaII
ApaI
HindIII
AUG
AUG
HindIII
PstI
A B C D
3 kb Processing Intermediate
A BC D
2.3 kb CNS Transcript
A C D
2.0 kb Epidermal Transcript
Intron
Exon A (untranslated leader)
Exon B (translated in CNS)
Exon C (translated)
Exon D (translated)

transcript(16). It appears that the B exon is spliced out in the epidermal transcript along with the introns and that this is the basis for the occurrence of the two tissue specific isoforms of DDC(16). Exons C and D comprise an ORF of 476 amino acids which is of sufficient size to encode the entire 54 kb DDC subunit(16). Furthermore, this ORF was demonstrated, via an expression test, to be sufficient for the production of DDC. There are two translational start signals, each specific for an alternate-splicing variant. The 2.3 kb C.N.S. transcript has a translational start site at position 1650 of the genomic sequence which places it within exon B (16). The 2.0 kb epidermal transcript has a translation start site at position 1750 within exon C (16). Other organizational elements include a termination codon at 4290, a polyadenylation signal at 4576 and the polyadenylation site at 4590 (16).

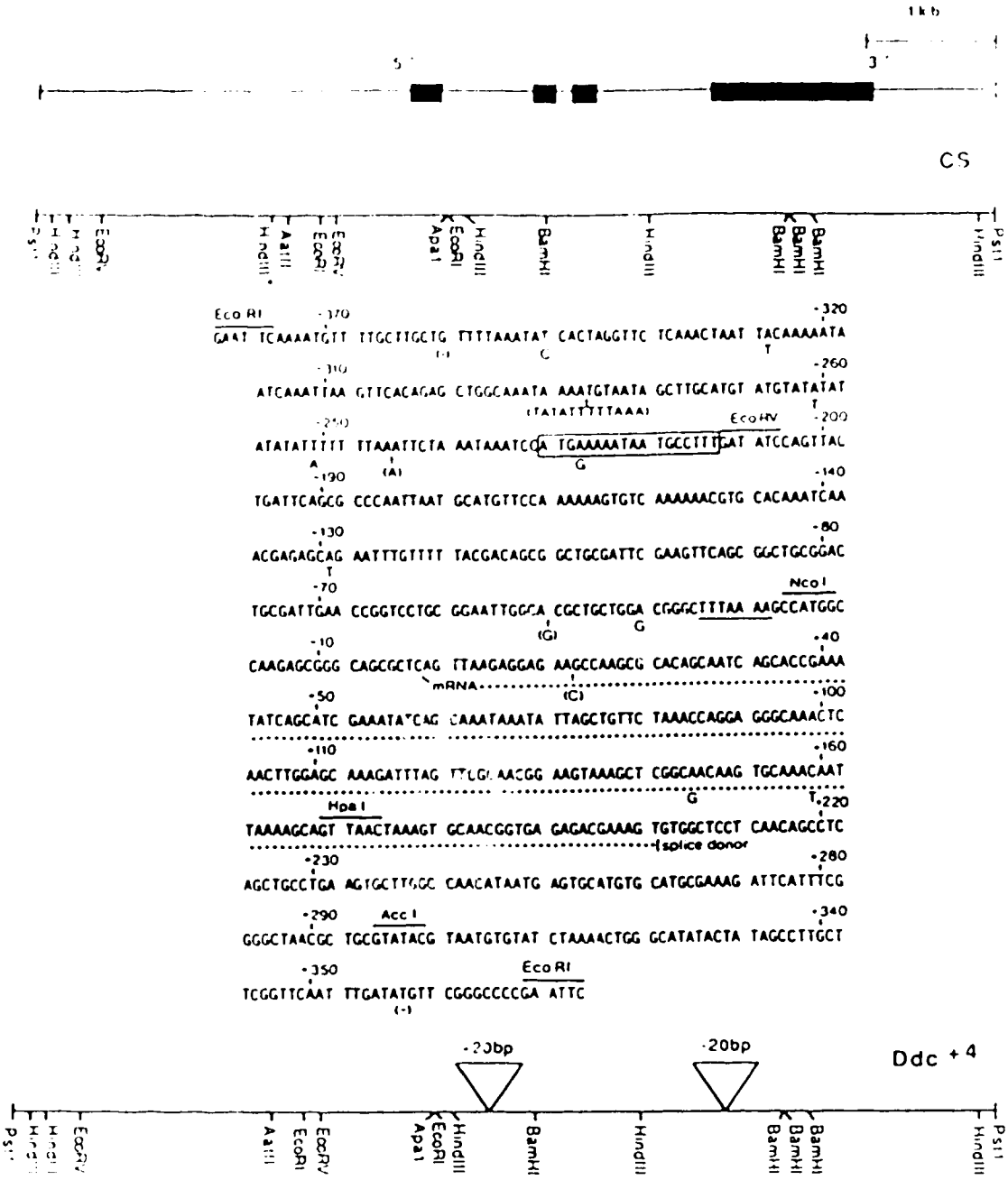
Within the 5' regulatory region upstream of the transcriptional start site, several putative regulatory elements (outlined in Figure 1) have been identified through consensus sequence homologies (52,54), deletion analysis (52) and analysis of the regulatory mutant Ddc^{+4} (54). Most of the above-mentioned analyses have focussed on the region extending from the TATA homology (-28) to -230. Deletion analysis of this region indicates the presence of multiple regulatory elements that appear to have additive effects on epidermal expression of *Ddc* (see Figure 1) (52). These elements (IIA,IIB,III, and IV) show strong homologies between *Drosophila* species (52) which is indicative of their conservation as control elements. None of these elements has been shown to affect the

pattern of expression, only the levels of expression and, as such, are not likely to be sites of ecdysteroid action. In addition to these putative control elements, sequence homology has indicated the presence of another putative regulatory element (see Figure 1) (54). This 18 bp stretch of DNA from -229 to -211 has significant homology with not only the canonical enhancer sequence of mammalian viruses(43), but also with sequences upstream of the hormonally regulated SGS-4 gene (see Figure 1)(57). Furthermore, this putative enhancer is the site of one of the DNA polymorphisms of the regulatory variant Ddc^{+4} (see Figure 2) (54). This base pair substitution (A→G transition) improves the homology with the mammalian enhancer and may be responsible for the overproduction observed at many developmental stages (54). Functional analysis of this enhancer-like element carried out by Charlotte Spencer revealed that this element does increase the level of expression. However, it does not confer any *Ddc* tissue specificity or temporal regulation to the *Adh* reporter gene to which it was fused (54). It should be stated that deletion of this entire region (-28 to -230), including all of the above-mentioned putative regulatory elements, does not entirely abolish *Ddc* expression at all stages (52). This points to the existence of regulatory sequences outside this region, a fact that is supported by the identification of DNase I hypersensitive sites both within and outside of this region (A. Sazci, personal communication).

C $\underline{Ddc^{+4}}$ - A regulatory variant

The existence of mutants, both structural and regulatory, can serve as useful tools in the analysis of ecdysteroid action on *Ddc*.

Figure 2. Comparison of Canton-S (CS-wild type) and Ddc^{+4} (variant) clones. Three major polymorphisms are observed outside of the sequenced EcoRI-EcoRI fragment (inset). These include: the loss of a HindIII restriction site at -768 (indicated by an asterisk), an apparent 20 bp insertion in the 0.6 EcoRI-BamHI fragment, and a 20 bp deletion in the 1.2 kb HindIII-BamHI fragment. The remaining polymorphisms (identified thus far) are illustrated within the EcoRI-EcoRI fragment sequenced in both the wild type and *Ddc*$^{+4}$ (see inset obtained from Charlotte Spencer)(54). The nucleotide sequence from CS is shown in the top line of the inset. Base pair substitutions in *Ddc*$^{+4}$ are shown immediately below the affected nucleotide. Insertions are indicated with an arrow and deletions with (-). The Ddc enhancer homology is outlined in a box. Other features represented include the transcriptional start site, splice donor site of intron 1, and the TATA homology. Nucleotides are numbered relative to the transcriptional start site.


1kb
5'
3'
CS
Pst I
Hind III
EcoRV
Aat II
EcoRI
Apa I
BamHI
Hind III
BamHI
BamHI
Hind III
EcoRI -370 -320
GAAT TCAAAATGTT TTGCTTGCTG TTTTAAATAT CACTAGGTTC TCAAACTAAT TACAAAAATA
-310 -260
ATCAAATTAA GTTCACAGAG CTGGCAAATA AAATGTAATA GCTTGCATGT ATGTATATAT
(TATATTTTTAAA)
-250 EcoRV -200
ATATATTTTT TTAAATTCTA AATAAATCCA TGAAAAATAA TGCCTTTGAT ATCCAGTTAC
-190 -140
TGATTCAGCG CCCAATTAAT GCATGTTCCA AAAAAGTGTC AAAAAACGTG CACAAATCAA
-130 -80
ACGAGAGCAG AATTTGTTTT TACGACAGCG GCTGCGATTC GAAGTTCAGC GGCTGCGGAC
-70 NcoI
TGCGATTGAA CCGGTCCTGC GGAATTGGCA CGCTGCTGGA CGGGCTTTAA AAGCCATGGC
-10 +40
CAAGAGCGGG CAGCGCTCAG TTAAGAGGAG AAGCCAAGCG CACAGCAATC AGCACCGAAA
mRNA
+50 +100
TATCAGCATC GAAATATCAG CAAATAAATA TTAGCTGTTC TAAACCAGGA GGGCAAACTC
+110 +160
AACTTGGAGC AAAGATTTAG TTCGCAACGG AAGTAAAGCT CGGCAACAAG TGCAAACAAT
HpaI +220
TAAAAGCAGT TAACTAAAGT GCAACGGTGA GACGCAAAAG TGTGGCTCCT CAACAGCCTC
splice donor
+230 +280
AGCTGCCTGA AGTGCTTGGC CAACATAATG AGTGCATGTG CATGCGAAAG ATTCATTTCG
+290 AccI +340
GGGCTAACGC TGCGTATACG TAATGTGTAT CTAAAACTGG GCATATACTA TAGCCTTGCT
+350 EcoRI
TCGGTTCAAT TTGATATGTT CGGGCCCCGA ATTC
-20bp
-20bp
Ddc +4
Pst I
Hind III
EcoRV
Aat II
EcoRI
Apa I
EcoRI
Hind III
BamHI
Hind III
BamHI
BamHI
Hind III

Two such mutant alleles which were used in selecting for and establishing transformed *Ddc* lines are Ddc^{n7} and Ddc^{ts2} (61). The expression of *Ddc* in these mutant strains was not analyzed *per se*, but they were of technical importance and are described in detail in Materials and Methods. The Ddc^{+4} mutant was isolated from a natural population in a screen of 109 independently isolated strains made isogenic for the second chromosomes (15). The Ddc^{+4} mutant is a regulatory variant with a complex phenotype. DDC activity profiles of this variant indicate that several stage specific changes occur (15). At pupariation, DDC activity is roughly 50% relative to the wild type Canton-S (C.S.) strain. In contrast to this, Ddc^{+4} is a 20% overproducer at adult eclosion and a 40-50% overproducer at the hatch and 2-3 larval moult. We have chosen to characterize this complex phenotype by calculating an "R value". R is the ratio of DDC activity at pupariation to that at adult eclosion. A wild type (C.S.) "R value" is typically 1.4 to 1.5 whereas the Ddc^{+4} "R value" is approximately .4 to .8 (6,7). "R values" do have limitations in that the complex phenotype may be the result of different independent mutations and thus the underproduction/overproduction components of the phenotype may be separable. Recombination mapping has indicated that the elements responsible for the variant phenotype map to the *Ddc* region (13). This observation has been confirmed at the molecular level by Adam Chen who showed that these elements (mutations) lie within a 7.6 kb PstI fragment containing the entire *Ddc* gene plus approximately 2 kb of 5' flanking sequence and 1 kb of 3' sequence.

Since the mutation(s) responsible for Ddc^{+4} affect the pattern of Ddc expression, identifying and characterizing the specific mutation(s) could provide information as to the site and mechanism of ecdysteroid action. To facilitiate this, *Ddc* genes from both C.S. and Ddc^{+4} have been cloned (14,25), restriction mapped (14) and sequenced (16,54). The differences between C.S. and Ddc^{+4} so far identified have been summarized in Figure 2. The known polymorphisms in Ddc^{+4} fall into several classes in both coding and non-coding regions. These include insertions, deletions, duplications and single base pair substitutions. Very little is known about the functional significance of these various polymorphisms with respect to regulation of *Ddc* gene expression. Certain assumptions can be made, however, as to the most likely location of the polymorphisms responsible for the variant phenotype. Most known regulatory elements are located in the 5' flanking region of genes. Furthermore, DDC activity levels in Ddc^{+4} are directly related to transcript levels (15) which makes it very unlikely that coding region polymorphisms are implicated in the Ddc^{+4} phenotype. Taken together, these observations suggest that the polymorphisms located in the 5' regulatory region are the ones likely to affect phenotypic expression. The strategy used to ascertain this was to manipulate segments of both Ddc^{+} and Ddc^{+4} genes so as to physically separate these polymorphisms. This would be followed by the reintroduction of these *in vitro* modified constructs into the genome of *Drosophila* using P-element mediated germ line transformation and subsequently analyzing their expression.

D P-element mediated germ line transformation

Hybrid dysgenesis is a term used to describe the genetic instability resulting from certain crosses involving two heterogenous families of mobile genetic elements (transposons) in *Drosophila melanogaster*; the P and I families (32). Certain mating schemes involving strains bearing P or I mobile genetic elements result in the occurrence of a series of correlated germ line abnormalities characteristic of hybrid dysgenesis (32). The dependence on the mating scheme indicates that the elements responsible for the occurrence of this phenomenon exist in either active or repressed states (32).

Dysgenic hybrids produced by the P family of transposable elements (P-M hybrid dysgenesis) have the following traits: a variety of unstable mutations, insertion, deletions, chromosomal rearrangements, excisions, temperature dependent sterility and male recombination (12). This phenomenon is observed only in the F_1 progeny of crosses involving paternal contributor (P) males and maternal contributor (M) females, and not in the reciprocal cross (32). P-M hybrid dysgenesis is the result of mass mobilization of multiple dispersed copies of a mobile genetic element termed a P-element (32). This high transpositional activity is entirely dependent on the cytotypic environment which can either suppress it (P-cytotype) or allow it to occur (M-cytotype) (12). These two cytotypes are believed to differ in the presence (P-cytotype) or absence (M-cytotype) of a repressor molecule that functions to inhibit transposition (12). The mass mobilization of P-elements producing hybrid dysgenesis is a germ line specific early zygotic

event, hence the dependence on the maternal cytoplasmic contribution to the developing zygote. Stabilization of these mobile genetic elements occurs after the zygotic genotype begins to influence cytoplasmic content - that is, until germ line cells begin to produce repressor molecules resulting in P-cytotype. P-element copy number in P strains is typically 30-50 copies per genome (2,50). P-elements fall into two general categories: defective and non-defective (46). The non-defective variety of P-elements are 2.9 kb in size containing four open reading frames believed to encode a polypeptide with transposase activity as well as the repressor responsible for conferring the P-cytotype. Current models suggest that the repressor molecule is a functional subunit of the transposase molecule (48). The defective P-elements range from 0.5-1.6 kb and are apparently products of a variety of internal deletions of a full length P-element (2.9 kb) (46). Defective P-elements are not autonomous but can be mobilized if transposase is supplied in trans (49). Thus, defective P-elements have lost the ability to synthesize transposase (49).

The structural organization of the 2.9 kb P-element is closely related to its functionality with four open reading frames separated by three introns which must be spliced out of the full length mRNA for the synthesis of the active 87 kd transposase protein (48). The third intron, in particular, must be spliced out in order to generate the active protein (87 kd) as it contains a translation stop signal. This intron is not spliced out in somatic tissue and results in the production of the 66 kd non-functional protein (48). The excision of the third intron is believed to be the basis for the

tissue specificity of transposition (48). Transposition of P-elements also has certain cis requirements. These are the presence of flanking 31 bp inverted repeats which are believed to be substrates for the transposase enzyme (48). The presence of the 31 bp inverted repeats confers mobility irrespective of the sequences contained between them as long as transposase is provided in trans (55). This is the basis for using P-elements as transformation vectors.

It is very typical of geneticists to use such elements as tools for conducting research and yet not fully understand their mechanism of action. P-element mediated transformation is the only method now available to generate viable DNA integrants into the germ line of *Drosophila melanogaster* and, as such, its use as a transforming vector is widespread. Several properties illustrating the viability of using P-elements as transformation vectors were demonstrated by Spradling and Rubin. They showed that not only can cloned intact P-elements transpose from injected bacterial plasmids to chromosomal DNA of germ line cells and be stably maintained but also that cloned non-autonomous defective P-elements can do the same if transposase is provided in trans. This is achieved by the coinjection of a functional "helper" P-element. Furthermore, they showed that non-autonomous P-elements carrying a cloned gene (ry^+) integrate into the genome and that such genes will maintain their integrity and be properly expressed. One technical problem to be overcome was how to prevent the "helper" P-element from integrating itself into the genome and causing the same instability seen in F1 dysgenic hybrids. This problem was solved by developing "helper" P-elements

like pπ 25.7 W.C. ("wings clipped") that were rendered non-autonomous by removing one of the 31 bp inverted repeats without impairing its ability to produce transposase (55).

The molecular events associated with P-element mediated germ line transformation can be summarized as follows: firstly, plasmid DNA containing a P-element construct bearing the gene of interest and a selectable marker, as well as helper P-element DNA, must be introduced into a developing zygote at pre-blastoderm stage prior to pole cell formation. Secondly, as the pole cells form, cytoplasm containing P-element DNAs will be packaged into the nuclei of these newly formed cells (55). This is followed by transcription of the extrachromosomal helper plasmid molecules and the subsequent transport of the mRNA into the cytoplasm where germ line specific processing and subsequent translation will result in the synthesis of a functional transposase in the germ line (55). The transposable molecules enter the nucleus where they catalyse the transposition of the P-element construct into the chromosome. The frequency of transposition is relatively low as both plasmid DNAs, and transposase mRNA are subject to degradation and, in the case of the plasmids, replication inhibition once inside the embryo (55). Typically, 1-10% of the subsequent gametes produced by a putative G_0 tranformant will bear P-element integrants and thus be used to establish stable homozygous "single insert" lines (55).

P-element mediated transformation as a tool to analyze the regulation of gene expression has several advantages and a few disadvantages over other strategies. The advantages are that genes are returned to a more typical environment as opposed to, say, *in*

vitro experiments. This means that they will more accurately reflect how the genes are regulated *in vivo* (56). Furthermore, the copy number can be controlled and determination of gene dose can be carried out. These integrants are stable yet they can be mobilized by making transposase available. The disadvantages are the fact that gene expression is subject to position effects. Since P-element insertion is essentially random, individual events may produce varying levels of expression and thus several transformants are needed to generate an average of the measured levels of gene expression which would be more accurate than any individual result. Another disadvantage is the low efficiency of transformation which is approximately 1-3% and frequent[illegible] 55).

There are several examples [illegible] P-element mediated germ line transformation has been successfully employed to study the expression of genes (19,22,24,37,62). Of particular interest are those transformations involving the Ddc gene (51). Several investigators have generated transformants with *in vitro* modified Ddc genes or gene constructs (7,51,52,54). These include a variety of deletion constructs (52), gene fusion constructs (54), and fragments carrying isolated variant alleles of Ddc. In all cases reintroduced Ddc genes are expressed, albeit at a wide range of levels generally lower (30-70%) than typical Canton-S (wild type) levels. P-element transformation, then, is a very feasible system to study the regulation of Ddc gene expression.

When undertaking work of this nature there are two broad approaches one can employ. The first is to modify normally

functional Ddc genes *in vitro* and determine the effects on expression by reintroducing them into the genome, i.e. *in vitro* mutagenesis, deletion analysis, gene fusions, etc. The other broad approach is to analyse naturally occurring regulatory variants and determine what polymorphisms are present to account for the aberrant phenotype. In this study my goal is to use a combination of the two. The specific polymorphisms of Ddc^{+4} which are responsible for the variant phenotype are to be determined via *in vitro* modifications of plasmids bearing intact and functional *Ddc* genes containing sequences from both C.S.and Ddc^{+4}. Two hybrid Ddc gene constructs were made and used in the transformations. The first one contains wild type sequences throughout except for a 1.0 kb AatII-ApaI segment, encompassing the region -640 to +360, which is Ddc^{+4} in origin. The second construct is the reciprocal of this having wild type sequences within the AatII-ApaI fragment and Ddc^{+4} sequences in the remainder of the gene. The goal was to measure the R-value in transformants derived from the two types of plasmids.This would permit conlusions to be drawn regarding the effect of polymorphisms located in the AatII- ApaI interval.

MATERIALS AND METHODS

A. *Drosophila melanogaster* stocks

Designation in Text	Ddc Allele	Strain	Function
C.S.	wild type(1)	+	served as contr l for DDC assays (activity set at 100%)
OREGON-R§	wild type(2)	+	M strain served as a control for gonadal dysgenesis ex iments
ts2 cn¶	Temp. sensitive lethal at 29°C Ddc activity 5-10% at perm. temp.	Ddc^{ts2} cn	host strain for P-element mediated germline transfor ation
n^7; Sb§ CyO Ubx	single copy wild type(1) single copy null allele of Ddc (heterozygous)	$1(2)Ddc^{n7}$ pr rdo hk In (2LR) O, dp^{lVI} Cy pr cn^2 Sb Ubx	served as 2nd & 3rd chromoso balancer stock. n^7 chromoso used to select transformant
π 2P§	wild type	+	P- strain used in gonadal dysgenesis experiments

¶ obtained from Dr. T. Wright; § obtained from Dr. J. Bell;

Note
individual mutants are described in Lindsley & Grell (36)

Maintenance

All stocks were continually on standard yeast-sucrose medium (45) modified to include a sodium phosphate buffer system at 22° to 25°C (ambient temp) except where indicated. Containers were either standard 250 ml milk bottles or 30 ml shell vials.

B. BACTERIAL STOCKS (*E. coli*)

Listing

Strain	Selection	Function
JM83	AMP^S TET^S $LacZ^-$	host for a variety of plasmid constructs
HB101	AMP^S TET^S $LacZ^-$	same

Maintenance

All bacterial stocks maintained in LB 15% glycerol in duplicate aliquots at -70 and -20°C.

Growth Media

Bacterial strains were grown in either LB (Luria Broth) containing 10 g/l Bacto-tryptone, 10 g/l NaCl, and 5 g/l Bacto-yeast extract. LB and AMP required the addition of ampicillin to a final concentration of 100mg/l. Addition of 15g/l of Bacto-agar was required for solid LB media. MacConkey agar media containing 17g/l Bacto-tryptone, NaCl 5g/l, proteose peptone 3g/l, Bacto-bile salts 1.5 λ/l,1% lactose Bacto-agar 13.5 g/l; Bacto-neutral red 0.03 g/l

and Bacto-crystal violet crystal violet 0.00[illegible] g/l plus 100 g/l ampicillin.

C. BACTERIAL TRANSFORMATION

Transformation of *E. coli* was carried out as described in Maniatis *et al.* (40) *E. coli* strains JM83 or HB101 were grown to saturation in LB (5ml) and then diluted 1/20 in LB and maintained in 37°C incubator/shaker until OD approximately .4 -.6. Cells were then harvested by centrifugation and washed in 50% of original volume of 50 mM $CaCl_2$. Subsequent to this, cells were harvested once again and resuspended in a 10% volume of 50 mM $CaCl_2$ and kept at 4°C overnight. Approximately 5-10 μl DNA solution was added per 0.2 ml aliquot of competent cells and kept on ice for at least one hour, followed by heat shocking at 42°C for 2 min. Upon addition of 0.8 ml LB, the solution was incubated for 1 hour at 37°C. Dilutions (0.15 ml or 0.2 ml) were plated on appropriate media (LB plus ampicillin or MacConkey lactose plus ampicillin) and incubated at 37°C for 12 to 24 hours.

D. LARGE SCALE PLASMID DNA EXTRACTION

Large scale plasmid isolation was carried out using the SDS lysis procedure described in Maniatis *et al* (40) with certain modifications. Cells were not amplified but rather grown to saturation and subsequently lysed. The second major change was the omission of phenol extractions. Quantitation of DNA samples was done by measuring OD 260/280 and/or running 1-5 μl samples on an agarose gel.

E. RAPID EXTRACTION OF PLASMID DNA

Small scale isolation of plasmid DNA was done using the boiling method described by Holmes and Quigley (27), with certain modifications. A 1.5 ml volume of saturated culture was centrifuged for 1 minute in an Eppendorf Microfuge. The pellet was resuspended in 0.35 ml of a solution containing 8% sucrose, 0.5% Triton X-100, 50 mM EDTA (pH 8.0) and 10mM TRIS-Cl (pH 8.0). 25 μl of a freshly prepared lysozyme solution (10 mg/ml in 10mM TRIS-HCl pH 8.0) was added and the mixture vortexed for 3 seconds. The tube was then placed in a boiling water bath for 40 seconds, followed by immediate centriguation in an Eppendorf centrifuge at room temperature. The pellet was removed with a toothpick and the supernatant extracted 1X with phenol, 2X with phenol/chloroform and 2X with chloroform. This was followed by the addition of 40 μl 2.5 M sodium acetate and 2 volumes of ice cold 95% ethanol. The pellet was washed several times with 70% ethanol, dried, then resuspended in an appropriate amount of TE (pH 8.0). Ribonuclease treatment was applied to samples that were to be restricted.

F. ISOLATION OF GENOMIC DNA FROM WHOLE FLIES

Extraction was done as follows. Adult flies (approx 200 mg) were homogenized in 3.0 ml of homogenizing buffer containing 10mM TRIS-HCl (pH 7.5), 60 mM EDTA, 0.15 mM spermidine, 0.2 mg/ml pronase E (fresh) and 0.15 mM spermidine. To this mixture, 3.0 ml lysis solution containing 0.2 M TRIS-HCl (pH 9.1), 30 mM EDTA, 2% SDS and 0.2 mg/ml pronase E (fresh) was added and the homogenate incubated at 37°C for one hour. The homogenate was then extracted 2X with

phenol, 1X with phenol/chloroform and 1X with chloroform. NaCl (5 M stock solution) was added to 0.2 M followed by addition of 2 volumes of ice cold 95% ethanol. The sample was thoroughly mixed and kept at -20°C for 30 min. The nucleic acids were pelleted and washed several times with 70% ethanol. The dried pellet was then resuspended in TE (pH 8.0) and the sample ribonuclease (25 μg/ml) treated at 37°C for 30 min. The nucleic acids were then once again precipitated with 95% ethanol, washed in 70%, dried, and resuspended in 1 ml TE. The DNA was further purified before restricting. See spermine precipitation.

G. RESTRICTION OF DNA SAMPLES

Manufacturer's recommendations were used for all restrictions with some general exceptions. The ratio of DNA (μg) to enzyme units was generally approximately 1:10. Spermidine (2 mM) was added to all restriction so as to improve cutting efficiency. All samples were heat killed for 10 min at 65°C to terminate reaction.

H. AGAROSE GEL ELECTROPHORESIS

DNA samples to be electrophoretically analyzed were mixed with 1/10 volume of tracking dye (0.03% xylene cyanol, 0.03% bromophenol blue and 10% glycerol). The samples were then loade[illegible]o the wells of a horizontally submerged (in TAE buffer) agaro[illegible]nesting of 0.75-1.0% agarose (Sigma Type II, Medium EEO) m[illegible](40 mM TRIS acetate, 2 mM EDTA pH 8.0) buffer. Gels w[illegible]the range of 25 to 80 V from anywhere between 1 and 1[illegible]c longer runs TBE (.089 M TRIS-Borate, 089M boric aci[illegible]EDTA

(pH 8.0)) was used instead of TAE. To visualize DNA fragments ethidium bromide (1 μg/ml) was added either to the buffer or to the melted agarose solution before pouring. Visualization required exposure to long wave-length UV light. Subsequent photography was done using a Polariod MP4 camera. Gels were run within the range of 25 to 80 V from anywhere between 1 and 14 hours.

I. POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide gel electrophoresis was carried out as described in Maniatis *et al.* (40) typically 8% acrylamide gels were used. The solution consisted of 26.6 ml of 30% acrylamide, 2.1 ml 3% ammonium persulfate in a total 100 ml TBE buffer. The solution was deaerated and 30 μl of TEMED (N-N-N'-N' tetra methylethyline diamine) was added prior to pouring. The solution was poured between two cleaned and siliconized glass plates held together by bulldog clips and separated by 2 mm spacers. The acrylamide was allowed to polymerize at room temperature for one hour. The electrophoresis apparatus was of the vertical type. The buffer used was TBE and gels were usually run at 250-350 v for 3 hours. The tracking dye used was 0.03% xylene cyanol, 0.03 bromophenol blue and 10% glycerol. It was mixed at 1/10 final volume of final DNA solution. The fragments were visualized by carefully separating the gel from one of the glass plates. The gel was then stained in a TBE solution containing 1 μg/ml ethidium bromide. Visualization and photography was the same as described above.

J. ISOLATION OF FRAGMENTS

3 MM Electroelution

3 MM electroelution was carried out as described in Maniatis *et al.* (40). Fragments run on a standard 0.8% agarose gel were isolated by making an incision immediately in front of the band of interest. A piece of Whatman 3 MM filter paper of appropriate size as well as a same-sized piece of dialysis membrane were inserted into the incison with the filter paper being closest to the band. The band was run into the paper which was then removed and washed 2X with 100 μl of elution buffer (0.2 M NaCl, 50 mM Tris-HCl (pH 7.6), 1 mM EDTA and 1% SDS). The eluate was extracted once with phenol/chloroform and once with chloroform. DNA was subsequently ethanol precipitated and, if to be used for ligation, drop dialysed. Fragments to be used in ligations were typically isolated in this manner.

Low melting point agarose

Fragments to be used as probes were typically isolated in this manner. A low melting point agarose gel (1%) was prepared as described above. DNA samples were run at 40 v for 3 hours at 4°C. The band of interest was excised and dissolved in a 90.8% w/v sodium iodide 2% w/v sodium sulfite solution. This was followed by the addition of 10 μl of silica slurry mix. The silica powder was maintained in suspension for five minutes to allow DNA to adsorb onto it. The mixture was then spun in an Eppendorf microfuge and the supernatant was discarded. The pellet was washed 2x with ethanol wash solution (48% ethanol in 2 mM Tris pH 7.4, 1 mM EDTA and 0.1 M NaCl). The purified DNA fragments were then eluted by

washing pellet with dH_2O and incubating for 30 min at 37°C. The sample was spun and the supernatant saved.

K. DNA LIGATION

DNA fragments were mixed in a 1:1 molar ratio of fragment ends in 50 µl reaction mixture containing 50 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 2 mM DTT, 1 mM adenosine triphosphate, 2 mM spermine, and 50 µg/ml bovine serum albumin. T4 DNA ligase (0.1 units) was added and the solution was incubated at 14°C for 8-16 hours.

L. SPERMINE PRECIPITATION

DNA samples containing a maximum of 100 µg/ml were precipitated by making solution 100 mM for KCl and 10 mM for spermine. The DNA was pelleted and washed twice in 75% ethanol, 100 mM Na Acetate, 10 mM Mg acetate for a 2-4 hour period. This was followed by pelleting and washing twice with 70% ethanol. The DNA was then resuspended in an appropriate volume of TE

M. OLIGO-LABELLING

For all Southern blots ^{32}PdCTP radioactive probes were made by the oligo-labelling described by Feinberg and Vogelstein (17,18). DNA fragments were either purified or in low melt agarose. A maximum of 0.1µg of DNA was used per labelling reaction. DNA samples were mixed with dH_2O up to 32.5 µl of solution and then heated to 100 °C for 3-5 minutes. This was followed by the addition of 10 µl of oligo-labelling buffer (OLB) made as follows. 1 ml of 1.25 M Tris-HCl (pH 8.0), 0.125 M $MgCl_2$ was added to 18 µl

2-mercaptoethanol solution containing 5 μl of each of the following, dATP, dGTP, dTTP (100 mM stock solutions in 3 mM Tris-HCl, 0.2 mM EDTA pH 7). These solutions were further mixed with 2 M hepes pH 6.6 and hexadeoxyribonucleotides (Pd[N]$_6$ Pharmacia) in TE (9000 units/ml) in a final ratio of 2:5:3. The addition of OLB was followed by the addition of 2 μl of 10 mg/ml BSA, 50 μ Ci of ^{32}P dCTP (typically 5 μl α^{32}PdCTP) and two units of Klenow fragment. The mixture was incubated at 37-42°C for 3 to 16 hours. The reaction was stopped by the addition of nick translation stop buffer (see Nick Translation). Specific activity was measured by running the sample through a Sephadex G-50 spin column equilibrated to pH 8 with TE as described in Maniatis *et al* (40). One μl sample was then spotted on Whatman #1 filter paper and washed in 0.3 M ammonium formate. Filters were then counted in an aliquot of dH_2O in a Beckman 7500 liquid scintillation spectrophotometer.

N. NICK TRANSLATION

Nick translation was employed exclusively in making ^{3}H probes for *in situ* hybridization. Radiolabelled nucleotide incorporation was carried out as described by Davis *et al.* (9) with a few modifications. Final reaction solution (50 μl) contained 50 mM Tris HCl (pH 7.2), 10 mM $MgSO_4$, 0.1 mM DTT, 50 μg/ml bovine serum albumin, 0.2 mM of each of dATP, dGTP and dTTP, approximately 10μCi ^{3}HTTP, 0.02 μg DNAse I and 5 units of DNA pol I. The sample was incubated at 14°C for 4-12 hours and stopped by the addition of nick translation stop buffer (20 mM EDTA, 2 mg/ml sonicated salmon sperm DNA, and 0.2% SDS). Specific activity was measured by running the

sample through a spin column (see Oligo-Labelling). 1-2 µl samples were mixed with 5 ml of aquasol and counted in a Beckman 7500 liquid scintillation spectrophotometer.

O. REPLICATING BACTERIAL COLONIES ONTO NITROCELLULOSE MEMBRANES

E. coli transformants were screened by filter colony hybridization according to Maniatis *et al.* (40). A piece of nitrocellulose membrane Biodyne™ was placed onto the surface of solid LB plus ampicillin media. Cells were patched onto the membrane and incubated at 37°C for 12-18 hours. With colonies facing up, the membrane was placed onto 3 MM filter paper and soaked with 0.5 M NaOH for 3 minutes. This step was repeated once more and subsequently transferred to another 3 MM filter paper soaked with 1 M Tris HCl (pH 7.4) for 5 minutes and repeated. The membrane was baked at 80°C in a vacuum oven for 2 hours.

P. SOUTHERN TRANSFERS OF GENOMIC AND PLASMID RESTRICTIONS

This method, which was modified from Davis *et al.* (9) was used to blot DNA samples from agarose as well as polyacrylamide gels. After electrophoresis, the gel was soaked in a denaturing solution of 1.5 M NaCl and 0.5 M NaOH for about 30 min (gentle agitation). This was followed by soaking in neutralizing solution of 3 M Na Acetate pH 5.5 for 15 min (gentle agitation). The gel was carefully placed on 3 sheets of 3 MM paper soaked with 10 x SSC. The filter papers were placed on glass plates with the ends dipped in 10 x SSC containing reservoirs. The nitrocellulose membrane was carefully placed on the gel and covered with two sheets of 3 MM filter paper

and 8-10 cm of paper towel. A 1 kg weight was applied to the paper towels. The transfer was allowed to proceed for 12-14 hours. The membrane was baked as described previously. For polyacrylamide gels, weight was increased and transfer time extended ~ 20 hours.

Q. HYBRIDIZATIONS

All Southern blots and colony transfers were hybridized according to Klessig's (33) method. Membranes were prehybridized 4 hours at 42°C in 30 prehybridization buffer containing 50% formamide, 500 μg/ml denatured salmon sperm DNA, 1 m NaCl, 50 mM PIPES pH 7.0, 0.5% sodium sarkosyl w/v, 5x Denhardt reagent, 20 μg/ml yeast tRNA, and 10 mM EDTA pH 8.0. Upon prehybridization buffer was replaced with fresh prehybridization buffer that was supplemented to 10% w/v with dextran sulfate. Denatured probe (to a final concentration of approximately 1 x 10^6 counts/ml) was added and hybridization allowed to proceed for 24-36 hours. Blots were washed 3x in 2 x SSC, 0.1% sarkosyl for 15 min at room temperature, 1x in 1 x SSC, 0.5% sarkosyl for 30 min at 55°C and 2x in 0.1 x SSC, 0.1% sarkosyl for 30 min at 55 °C. Blots were wrapped in plastic and Kodak X-ray film was exposed to them in X-ray film cassettes with intensifier screens. Cassettes were kept at -70°C.

R. STRIPPING BLOTS FOR REPROBING

Blots were stripped according to Biodyne™ technical recommendations. Blots were incubated at 65°C for 1 hour in solution of 10 mM Na Phosphate pH 6.5, and 50% formamide. Membranes were then washed for 15 min at room temperature in a solution of 2X

SSC and 0.1% (w/v) SDS. Prehybridization times we doubled for rehybridized or stripped blots.

S. *IN SITU* HYBRIDIZATIONS TO POLYTENE CHROMOSOMES

In situ hybridization was carried out as described by Pardue and Gall (47). Third instar larvae grown at 18°C on yeast rich medium and maintained at low density were collected and their salivary glands dissected in 45% acetic acid. The gland were then transferred to gelatinized (gelatin: Fisher G-8 U.S.P. Granular, 270 Bloom) slides (4 glands/slide). The glands were covered with a siliconized cover slip (#2) and squashed by applying pressure. Sufficient pressure was required to assure proper spread. The slides were dipped in liquid nitrogen for 10 seconds and the cover slips removed. The slides were then immediately submerged for 2 min in a 3:1 solution of ethanol to acetic acid followed by dehydration by dipping once in 70% and once in 95% ethanol (5 min each time). Slides were air dried and stored at 4°C. This was followed by heat treatment for 30 min at 55° in 2x SSC. The dehydration step was again repeated as described above.

The chromosomes were then denatured by immersing slides in 0.07N NaOH for 3 min again followed by dehydration. Slides were dried and stored as described above. Hybridization was performed as follows: 30 μl of hybridization mixture was added onto the region of the slide bearing chromosomes and covered with a cover slip. The hybridization solution contained equal volumes of denatured ^3H labelled probe (~3x 10^4 CPM/μl) and sonicated salmon sperm DNA (0.4 mg/ml) mixed with two volumes of hybridization buffer containing .2

ml 50x Denhardt's solution, 1.0 ml 50% dextran sulfate, 0.66 ml 5mm NaCl, 0.1 ml 1mm $MgCl_2$, 0.2 ml, 0.5 M $NaPO_4$ (pH 7.0) and 0.34 ml dH_2O to a total of 2.5 ml. Slides were then incubated at 65°C 12-16 hours in a high humidity container. The washes were carried out as follows: 3x at 65°C in 2x SSC, 2x at room temperature in 2x SSC and again followed by dehydration, drying and storage. Visualization was performed by dipping slides in autoradiographic emulsion (Kodad NTB 2 liquid emulsion diluted 1:1 with dH_2O) and exposing at 4°C for 5 to 7 days. Slides were developed as follows: 1 in in Kodak Dektol developer (diluted 1:1 with dH_2O), stopped in dH_2O for 30 sec., and fixed for 5 min in Kodak Rapid-Fix. The slides were then rinsed, stained with Giemsa stain, and finally made permanent by adding 1 drop of Permount (Fisher) and covering with a cover slip.

T. DDC ENZYME ASSAYS

Assays were performed as described by McCaman *et al.* (42) with modifications. Tissues (white pre-pupae and young adult [0-2 hrs] flies) were collected and homogenized (10 mg/ml) in extraction buffer containing 0.3 M sucrose, 0.2 mM phenylthiourea and 0.1 M sodium phosphate pH 7.2. Aliquots of these extracts were he killed at 65°C for 30 min. These served as blanks.

As were done in triplicate for all samples except blanks. In each assay, 10 μl of the extract was added to 10 μl of hot reaction mix in an Eppendorf tube. The reaction mix consisted of 0.1 M Na phosphate pH 7.0 0.1 mM pyridoxal 5' phosphate, 0.5 mM cold (unlabelled) L-DOPA, and 0.4 mM ^{3}H L-DOPA 10μCi. The reaction mix was incubated for 30 min at 30°C. The reaction was stopped by

the addition of 100 μl of 0.1 M diethylhexylphosphoric acid in $CHCl_3$. Labelled reaction product (L-Dopa) was measured by washing the organic phase twice with 300μl 0.01 M Na phosphate pH 7.0. The remaining organic phase (50 μl) containing ^3H dopamine was transferred to a scintillation vial containing 5 ml Aquasol (scintillation cocktail) and the sample was counted in a Beckman 7500 scintillation spectrophotometer. The total protein concentration was determined using Bradford's Coomassie blue method as modified by Spector *et al.* (4,53).

U. GONADAL DYSGENESIS-OVARY DISSECTION

Appropriate crosses were set up and incubated at 29°C. Once sufficient eggs hatched, parents were cleared or transferred to a fresh vial. F_1 female progeny were subsequently transferred to a fresh vial and maintained at room temperature for 2-5 days. Using fine forceps, ovaries were dissected while females were submerged in distilled water in a shallow petri dish. Approximately 20-50 females were dissected per cross. The number of abnormal and normal ovaries were scored.

V. MICROINJECTIONS

The method used was as described by Rubin and Spradling (49) with modifications. Collections were made from a 2700 cm^3 population cage (Tyler Research) containing several hundred 3-5 day old flies. All collections were made at 20°C as a requirement of using ts2 cn as the host strain. Collections were made at one hour intervals by placing 35mm petrie plates containing 1.5% age, 1%

grape juice concentrate and smeared with fresh live yeast culture. The eggs were washed with dH_2O and dechorionated following either of the following two regimes.

Chemical: Eggs were collected on Nitex nylon filters and dipped in the following solutions; 1x in 50% (1:1 with H_2O) bleach solution (commercially available) for 60 sec, 1x in dH_2O for 60 sec, 1x in 0.05% Triton-x in insect Ringer's solution for 30 sec, followed by extensive washing in dH_2O.

Mechanical: Eggs were lifted directly from collection agar and individually dechorionated by rolling on double-sided sticky tape. This was done at 18-20°C in a chamber with ambient humidity at approximately 80%.

Dechorionated eggs were mounted on slides by placing them along the edges of double-sided sticky tape such that the posterior ends protruded slightly. Eggs were slightly dehydrated 1-5% by placing them in a sealed container containing Dri-rite H_2O absorbant for approximately 20 min. Dessication was carried out at 20°C. When sufficiently dessicated, the eggs were covered in Halocarbon oil (Series 700). Eggs that were found to be older than 0-4 hours were destroyed at this point.

The injections were carried out using a Leitz micromanipulator-microscope assembly. Injection solution was hydraulically forced into the very posterior end of the embryos. The glass needles were made by pulling 25 μl Drummond micro cap pippettes with an electrode puller (Aloe Scientific). The sharp points were broken to a diameter of approximately 1 mm at 100x magnification by gently forcing needle tips up against the edge of a

cover slip. The DNA injection solution was prepared as follows: appropriate DNA was co-precipitated with pπ25.7 wings clipped helper plasmid, obtained from Dr Larry Marsh, in a ratio of 5:1 construct to helper. The total DNA concentration (final concentration) was 300-400 μg/ml. The DNA was resuspended in injection buffer (5 mM KCl and 0.1 mM sodium phosphate ph 6.8). The volume injected was 1-3% of the total volume of the embryo. Injected embryos were maintained at 18-20°C under high humidity. The hatched 1st instar larvae were transferred to standard media covered with live yeast culture. The subsequent G_o adults were crossed as described in Results.

RESULTS

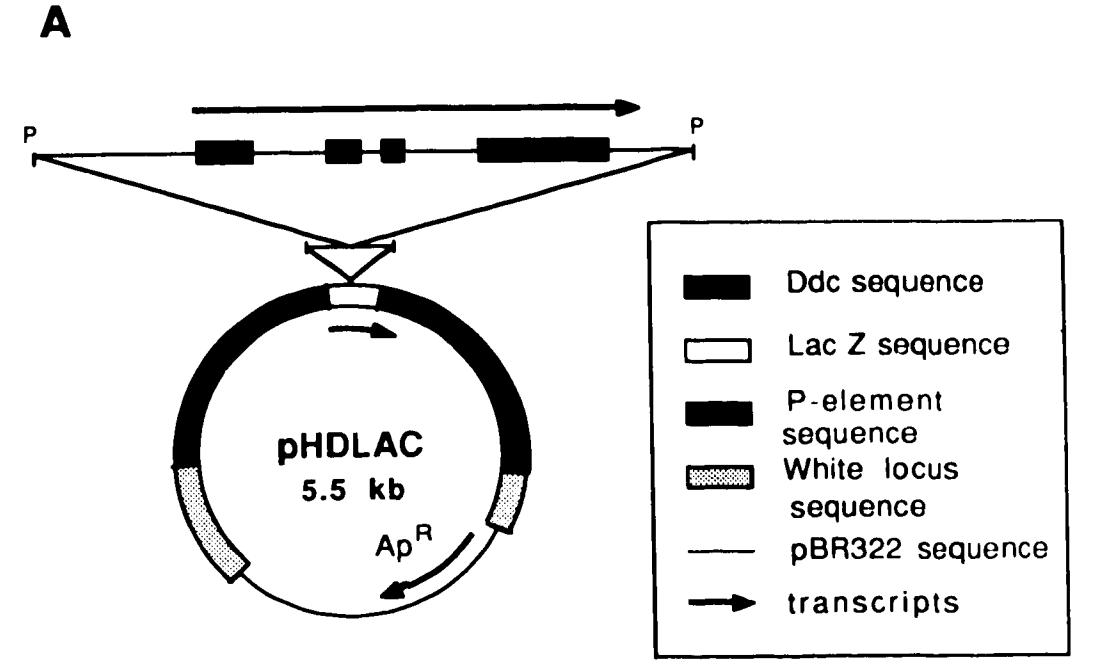
A. Construction of hybrid *Ddc* gene P-element vectors

Figure 3A illustrates the organization and composition of a *Ddc*-bearing derivative of the P-element vector pHDLAC into which the genes from C.S. and Ddc^{+4} were cloned. While this vector is suitable for P-element transformation, it is not practical for manipulating fragments. This is because the extraneous sequence present (see Figure 3A) makes it difficult to find unique restriction sites. I set out to make two constructs which differed in the origin of the DNA (C.S. or Ddc^{+4}) within the 1.0 kb AatII-ApaI segment (see Figure 1). Four fragments are generated upon AatII-ApaI restriction (see Figure 4) making it prohibitively difficult to reassemble (via ligation) the intended constructs. This problem was circumvented by employing a different strategy as is outlined in Figure 5. The approach involved making the two desired constructs (pLuz & pMil) in two steps via the intermediate constructs (pApr & pAma).

The first step involved ApaI restrictions of p10LAC and pZ (see Figure 5), generating two fragments 9 and 4 kb in size. These fragments were isolated following agarose gel electrophoresis, combined as shown by the arrows and ligated together. Because the larger (9 kb) fragment could re-ligate to form a functional plasmid, a high proportion of the ligation products were the undesirable recircularized 9 kb fragments. These 9 kb plasmids transform bacteria at a higher efficiency than the desired 13.1 kb plasmids resulting in a very small proportion of transformants being useful. To identify bacterial transformants with the 13.1 kb plasmids,

Figure 3A. The organization and sequence content of the P-element vector pHDLAC obtained from Dr. Larry Marsh. The relative proportions of the various segments are approximations only. The orientation of the PstI insert bearing the *Ddc* transcriptional unit relative to the vector transcriptional units (LAC Z and AMP^R) is indicated. Not shown is the composition of the polylinker which is from pUC19. P=PstI, Ap^R=ampicillin resistance gene.

Figure 3B. A circular restriction map of a p[$Ddc^{+/+4}$] vector, derived from pHDLAC, illustrating the fragments used as probes for Southern and/or *in situ* analysis. The relative sizes as indicated by this diagram are approximations only. Although not indicated, the entire plasmid itself was used as a probe for certain experiments.


A
P
P
pHDLAC
5.5 kb
Ap^R
Ddc sequence
Lac Z sequence
P-element sequence
White locus sequence
pBR322 sequence
transcripts

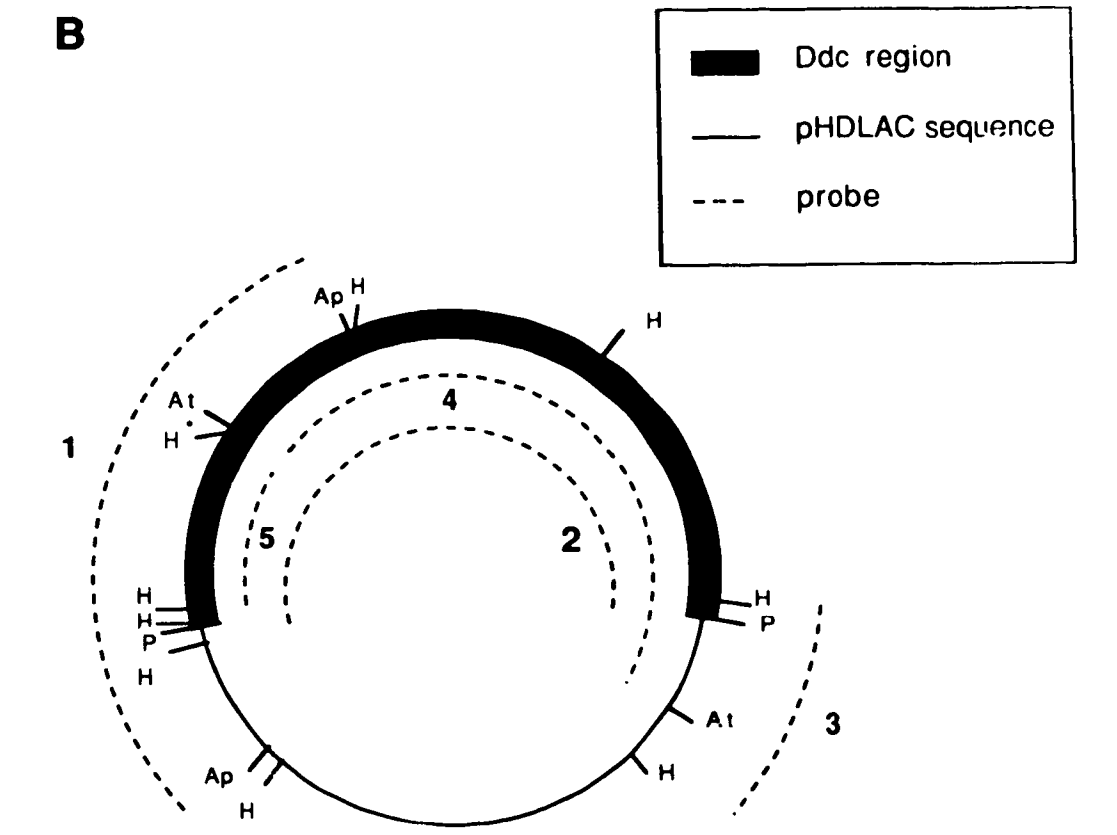

B
Ddc region
pHDLAC sequence
probe
Ap H
H
At
H
1
4
5
2
H
H
P
H
H
P
At
3
Ap
H
H

Figure 4. The organization and structure of p10LAC and pZ constructs. The boxes represent the 7.6 kb PstI fragments and their origin (C.S. or Ddc^{+4}). Restriction maps of these plasmids indicate those sites relevant to the making of hybrid *Ddc* constructs. The fragments produced by HindIII digestion are shown as lettered (A-G) segments within each plasmid and are displayed in the photograph of an agarose gel in which approximately 1.5-2.0 μg of DNA were loaded per lane. Size estimates were made based on a 1 kb ladder marker DNA (BRL) that was also run on these gels but is not shown here. Standard curves were drawn and used to interpolate fragment sizes.

The two polymorphisms that distinguish pZ from p10LAC are denoted by an asterisk (*) in both the diagrams and their corresponding restriction patterns. The HindIII restriction size polymorphism results in the disappearance of fragments F and G (seen in p10LAC) and the appearance of the larger fragment FG (seen in pZ). The other polymorphism affects the migration of fragment C due to an extra 150-200 bp insertion of DNA. This fragment is approximately 1.0 kb in size in pZ and .8 kb in p10LAC.

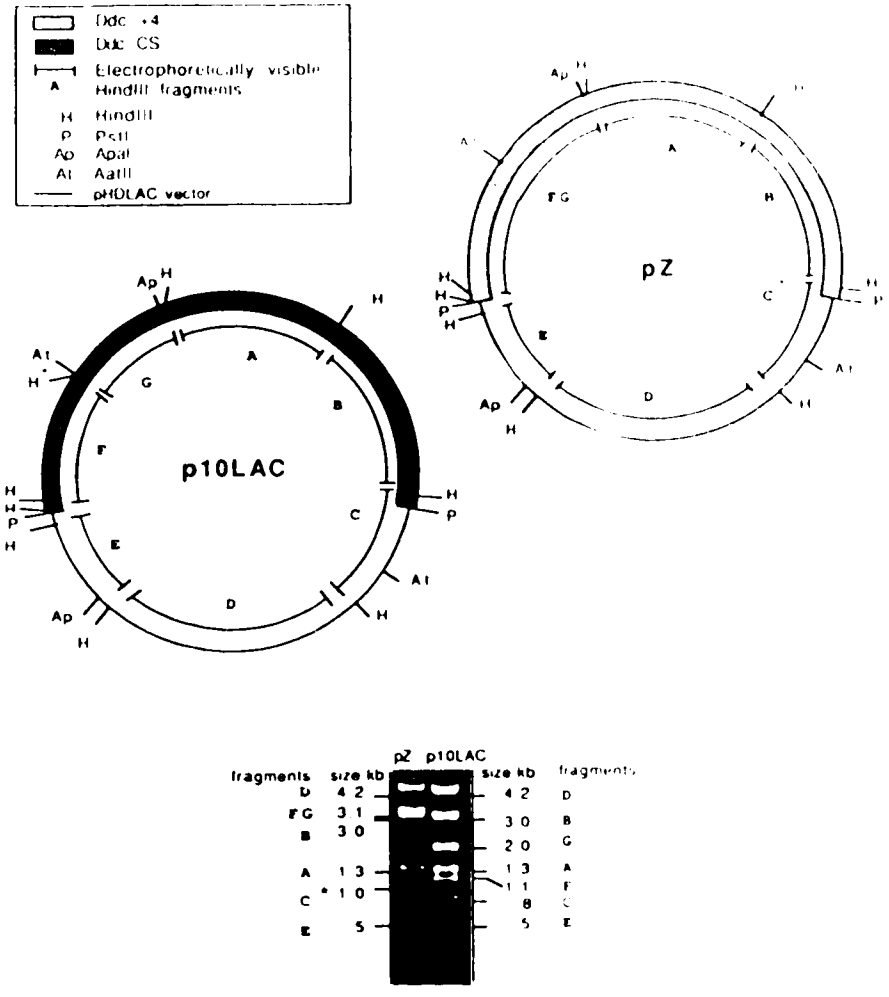
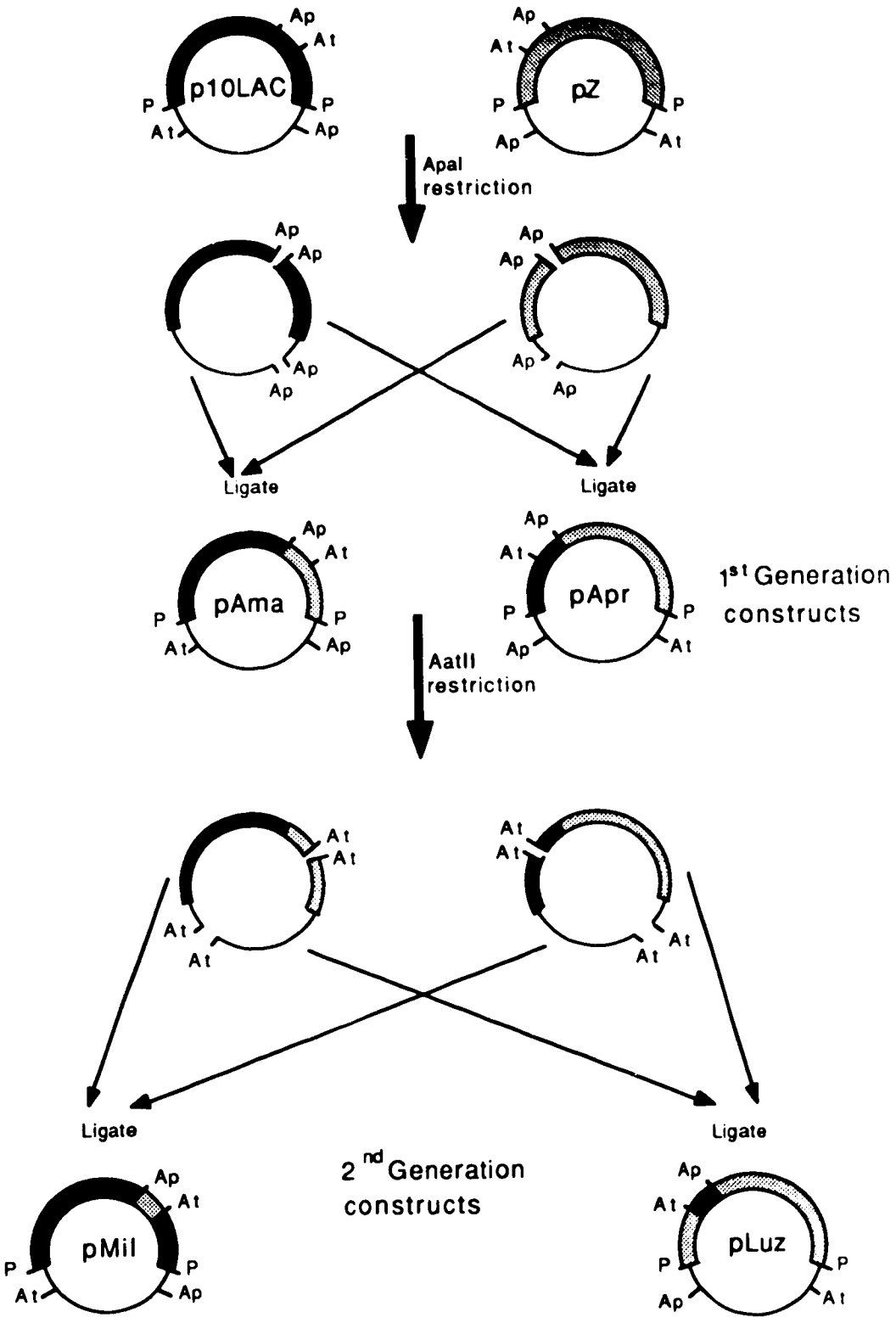

Ddc +4
Ddc CS
Electrophoretically visible HindIII fragments
H HindIII
P PstI
Ap ApaI
At AatII
pHDLAC vector
p10LAC
pZ
A
B
C
D
E
F
G
Ap
At
H
P
pZ p10LAC
fragments size kb
size kb fragments
D 4 2
FG 3 1
B 3 0
A 1 3
C * 1 0
E 5
4 2 D
3 0 B
2 0 G
1 3 A
1 1 F
8 C
5 E

Figure 5. A diagramatic representation of the strategy employed to make the desired hybrid gene constructs. The boxed regions represent DNA from the *Ddc* region (7.6 kb PstI fragment): sequences from the wild type (C.S.) are dark whereas those from the Ddc^{+4} variant are light. The thin lines represent pHDLAC vector sequence. Six restriction site are indicated with P=PstI, At=AatII and Ap=ApaI. Sites not involved in a particular restriction are omitted from the resulting fragments for simplicity. The thin arrows illustrate the combinations of fragments which were ligated together. Only the desired ligation products are shown (see text).

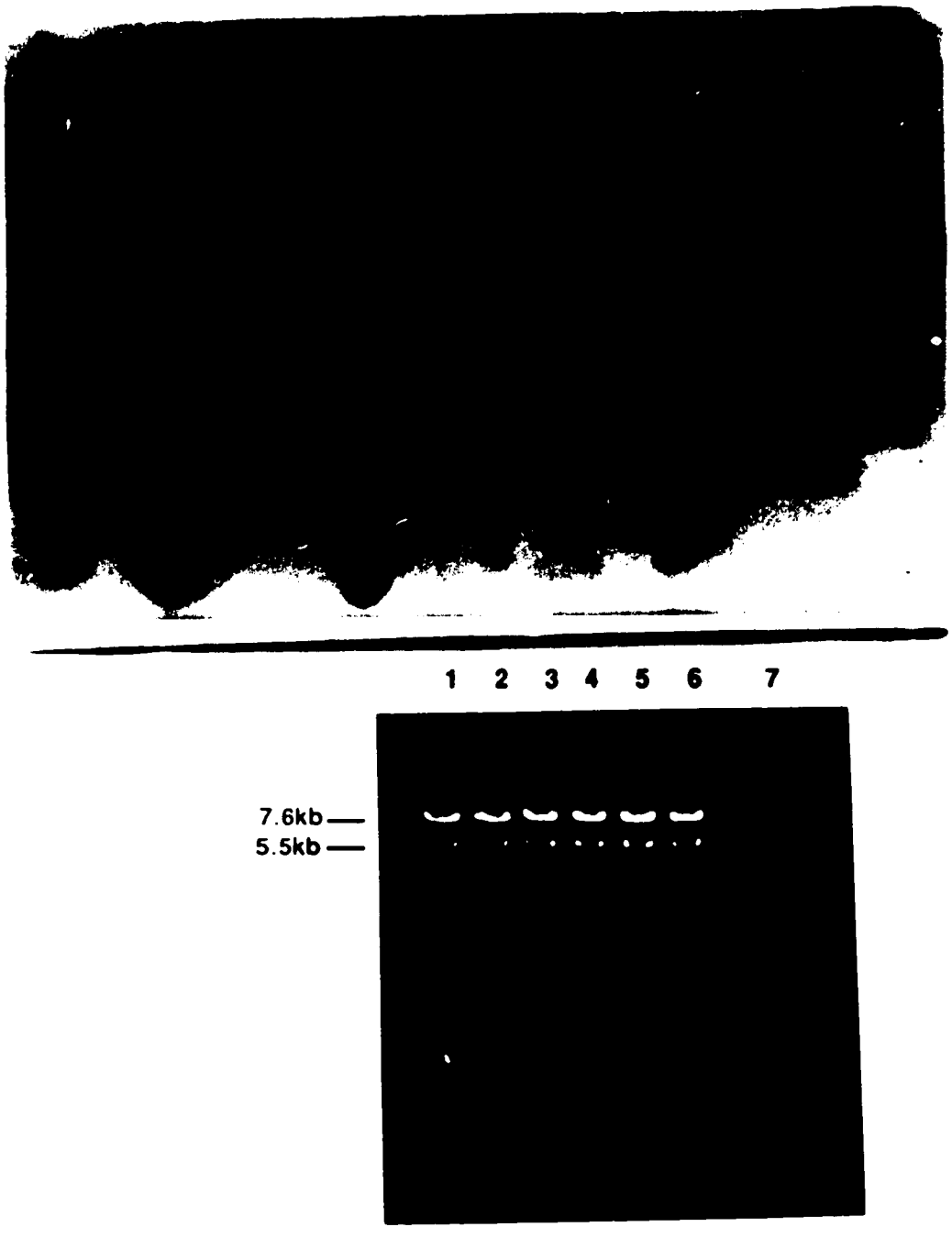

p10LAC
pZ
ApaI restriction
Ligate
Ligate
pAma
pApr
1st Generation constructs
AatII restriction
Ligate
Ligate
2nd Generation constructs
pMil
pLuz

filter colony hybridization was carried out (see Figure 6A) using the smaller (4 kb) fragment as a probe. Several hundred colonies were screened in this manner and a sample of the results is shown in Figure 6A. The fragments that make up the 13.1 kb plasmid can ligate in either of two orientations, one of which will result in a construct having a discontinuous *Ddc* fragment which must be selected against. Fully 50% of the positives isolated from filter colony hybridization will have the incorrect structure described above. To identify plasmids produced from fragments ligated in the correct orientation, PstI restriction analysis was carried out. The two possible orientations can be resolved by virtue of the fact that each will produce a unique PstI restriction pattern. This is due to the fact that the two ApaI sites are not equidistant from the 5' PstI site (see Figure 4). In the correct orientation, the fragments produced by PstI restriction are 7.6 kb (*Ddc* insert) and 5.5 kb (pHDLAC vector) in size. The incorrect orientation will result in 8.2 and 4.9 kb fragments being produced. These differences can easily be resolved on 0.75% agarose gels (data not shown). The two first generation constructs (pApr and pAma) were isolated and identified in this manner (Figure 6B).

The two desired second generation constructs (see Figure 5) were made in an analogous fashion. The starting materials were the plasmids pApr and pAma. AatII restrictions produced fragments incapable of existing as functional plasmids upon recirc[illegible]ion. Therefore, it was not necessary to screen positives by filter colony hybridization as described above. The same difficulties with orientation of ligated fragments persisted, however, but were

Figure 6A. The sample of the results of filter colony hybridization. Hybridization was carried out to screen transformants for those that contained the desired 13.1 kb plasmids. The probe used was #1 from Figure 3B. Several hundred colonies were screened per transformation and approximately 40 positives isolated. The positives were restreaked on LB plus Amp plates.

Figure 6B. Positives isolated from the screens described above were screened for correct orientation of fragments by PstI digestion analysis of DNA isolated by miniprep procedure. Samples were run on 0.75% agarose gels with approximately 1.5-2 μg/slot of DNA. Lanes 1, 2, 3, 4, 5, 6, and 7 contained DNA from pZ, p10LAC, pAma, pApr (first generation constructs), pLuz, pMil (second generation constructs) and 1 kb ladder marker DNA, respectively.


1 2 3 4 5 6 7
7.6kb
5.5kb

resolved through PstI restriction analysis as previously described. The second generation constructs pMil and pApr were isolated in this manner. The PstI restriction pattern of all four constructs as well as the starting plasmids plOLAC and pZ illustrating the correct fragment composition and orientation are shown in Figure 7.

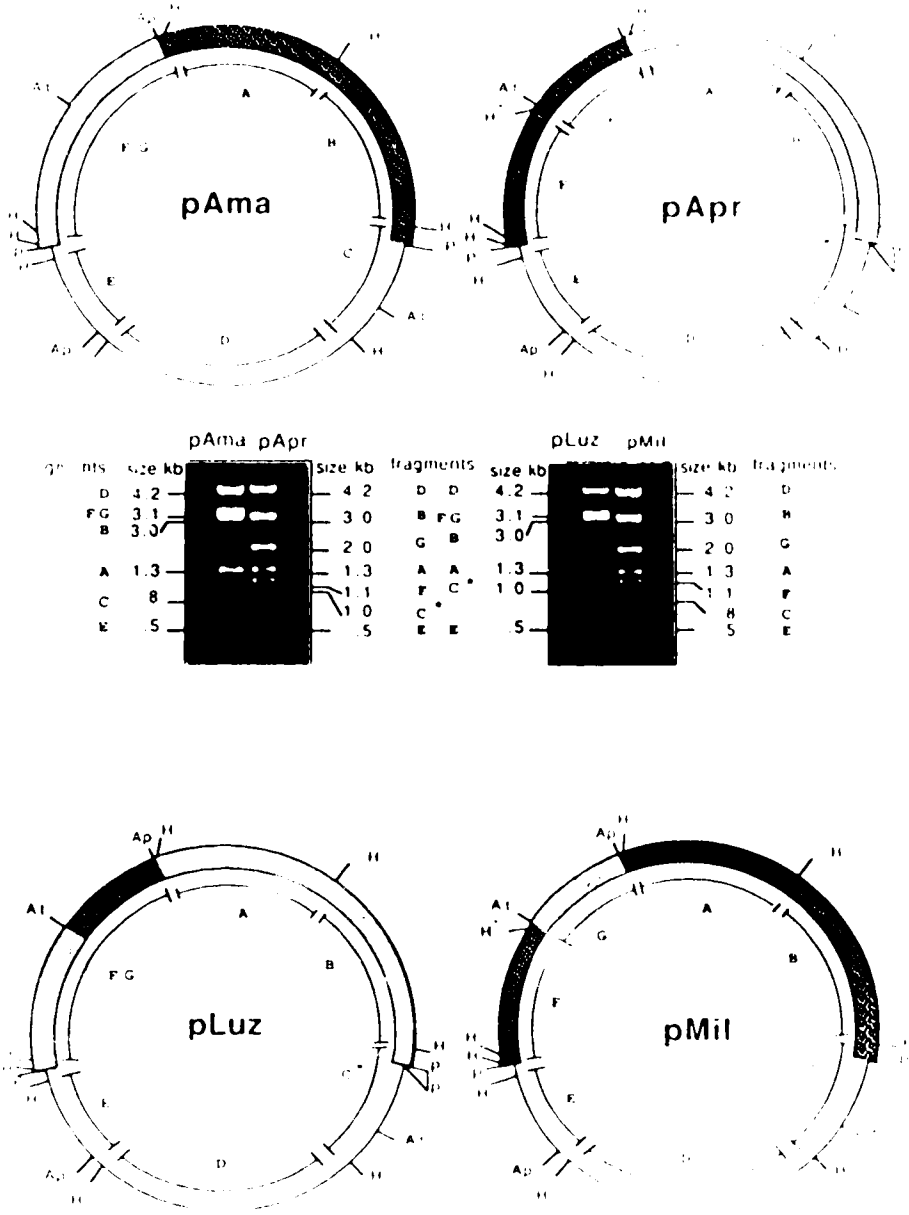
B. Confirmation of the correct structure of the four constructs

The presence of HindIII restriction fragment length polymorphisms both 5' and 3' of the AatII-ApaI interval enabled a verification of the constructs shown in Figure 5. *Ddc*$^{+4}$ sequence upstream of the AatII-ApaI interval can be identified by a 3.1 kb HindIII fragment (FG from Figure 4). The corresponding region in Canton-S is characterized by two smaller fragments (F 2.0 kb and G 1.1 kb from Figure 4). *Ddc*$^{+4}$ and C.S. sequences downstream of the AatII-ApaI interval can likewise be distinguished by the different mobilities of fragment C (see Figure 4). In *Ddc*$^{+4}$ this fragment migrates slower due to an extra 150-200 bp of sequence being present. In the first generation construct pAma, the 3.1 kb FG fragment has been "uncoupled" from the 1.0 kb C* fragment while in pApr fragments F and G are now found with the 1.0 kb C* fragment as shown in Figure 7. In the second generation constructs pLuz and pMil, the *Ddc*$^{+4}$ and Canton-S specific polymorphisms are re-coupled (see Figure 7), thus confirming the desired structures.

C. Determination of the nature and origin of the 3' polymorphism

The 3' polymorphism seen in fragment C was instrumental in confirming the structure of the hybrid constructs (Figure 7). Because this difference between *Ddc*$^{+4}$ and C.S. had not been detected prior to the subcloning of the 7.6 kb PstI fragment from the *Ddc*$^{+4}$

Figure 7. Diagrammatic representation of the first (pAma, pApr) and second (pLuz, pMil) generation hybrid *Ddc* constructs and their corresponding HindIII restriction patterns. The dark boxes represent wild type *Ddc* sequences from p10LAC and the light boxes Ddc^{+4} sequences from pZ. Lettered (A-G) segments within each diagram represent the electrophoretically visible HindIII fragments, shown on the agarose. Approximately 2.5 μg were loaded in the pAma and pApr slots and 1.5 μg in the pLuz and pMil lanes. The polymorphisms (described in Figure 4) are denoted by an asterisk (*).


pAma
pApr
pAma pApr
pLuz pMil
size kb
fragments
pLuz
pMil

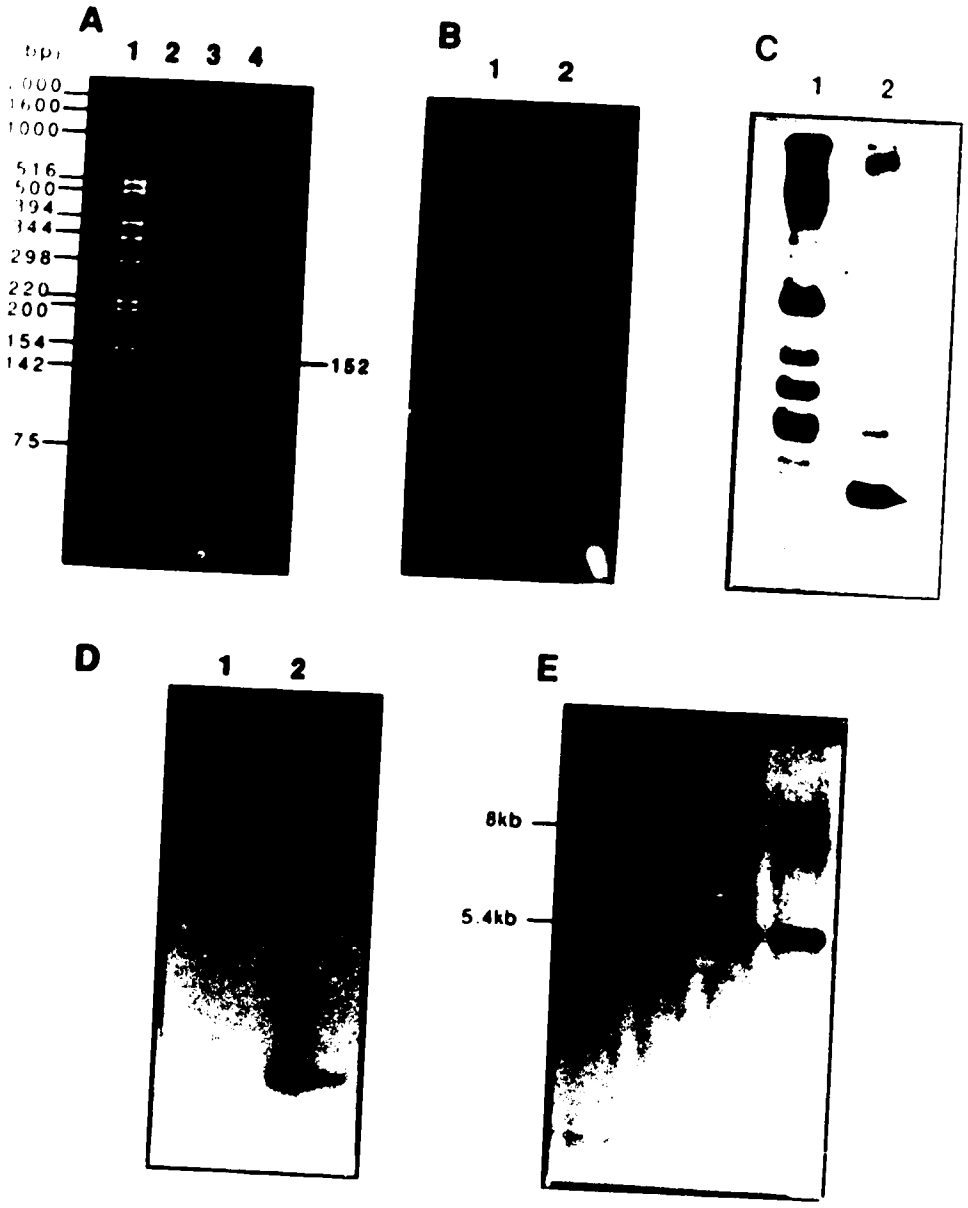
clone λ15(15), it was felt that the nature of this polymorphism should be investigated. The most likely possibility was that the 150-200 bp insertion in fragment C seen in *Ddc*$^{+4}$ was a cloning artifact, produced when the 7.6 kb PstI fragment was subcloned from λ15 into pHDLAC. The subcloning was done using a "shotgun" strategy, meaning the entire λ15 clone was restricted with PstI and all the ensuing fragments were used in the ligation with pHDLAC DNA. Thus it is possible that a small PstI fragment (150-200 bp) co-ligated with the larger 7.6 kb PstI fragment in a position between the 3' end of the larger fragment and the vector-A region spanned by fragment C (Figure 4). This possibility can be easily distinguished by PstI restriction analysis as the small 150-200 bp fragment would be liberated. Figure 8A clearly shows a 152 bp fragment is liberated upon PstI restriction of pZ, thus confirming the 3' "polymorphism" is, in fact, a cloning artifact.

It was important to determine the origin of this fragment as well because of the possibility that it might contain regulatory elements (e.g., enhancers) that might interfere with the expression of *Ddc*. This fragment had two possible origins; one is the genome of lambda and the other is the *Ddc* region included in the 20 kb Sau3A insert of λ15, excluding, of course, the 7.6 kb PstI fragment. Southern analysis was performed to resolve the two possibilities and the results are presented in Figures 8B, C, D, and E. No cross hybridization was detected between the small PstI fragment and the genome of wild type lambda. This rules out the first possibility. The second one was confirmed by hybridizing a genomic blot of BamHI restricted *Drosophila* DNA with probe #3 from Figure 3B.

Figure 8A. Restriction analysis of C* polymorphism. Lanes 2, 3, and 4 contained 2.0 μg of PstI restricted pZ, pl0LAC and pHDLAC DNA, respectively. The samples were run on an 8% polyacrylamide gel. The other expected restriction products (Figure 3,4) are too big to enter gel. Lane 1 contained 1kb ladder DNA. Size estimates were carried out as described in Figure 4 legend

Figure 8B, C, and D. Southern analyses of C* polymorphism. DNA was restricted with PstI, electrophoresed on an 8% polyacrylamide gel,and blotted onto a nitrocellulose membrane. Lane 1 contained 3.0 μg of DNA from the genome of wild type lambda and lane 2 contained 2.5μg of DNA from pZ. Blots were hybridized to three different probes. The probes were as follows:B- the entire genome of wild type lambda, C- the genome of the *Ddc*$^{+4}$ clone, lambda 15, which was the source of the 7.6kb PstI fragment cloned into pZ, and D- the 152 bp fragment identified in A.

Figure 8E. Southern analysis of *Drosophila* genomic DNA for the presence of the 152 bp PstI fragment. Each lane contained approximately the same amount (1.5-2.0 μg) of BamHI restricted genomic DNA. The probe used was #3 from Figure 3B. This fragment contained sequences from the 3' end of the *Ddc* gene as well as the 152 bp PstI fragment and about 1.5 kb of pHDLAC vector sequence. Size estimates were made as described in Figure 4 legend


A
1 2 3 4
bp
1600
1000
516
500
394
344
298
220
200
154
142
75
152
B
1 2
C
1 2
D
1 2
E
8kb
5.4kb

This probe contained sequences from the 3' end of the 7.6 kb PstI *Ddc* fragment extending into the vector and including the cloning artifact. Two hybridization signals were detected (see Figure 8E). The 8.0 kb fragment was anticipated as it extends from just within the 3' end of the 7.6 kb fragment to a BamHI site 8.0 kb downstream. This fragment would hybridize with the *Ddc* portion of the probe. The cloning artifact hybridized with a 5.4 kb BamHI fragment. A band of exactly this size extends upstream of *Ddc* to a BamHI site that lies beyond the PstI site in the unsequenced portion of the intergenic region between *Ddc* and the upstream Cc (48) gene.

D. P-element mediated germ line transformation

P-element mediated germ line transformation was carried out as described by Spradling and Rubin (55). The mating schemes used to establish transformed lines and determine the site (or sites) of insertion are outlined in detail in Figures 9 and 10, respectively. The selection for transformants was based entirely on the rescue of a lethal combination of *Ddc* alleles: Ddc^{ts2}/Ddc^{n7}. The host strain for transformation was Ddc^{ts2} cn which had been previously shown by Adam Chen (6) to be very sensitive to the manipulations required for microinjection (see Materials and Methods) resulting in very low survival rates. These were estimated to be on the order of 5-10% which is well below commonly reported rates of 25-30% (30). Several modifications were made (described in Materials and Methods) to the microinjection procedure so as to enhance survival rates. The results presented in Table 1 indicate a survival rate to hatching of approximately 25%. It is clear that difficulties with Ddc^{ts2} cn

Figure 9. Mating scheme used to generate and select for putative transformants. The host strain for transformation was ts2 cn. Embryos (1-4 hr) were microinjected (striped arrow) with plasmid constructs plus "wings clipped" helper and maintained at the permissive temperature, 20°C. Surviving G_0 adults bearing a potential *Ddc* transposon (*) were mated individually as shown and transferred to the restrictive temperature, 29°C. Straight winged individuals will be observed only if the lethal heterozygote n^7/ts2 cn(a) is rescued by a newly integrated *Ddc*$^+$ gene. Since only a few G_1's were produced per single pair mating, the curly-winged progeny (b) were also saved and allowed to self at 29°C. In this way, transformants that might otherwise have been discarded were recovered as straight winged survivors (ts2 cn/ts2 cn). These flies were further crossed to the balancer strain and the n^7/ts2 cn straight wing flies recovered. These individuals, as well as the n^7/ts2 cn G_1's (a) were further crossed as described in Figure 10.

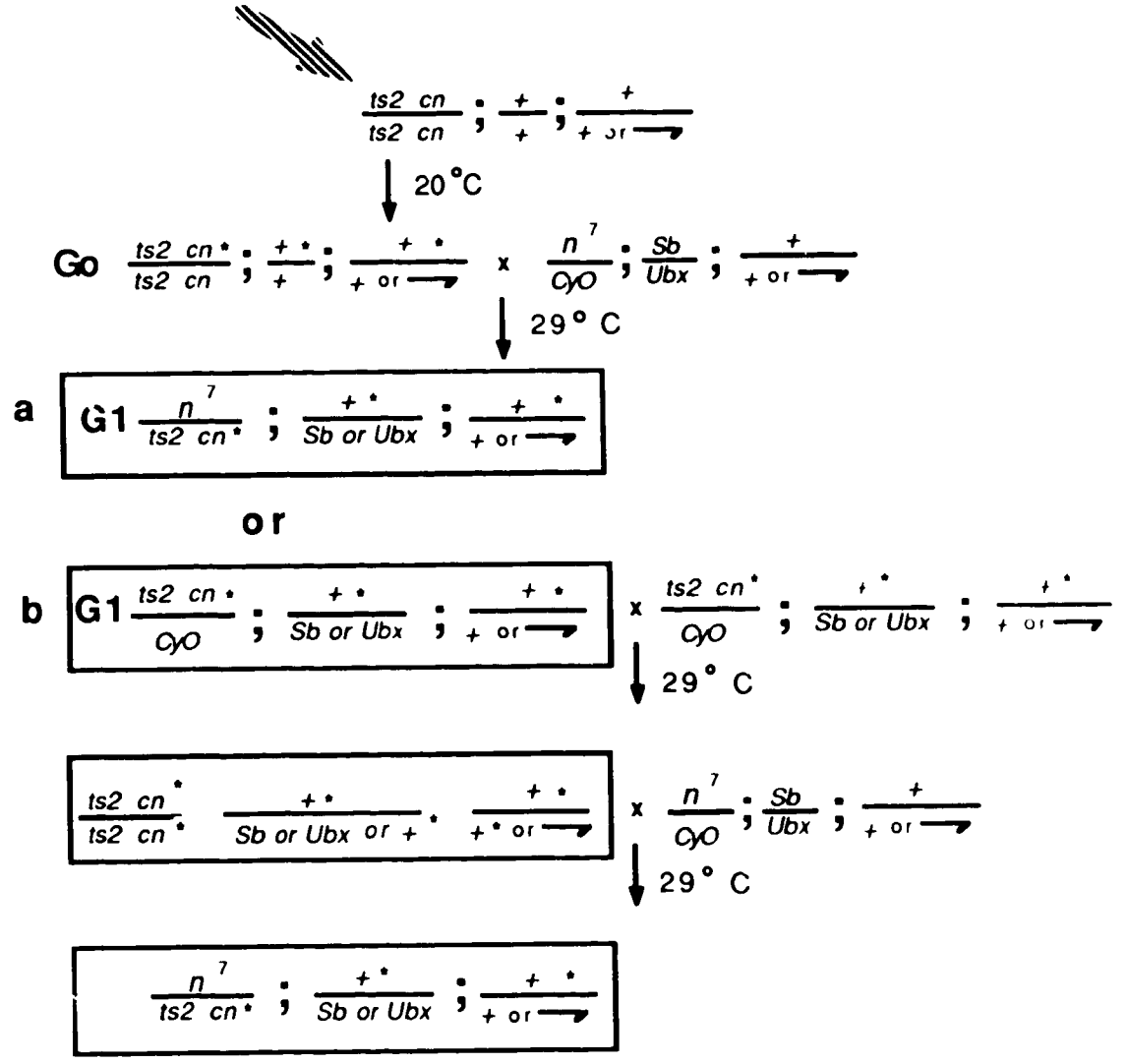

ts2 cn / ts2 cn ; + / + ; + / + or
20 °C
Go ts2 cn* / ts2 cn ; + * / + ; + * / + or x n 7 / CyO ; Sb / Ubx ; + / + or
29 ° C
a
G1 n 7 / ts2 cn* ; + * / Sb or Ubx ; + * / + or
or
b
G1 ts2 cn* / CyO ; + * / Sb or Ubx ; + * / + or x ts2 cn* / CyO ; + * / Sb or Ubx ; + * / + or
29 ° C
ts2 cn* / ts2 cn* + * / Sb or Ubx or + * + * / + * or x n 7 / CyO ; Sb / Ubx ; + / + or
29 ° C
n 7 / ts2 cn* ; + * / Sb or Ubx ; + * / + or

Figure 10. Genetic determination of chromosomal location of P-element insertion. In order to determine into which chromosome(s) the P-element had integrated, individual G1's were crossed back to the balancer and the ensuing progeny analysed. Three distinct possibilities prevail. If no n^7/n^7 progeny are observed then the insert must be on the second chromosome (ts2 cn*). If n^7/n^7 progeny are observed, then inserts are either on the third chromosome or on the X. These two possibilities can be resolved by virtue of the fact that two third chromosome balancers are present. If no n^7/n^7;Sb/Ubx individuals are observed, then the P-element must be on the third chromosome. If such flies are present, the insert must be on the X. Note that crosses involving G_1 males would result in no male progeny if P-element is on the X. n^7 can be distinguished from ts2 cn by virtue of the markers outlined in the stock listing of the Materials & Methods.

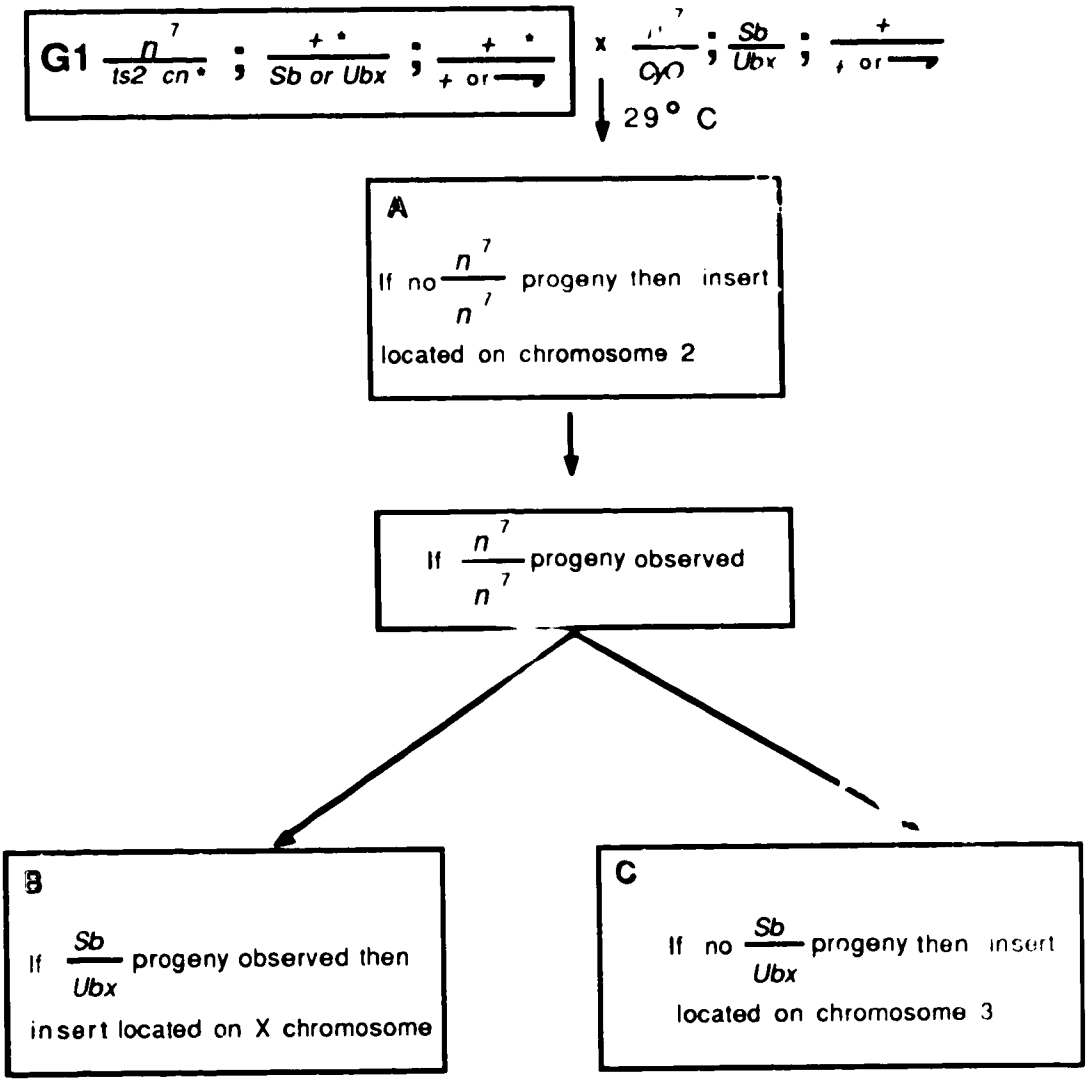

G1 n7 / ts2 cn* ; + * / Sb or Ubx ; + * / + or
x
Sb / Ubx ; + / + or
29° C
A
If no n7 / n7 progeny then insert
located on chromosome 2
If n7 / n7 progeny observed
B
If Sb / Ubx progeny observed then
insert located on X chromosome
C
If no Sb / Ubx progeny then insert
located on chromosome 3

Table 1 Results of microinjections

Plasmid	# Embryos Injected *	# Larvae Hatched	# Adults Eclosed	# Fertile Adults	# Transformants
pLuz	2564	615	498	286	1
pMil	2372	569	401	220	0

* 30% of injected embryos were chemically dechorionated the remainder were mechanically dechorionated (as described in Materials & Methods)

survival can be overcome, since both survival to adulthood and adult fertility rates were comparable to what has been observed elsewhere (30).

Transformation efficiencies are typically observed to be 1-3% (30) but are frequently lower (30). The transformation efficiency obtained here was two orders of magnitude lower than the reported efficiencies. With only one transformed line generated (see Table 1), the efficiency was estimated to be 0.02%. Possible explanations for this extremely low efficiency are presented in the discussion. The transformed line, designated TL-1, carried a pLuz hybrid *Ddc* P-element insert. The site of insertion was genetically determined to be on chromosome 2 by using the mating scheme outlined in Figure 10. This assignment was made since straight-winged progeny homozygous for the multiply marked n^7 chromosome could not be recovered (see Stock Listing in Materials and Methods for summary of second chromosome markers). Not shown in Table 1 are four instar where putative G_1 transformants were recovered but failed to produce an n^7/ts2 cn G_2 generation. These were classified as "escapers" of the Ddc^{ts2} temperature sensitive lethality.

E. Southern analysis of transformed line TL-1

The copy number of inserted transposon in TL-1 was determined by genomic Southern hybridization. Genomic DNA was isolated from TL-1 as well as from the host strain ts2 cn and the n^7/CyO;Sb/Ubx balancer strain. The analysis was carried out using four different restriction enzymes (EcoRI, ApaI, BamHI and SalI) and the results are presented in Figures 11 and 12. The autoradiographs were probed with fragment #5 from Figure 3B. This fragment, produced by HindIII

Figure 11. Southern Analysis of transformed Line TL-1, Host Strain ts2 cn, and balancer strain n^7/Cyo;Sb/Ubx. High molecular weight genomic DNA was restricted with EcoRI, electrophoresed on .75% agarose gel and blotted onto a nitrocellulose membrane. The blot was hybridized with 32P oligo-labelled probe #5 (from Figure 3B) and autoradiographed. Slots 1, 2, 3 and 4 contain the ts2 cn (5 μg), n^7/Cyo;Sb/Ubx (5 μg),TL-1 (2 μg), and TL-1 (4 μg) DNA, respectively. Molecular weight determinations were made as described in legend of Figure 4.

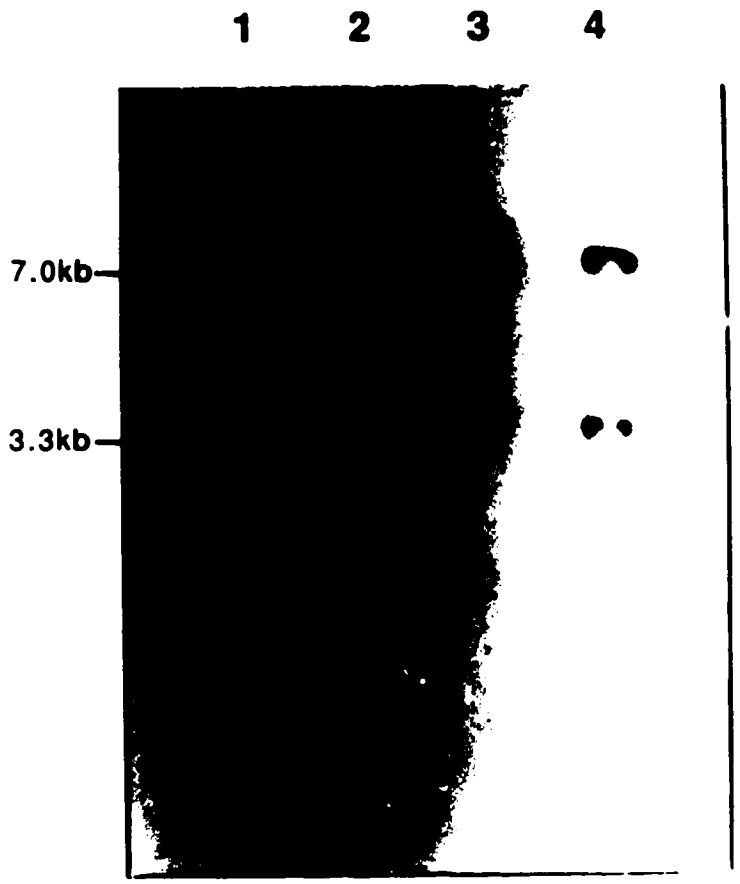
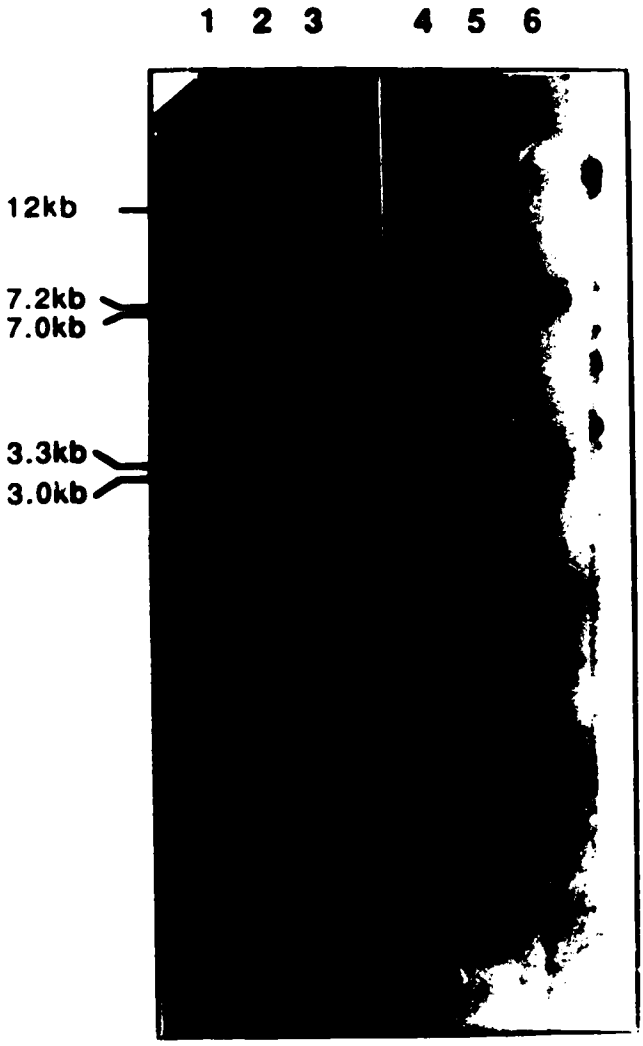

1
2
3
4
7.0kb
3.3kb

Figure 12. Southern analyses of transformed line TL-1 and host strain ts2 cn. Restricted high molecular weight DNA was electrophoresed on .75% agarose gel and blotted onto a nitrocellulose membrane. The blot was hybridized with ^{32}P oligo-labelled probe #1 (from Figure 3B) and autoradiographed. Lanes 1 and 4 contained DNA restricted with ApaI, 2 and 5 with BamHI, and 3 and 6 with SalI. Lanes 1, 2, and 3 contained DNA from ts2 cn (5 μg) and 4, 5, and 6 from TL-1 (2 μg). Molecular weight determinations were made as previously described (Figure 4 legend).


1 2 3 4 5 6
12kb
7.2kb
7.0kb
3.3kb
3.0kb

restriction, extends 1.1 kb upstream of the HindIII site at -768, and thus will hybridize to fragments of variable length depending on the distance between the EcoRI site in the transposon and the nearest EcoRI site in the flanking region. In all likelihood, this distance would be different from that of the endogenous *Ddc* gene. Thus, inserts can be clearly distinguished from the endogenous *Ddc* gene as well as each other.

Hybridization with EcoRI restricted samples from TL-1 (Figure 11) show a unique 7.0 kb fragment that was present in the control lanes as well. This band represents the endogenous *Ddc* gene located at 37 C1-2 of 2L. The extra band (3.3 kb) seen in the TL-1 lanes of the autoradiograph in Figure 11 represents an integrated *Ddc* gene. Only one such additional band was observed indicating the *Ddc* insert must be single copy. The same type of analysis was carried out with the results shown in Figure 12. It was initially perplexing to note that all four of the enzymes used in this particular analysis produced additional bands corresponding to a *Ddc* insert, that were of similar size (3.0 to 3.3 kb). In all cases, however, the insert was confirmed to be present in single copy.

F. *In situ* analysis of transformed line TL-1

In situ analyses was performed on TL-1 so as to identify the precise cytological location of the *Ddc* insert. The probe used for this was the entire pl01AC plasmid which contained *Ddc*, P-element, and white sequences. Three hybridization sites were anticipated; these were 37C1-2 (the endogenous *Ddc* locus), a second one at either of the second chromosome (*Ddc* insert), and a third at [illegible] (the X chromosome white locus). Two unexpected

observations were made (see Figure 13, A, B, and C). Firstly, no additional hybridization site was detected on the second chromosome as was expected from the genetic data. Secondly, an additional hybridization signal at 5 EF was detected. We believe the additional hybridization signal on the X chromosome is due to a contaminating P-element of unknown origin. We felt that this P-element should be investigated further as it may have contributed to the low frequency of transformation observed, particularly if its origin was the ts2 cn host strain.

G. Southern analysis of TL-1 for additional P-element sequences

Southern hybridization was carried out in order to confirm the presence of additional P-element sequences in TL-1 and also to determine the origin of such sequences. DNA samples from TL-1, ts2 cn, and n^7/Cyo;Sb/Ubx were restricted, electrophoresed on .75% agarose and blotted. The probe used was a 1.0 kb HindIII fragment from the 5' end of an intact P-element excluding the 31 bp inverted repeat. The autoradiograph is presented in Figure 14. The results show no P-elements were present in ts2 cn, as anticipated. Unexpected, though, a 2.5 kb band was observed in the n^7/CyO;Sb/Ubx lane. This same band was seen in TL-1 along with a 6.1 kb band which we ascribe to partial digestion of the TL-1 DNA samples. Based on these observations, we concluded that the 2.5 kb band must represent the contaminating P-element, and its origin was the n^7/CyO;Sb/Ubx balancer stock to which all Go survivors were crossed (see Figure 9). The absence of an additional band corresponding to the 5' P-element portion of the *Ddc* insert in TL-1 was puzzling. Given the apparent insertion of the *Ddc* transposon into the

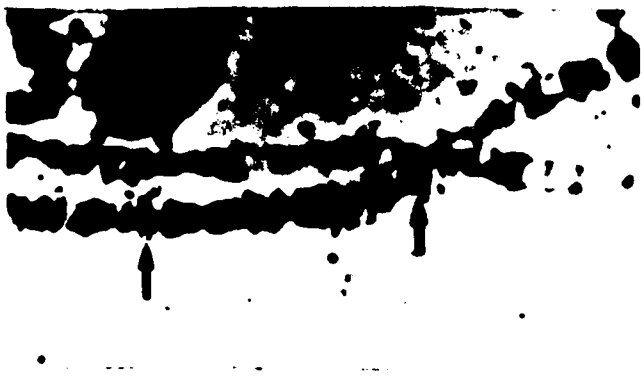
Figure 13. *In situ* analysis of transformed Line TL-1. *In situ* analysis, autoradiography and photography were performed as described in Materials and Methods. Chromosomes were hybridized with nick translated ^{3}H p10LAC (see Figure 4). Arrows indicate areas of hybridization.

A - Intense hybridization over 37 C 1-2 on chromosome 2L, the endogenous location of *Ddc*.

B - Hybridization over 3 C-3 (white gene) and 5 EF of the X chromosome.

C - Hybridization over 3 C-3 (white gene) and 5 EF of the X chromosome with tip clearly visible.

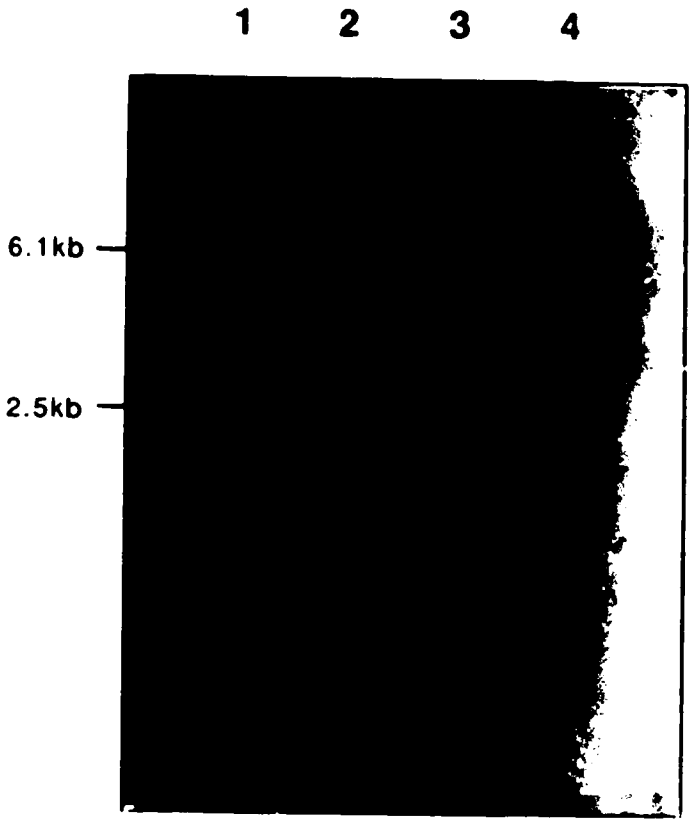
A



B

C

Figure 14. Southern analysis of transformed line TL-1, host strain ts2 cn and balancer stock n^7/CyO;Sb/Ubx for the presence of P-elements. Genomic DNA was restricted with EcoRI and electrophoresed on a .75% agarose gel and blotted onto a nitrocellulose membrane. The probe used was a 1.0 kb HindIII fragment from the 5' end of the P-element contained within π25.7 W.C. excluding the 31 bp repeat. This fragment was ^{32}P oligo-labelled and hybridized to the DNA on the membrane. Lanes 1, 2, 3 and 4 contained ts2 cn, n^7/cyO;Sb/Ubx, TL-1 (4 μg), and TL-1 (2 μg) DNA, respectively. Size estimates were carried out as previously described (Figure 4 legend).


1
2
3
4
6.1kb
2.5kb

endogenous gene, it is possible that a mechanism other than P-element transposition was responsible for insertion. Such a mechanism may have resulted in the loss of one or both P-element ends and, thus, explain the failure to detect them by Southern analysi

H. Analysis of TL-1 and n^7/Cyo;Sb/Ubx for P-element repressor activi'

Assays were carried out to determine if the P-element was producing repressor in the transformed line TL-1 and the balancer stock n^7/Cyo;Sb/Ubx. A standard gonadal dysgenesis (GD) or GD sterility test was used to measure repressor activity. The basis for this test is the fact that any repressor activity will be detected as a decrease in the occurrence of GD sterility (determined by the presence or absence of ovaries) below 100% when females of the strain to be tested are crossed to known P males (π2P). The results of this experiment are presented in Table 2. The control crosses involving known P males crossed to known M females produced dysgenic offspring without exception. That is, no ovaries were detected in any of the offspring from these crosses, and this is typically the case (J. Williams, pers. comm.). Repressor activity was detected in both TL-1 and n^7/CyO;Sb/Ubx albeit at much different levels. Why the two should differ is not known. The repressor activity in the strain n^7/CyO;Sb/Ubx is estimated at 65% which can be classified as moderate to strong (37) whereas in TL-1 it was weak (5%).

Table2 P-element repressor assay

Crosses ♂ x ♀		# Individuals scored	% Gonadal dysgenesis
♂ 2P (P-strain)	Oregon-R (M-strain)	61	100
	$\frac{n^7}{Cyo}; \frac{Sb}{Ubx}$ (Unknown)	44	35 *
	TL-1 (Unknown)	64	95 *
Oregon-R (M-strain)	♂ 2P (P-strain)	48	0
$\frac{n^7}{Cyo}; \frac{Sb}{Ubx}$ (Unknown)		52	0
TL-1 (Unknown)		38	0

* Individuals with remnant ovaries having one or two eggs were observed and scored as dysgenic. Such individuals were not observed in control cross.

I. DDC enzyme and protein assays of transformed line TL-1

To determine the *Ddc* phenotype produced by the integrated transposon, pluz, DDC specific activity of TL-1 was assayed. White prepupae and young adults (0-12 hours) were collected and the DDC activity and protein content were determined as described in Materials and Methods. Corrected DDC specific activity was calculated and the results are presented in Table 3. DDC activity was estimated to be 70-77% that of the wild type control (Canton-S) at pupariation and adult eclosion. The phenotype of TL-1, which is determined by the calculation of "R-values" (described in Introduction), was estimated to be 1.34. The wild type Canton-S value of 1.48 agrees well with what our laboratory has reported elsewhere(6,7).

Table 3 DDC activity of TL-1 and C.S.

Strains	DDC activity (CPM/ug protein)*		Ratio of Pupariation/Eclosion (R-value)
	Pupariation	Eclosion	
TL-1	1139 +/- 29	850 +/- 23	1.34 +/- .29
Canton-S	1631 +/- 16	1103 +/- 33	1.48 +/- .24

* Average of 4 determinations from one +/- standard deviation

Standard deviation for ratios was calculated using the following equation:

$$SD = \sqrt{\frac{\sum (Yi - RXi)^2}{n(n-1)}} \qquad \frac{\bar{Y}}{\bar{X}} = R$$

DISCUSSION

A. Location and structure of pLuz insert

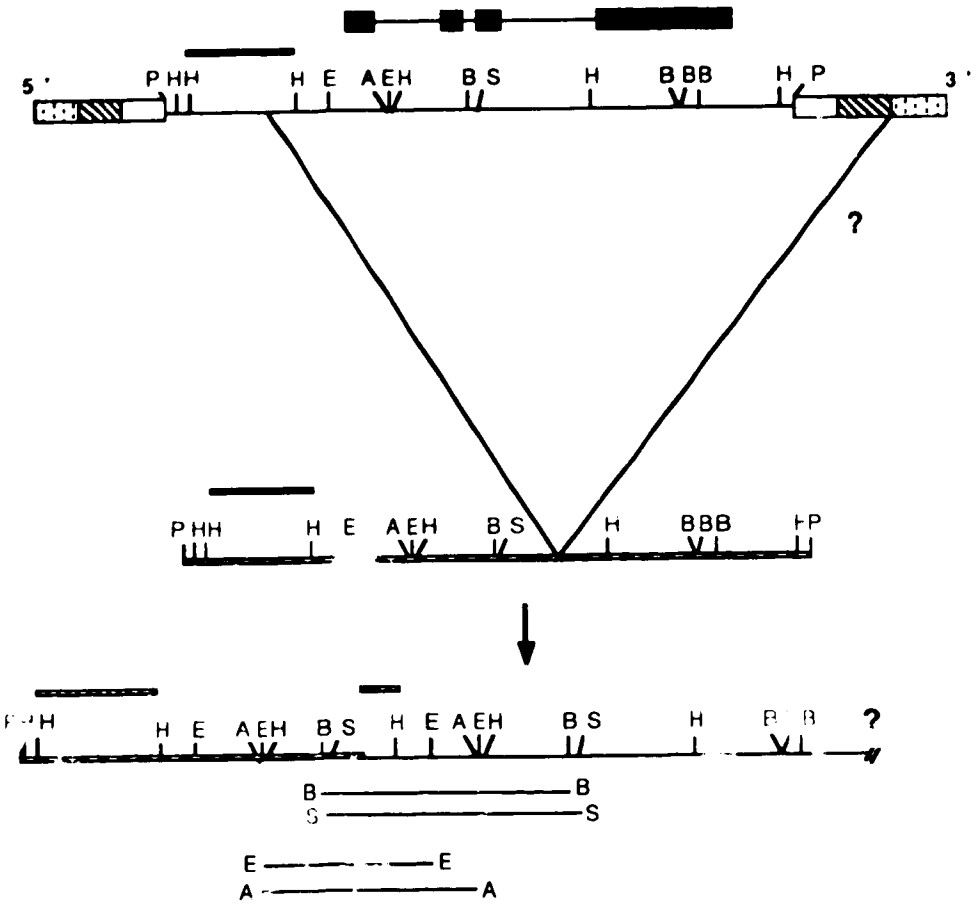
In an attempt to locate the site of pLuz insertion, two different approaches were employed. An assignment to the second chromosome was made on the basis of genetic data as outlined in Figure 10. This conclusion was reached on the basis of my inability to generate n^7/n^7 homozygotes from the appropriate cross (see Figure 10). The only other explanation for the absence of n^7/n^7 homozygotes would be if recessive lethals other than Ddc^{n7} were present on the n^7 chromosome. Recessive lethal assays on n^7/CyO;Sb/Ubx were carried out on several occasions by establishing homozygotes for the n^7 chromosome in the presence of a Ddc^+ transposon on the 3rd chromosome and no such lethals were found. On the basis of the genetic data, we fully expected to see two sites of hybridization of a *Ddc* gene probe on the chromosome second, since the endogenous gene lies on 2L. However, no hybridization signal other than at the endogenous *Ddc* locus (37 C1-2) was detected in the transformed line TL-1. We considered three possible explanations for this observation. The first was that the transposon inserted into a heterochromatic region of the second chromosome. *In situ* hybridizations to heterochromatic DNA are known to be very poor (diffuse grains) and thus difficult to detect (B. Wakamoto, personal communication). This possibility was ruled out on the basis of the fact that the insert was producing DDC activity at reasonably high levels (see Table 3) which is not characteristic of genes located in heterochromatin. The second possibility was that the P-element transposed (in a normal fashion) into the endogenous *Ddc* locus.

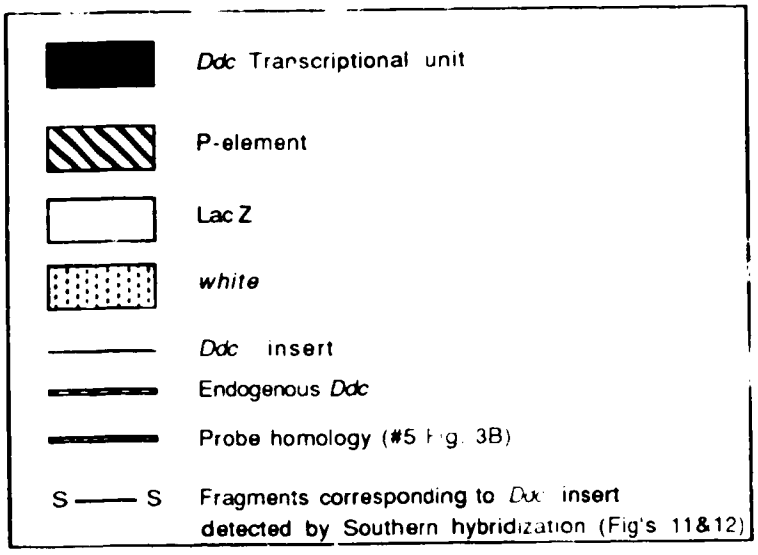
This is highly unlikely since primary P-element insertions are relatively random in terms of location. The probability of a random P-element insertion into *Ddc* is very low; none have been detected among the 20 or so *Ddc* transformants generated so far (6, 7, 52, 54) The third possibility, and the one currently favoured, is that the pLuz transposon p[*Ddc*^{Luz}] inserted into the endogenous *Ddc* via a recombinational event akin to standard homologous recombination. This phenomenon is not typically observed in *Drosophila*. However, recent instances have been reported (23) in which engrailed transposons were found to insert into the engrailed region at high frequency. Several of the inserts, scattered throughout this region, had integrated into the coding portions of the *engrailed* and *invected* genes, thus disrupting them. In an attempt to obtain evidence supporting this explanation, an *in situ* hybridization with a P-element probe was carried out. However, the experiment was unsuccessful as even the site on the X chromosome (the contaminating P-element) was not visualized. While this experiment is worth repeating, it should be noted that this approach may not be useful The possibility exists, given the uncertainty concerning the mechanism of insertion, that no P-element sequences are present at 37 C1-2 - the postulated site of insertion (discussed below). This type of analysis should also be carried out using a pure *Ddc* probe so as to rule out the possibility that the signal observed on the X is due to a *Ddc* insert.

The Southern analyses proved to be more useful in unravelling the sequence of events which produced the TL-1 strain. Initially, we were puzzled by the fact that the fragments corresponding to the

p[*Ddc*^{Luz}] insert were similar in size (3.0-3.3 kb), irrespective of which of the four restriction enzymes (ApaI, EcoRI, SalI and BamHI) was used (see Figures 11 and 12). The size of these fragments would normally be determined by the distance between the transposon end and the nearest site in the flanking region and would be expected to vary. Furthermore, the observed sizes (3.0-3.3 kb) are smaller than the minimum lengths of 3.8 to 5.0 kb which are known to span the distance between the particular site in the *Ddc* region and the 5' boundary of the P element. This result suggests that a portion of the 5' end of the transposon is missing - a fact that is supported by the results of the P-element Southern shown in Figure 14. Two hybridization signals were anticipated. However, only the one corresponding to the contaminating P-element on the X chromosome was detected in the TL-1 lanes. The absence of an additional band indicates a substantial portion, if not all of the 5' P-element end of the transposon, has been deleted. A possible explanation for these observations is illustrated in Figure 15. We postulate that the transposon (TOP) was inserted into the endogenous *Ddc*^{ts2} gene, downstream of the SalI site. The insertion event resulted in the deletion of a part of the 5' end of the transposon, including the P-element sequence. By varying the exact site of insertion and the extent of the deletion, it is possible to account for the observed sizes of the transposon bands. Quite coincidentally, the four enzymes produce nearly identical fragments as shown in the figure. It is not known if the 3' end of the transposon was similarly deleted. Clearly, genomic Southern analysis of the 3' end will have to be carried out. It is important to note that the bands observed

Figure 15: Postulated location and structure of pLuz insert with the question marks denoting the uncertainty as to the structure of the 3' end of the pLuz insert. Note that the distance from the insertion point to the SalI site, as well as the extent of the 5' end truncation, are also approximations. The magnitude of these were chosen with a view to accommodate the estimated sizes of the bands in Figures 11 and 12 that corresponded to the *Ddc* insert.


5'
P H H
H E A E H
B S
H
B B B
H P
3'
?
P H H
H E
A E H
B S
H
B B B
H P
H E A E H
B S
H
?
B
B
S
S
E
E
A
A


Ddc Transcriptional unit
P-element
Lac Z
white
Ddc insert
Endogenous *Ddc*
Probe homology (#5 Fig. 3B)
S —— S
Fragments corresponding to *Ddc* insert detected by Southern hybridization (Fig's 11&12)

in Figures 11 and 12 that correspond to the endogenous *Ddc* gene are unaffected as they lie upstream of the insertional event. As predicted, these bands exhibit the same size in TL-1, *ts2 cn*, and n^7/CyO;Sb/Ubx (Figures 11 and 12).

B. Analysis of TL-1 for DDC activity

Transformed TL-1 homozygotes were established and *Ddc* enzyme activity measurements were made. The results are presented in Table 3. This strain contained two copies of the pLuz transposon. The endogenous Ddc^{ts2} genes derived from the host strain are believed to be non-functional as a result of the insertion of p[Ddc^{luz}] (See Figure 15). Therefore, we assume that the Ddc^{ts2} genes did not contribute to the activity level measured in TL-1. It is reassuring to note that DDC activity levels in TL-1 are nearly normal, as would be expected if the site of insertion is in the *Ddc* region. It is well documented that *Ddc* transposons in other locations can be subject to position effects which significantly reduce *Ddc* expression (6). The ratio of DDC activity at pupariation to eclosion was determined for TL-1 and Canton-S (wild type) since these "R-values" have proven to be useful parameters to distinguish the enzyme phenotype of Ddc^{+4} from that of Canton-S (10). Quite clearly, TL-1 exhibits an "R-value" indistinguishable from that of Canton-S (C.S.). The *Ddc* region in pLuz contained Ddc^{+4} sequence throughout except for the AatII-ApaI interval which was wild type (see Figure 7). Therefore, I conclude that this interval contains all the significant regulatory differences between C.S. and Ddc^{+4}. Unfortunately, this conclusion could not be confirmed since transformed lines of the reciprocal product, pMil (Figure 7) were

not obtained. However, we expect that lines transformed with pMil will exhibit "R-values" of 0.4 to 0.8, characteristic of *Ddc*[14]. This interpretation must be tempered for two reasons. One is the fact that the 5' end of the transposon including upstream portions of the 7.6 kb PstI fragment appears to have been deleted. It is possible that as yet unidentified regulatory elements may have been removed. This deletion cannot extend beyond (3' direction) the HindIII site at -768 since that would abolish hybridization with the 5' end probe (Figure 15). No regulatory elements (putative or otherwise) have been detected upstream of -229. Therefore, I feel it is unlikely that the deletion affected the expression of p[*Ddc*^{Luz}]. The second potential problem is that the expression of the *Ddc* insert may have been influenced by the regulatory sequences of the endogenous *Ddc*^{ts2} which are clearly unaffected by the insertional event (see Figure 15). The endogenous regulatory elements are estimated to be 3 to 3.5 kb away from the transcriptional start site of the *Ddc* insert. Since powerful regulatory elements of the kind required to influence gene expression over large distances (1.0 kb plus) have not been detected in *Ddc*, I feel it is unlikely that the expression of p[Ddc^{luz}] was significantly altered by endogenous elements.

C. A model for the absence of P-element mediated transformants

It is apparent that the transformed line TL-1 was not obtained by P-element mediated germ line transformation but rather by a rare "site directed" insertional event. In fact, no P-element mediated transformants were generated although on the order of 30-50 might have been expected from the roughly 5,000 embryos that were injected

(30). Based on the data accumulated on TL-1, I have developed a satisfactory model outlining a series of events which could have led to its establishment. The model is predicated on the presence of the contaminating P-element found on the X chromosome of the n^7/Cyo;Sb/Ubx balancer. As shown in Figures 9 and 10, the selection scheme for the isolation of putative transformants was based on the "rescue" of lethal *Ddc* alleles present in both the host and background strains. P-element inserts that fail to express DDC activity would, thus, not be detected. I propose that the contaminating P element produces repressor which is interferring with the expression of P-element borne *Ddc* inserts, resulting in a failure to detect them using our selection system. This is akin to the cytotype dependent suppression of P-element induced mutations described for certain mutants of the vestigial (*vg*) locus (58). These are suppressed when stabilized in a P-cytotype. Both transposase and repressor activity are detectable in P-cytotype strains. It is unlikely, however, that transposase, which is germ line specific (58), mediates the effects observed with *vg* (58) and *Ddc*, as these genes are somatically expressed. The repressor, which is expressed in germ line and somatic tissue, interacts with P-element ends (its "natural" substrates) in such a way as to interfere with the expression of the interjacent *vg* and *Ddc* genes. It is interesting to note that repressor mediated expression interference involves two sharply contrasting effects. Suppression of *vg* mutant alleles likely involves an <u>increase</u> in *vg* transcription (58), whereas the effect we suggest here involves a <u>decrease</u> in gene expression from p[Ddc^{Luz}] inserts to a level insufficient to sustain variability in

the absence of endogenous gene expression. A key component of the model we propose here is why and how was p[Ddc^{Luz}] able to "escape" this repressor interference. Once again, observations with the cytotype sensitive *vg* mutant alleles are helpful. It was clearly demonstrated that <u>both</u> P-element ends are absolutely required for the repressor mediated suppression of *vg* mutants (58). We believe that p[Ddc^{Luz}] was immune to this because at least one P-element end was missing (see Figure 15). The absence of P-element mediated germ line transformants, then, was not due to a failure in the molecular aspects of the transpositional event, but rather due to the inability to detect these events once they occurred.

Many aspects of the model are easily testable and some preliminary experiments have been undertaken already. Since the presence of the repressor molecule is an essential feature of this model, P-element repressor assays were carried out on TL-1 and n^{1}/CyO;Sb/Ubx. As indicated in Table 2, there is repressor activity associated with the P-element bearing chromosome present in TL-1 and n^{1}/CyO;Sb/Ubx. Significant amounts of repressor activity were discovered in both strains albeit at different levels. We have no satisfactory explanation as to why different repressor levels were detected in TL-1 and n^{1}/CyO;Sb/Ubx.

We have envisaged several experiments designed to test the effect of repressor on the expression of *Ddc* transposons. We are, at present, attempting to introduce the P-element bearing X chromosome into previously established *Ddc* transformants in an attempt to see whether DDC enzyme levels are reduced as would be predicted by the model. To test whether the model is applicable to

repressor molecules in general, or is specific to the repressor produced by the contaminating P-element, a variation of the above-described experiment is being undertaken. This experiment involves introducing a chromosome carrying P-element borne *Ddc* inserts into a known P-cytotype background and again assaying for DDC activity. Further experiments include a molecular characterization of the pLuz insert to determine its structure as well as a precise site of insertion. This could be done by genomic southern mapping of the *Ddc* region in TL-1 or by cloning the pLuz transposon entirely and restriction mapping and/or sequencing it. Alternatively, polymerase chain reaction (pcr)(44) experiments can be utilized to map the exact breakpoints caused by the insertional event. Similar kinds of studies should also be undertaken with the contaminating P-element as it may have unique properties useful in elucidating the mechanisms of cytotype establishment. If as proposed, *Ddc* bearing P-element transposons are reactive to repressor molecules, this may provide a very sensitive assay for repressor activity. A second chromosome with three *Ddc* P-element inserts on it exists with each insert producing normal levels of enzyme. It will be possible to monitor repressor levels by noting the decrease in DDC activity caused by the negative interference on p[*Ddc*$^+$] expression. This can be done without fear of causing lethality as the endogenous gene present on this chromosome should be unaffected. A quantitative assay of this accuracy could be used to select for mutants that overproduce repressor at high levels thus facilitating the isolation and characterization of these molecules.

BIBLIOGRAPHY

1. Beall, C.J. and J. Hirsh [illegible]. High levels of intron containing RNA's are associated with expression of the *Drosophila dopa decarboxylase* gene. Mol. Cell. Biol. 4: 1669-1674

2. Bingham, P.M., M.G. Kidwell and G.M. Rubin, 1982. The molecular basis of P-M hybrid dysgenesis: the role of the P-element, a P-strain specific transposon family. Cell *29*: 995-1004.

3. Birnboim, H.C. and J. Doly, 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. *7*: 1513-1523.

4. Bradford, M., 1976. A rapid and sensitive method for quantification of microgram amounts of protein utilizing the principle of protein-dye binding. Anal. Biochem. *72*: 248-252.

5. Clark, W.C., J. Doctor, J.W. Fristrom and R.B. Hodgetts, 1986. Dopa decarboxylase from *Drosophila melanogaster*. Mol. Gen. Genet. *162*: 287-297.

6. Chen, Zhi-Qing, 1986. A functional study of a variant dopa decarboxylase gene of *Drosophila melanogaster*, using DNA-mediated germ line transformation. M.Sc. Thesis, University of Alberta, Edmonton, Alberta.

7. Chen, Zhi-Qing, and Hodgetts, R. 1987. Functional analysis of a naturally occurring variant dopa decarboxylase gene in *Drosophila melanogaster* using Pelement mediated germ line transformation. Mol. Gen. Genet. (1987) 207:441-445

8. Clark, W.C., J. Doctor, J. Fristrom and R. Hodgetts, 1986. Differential responses of the dopa decarboxylase gene to 20-OH-ecdysone in *Drosophila melanogaster*. Dev. Biol. *114*: 141-150.

9. Davis, R.W., D. Botstein and J.R. Roth, 1980. Advanced bacterial genetics (Cold Spring Harbor Laboratory). New York.

10. Dewhurst, S.A., S.G. Croker, K. Ikeda and R.E. McCaman, 1972. Metabolism of biogenic amines in *Drosophila* nervous tissue. Comp Biochem Physiol. *43*: 975-981.

11. Dickinson, W.J. and D.T. Sullivan, 1975. Enzymes of tyrosine metabolism. From "Results and problems in cell differentiation series" 6: 97-113. (Spring-Verlag, New York, Heidelberg,

12. Engels, W.R., 1979. Hybrid dysgenesis in *Drosophila melanogaster*: rules of inheritance of female sterility.

Genet. Res. *33*: 219-236.

13. Estelle, M.A. and R.B. Hodgetts, 1984a. Genetic elements near the structural gene modulate the level of dopa decarboxylase during *Drosophila* development. Mol. Gen. Genet. 434-441.

14. Estelle, M.A. and R.B. Hodgetts, 1984b. Insertion polymorphisms may cause stage specific variation in mRNA levels for dopa decarboxylase in *Drosophila*. Mol. Gen. Genet. *195*: 442-451.

15. Estelle, M., 1983. An analysis of a dopa decarboxylase activity variant in *Drosophila melanogaster*. Ph.D. Thesis, University of Alberta, Edmonton, Alberta.

16. Eveleth,D.D.,Gietz,R.D.,Spencer,C.A.,Nargang,F.E.,Hodgetts,R.B., Marsh,J.L., 1986. Sequence and structure of the dopa decarboxylase gene of *Drosophila*: evidence for novel RNA splicing variants. EMBO J. 5: 2663-2672.

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

18. Feinberg, A.P. and B. Vogelstein, 1984. Addendum of "A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity." Anal. Biochem. *137*: 266-267.

19. Gehring, W.J., R. Klemenz, U. Weber, U. Kloter, 1984. Functional analysis of the *white*$^{+}$ gene of *Drosophila* by P-factor-mediated transformation. EMBO J. 3: 2077-2085.

20. Gietz, R.D. and R.B. Hodgetts, 1985. An analysis of dopa decarboxylase expression during embryogenesis in *Drosophila melanogaster*. Dev. Biol. *107*: 142-155.

21. Gietz, R.D.H., 1984. The analysis of dopa decarboxylase expression during embryogenesis in *Drosophila melanogaster*. Ph.D. Dissertation, University of Alberta, Edmonton, Alberta

22. Goldberg, D.A., J.W. Posakony and T. Maniatis 1983. Correct developmental expression of a cloned alcohol dehydrogenase gene transduced into the *Drosophila* germ line. Cell 34: 59-73.

23. Hama, C., Kornberg, T., 1988. The study of *engrailed* expression in the posterior compartment. Abstracts 29th Annual Drosophila Research Conference: 13.

24. Hazelrigg, T., R. Levis and G.M. Rubin, 1984. Transformation of *white* locus DNA in *Drosophila*: dosage compensation, zeste interaction, and position effects. Cell 36 [illegible]

25. Hirsch, J. and N. Davidson, 1981. Isolation and

characterization of the dopa decarboxylase gene of *Drosophila melanogaster*. Mol. Cell. Biol. *1*: 475-485.

26. Hodgetts, R.B., 1975. The response of dopa decarboxylase activity to variations in gene dosage in *Drosophila*: a possible location of the structural gene. Genetics *79* [illegible]

27. Holmes, D.S. and M. Quigley, 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal Biochem. [illegible]: 193-197.

28. Hirsh, J., B. Morgan and S. Scholnick, 1986. Delimiting regulatory sequences of the *Drosophila melanogaster* [illegible] gene. Mol. Cell. Biol. *6*: 448-457.

29. Karess, R.E. and G.M. Rubin, 1984. Analysis of P transposable element function in *Drosophila melanogaster* [illegible] *38*: 135-146.

30. Karess, R., 1985. P element mediated germ line transformation of *Drosophila*, In, DNA cloning, Vol. II, Ch. 5, pp. 121-141, D.M. Glover, Editor, IRL Press.

31. Karlson, P. and C.E. Sekeris, 1966. Ecdysone, an insect steroid hormone and its mode of action. From "Recent Progress in Hormone Research" *22*: 473-502. Ed. by Princus, G., Academic Press, New York and London.

32. Kidwell, M.G., J.F. Kidwell and J.A. Sved, 1977. Hybrid dysgenesis in *Drosophila melanogster*: a syndrome of aberrant traits including mutation, sterility and male recombination. Genetics *86*: 813-833.

33. Kless[illegible] D.F. and J.O. Berry, 1983. Improved filter hybridization method for detection of single copy sequences in large eukaryotic genomes. Plant Mol. Biol. Rep. *1*: 12-18.

34. Kraminsky, G.P., W.C. Clark, M.A. Estelle, R.D. Gietz, B.A. Sage, J.D. O'Connor and R.B. Hodetts, 1980. Induction of translatable mRNA for dopa decarboxylase in *Drosophila*: an early response to ecdysone. Proc. Natl. Acad. Sci. USA. *77*: 4175-4179.

35. Laski, F.A., D.C. Rio and G.M. Rubin, 1986 Tissue specificity of *Drosophila* P element transposition is regulated at the level of mRNA splicing. Cell 44: 7-19.

36. Lindsley, D. and E.H. Grell, 1967. Genetic variations of *Drosophila melanogaster*. Carnegie Institution of Washington Publication

37. Lis, J.T., J.A. Simon and C.A. Sutton, 1983. New heat shock puffs and β-galactosidase activity resulting from transformation of *Drosophila* with an *hsp 70-lacZ* hybrid gene.

Cell 35: 403-410.

38. Livingstone, M.S. and B.L. Tempel, 1983. Genetic dissection of monoamine neurotransmitter synthesis in *Drosophila*. Nature *303*: 67-70.

39. Lunan, K.D. and H.K. Mitchell, 1969. The metabolism of tyrosine-O-phosphate in *Drosophila*. Arch. Biochem. Biophys. *132*: 450-456.

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

41. Marsh, J.L. and T.R.F Wright, 1980. Developmental relationship between dopa decarboxylase, dopamine acetyl transferase, and ecdysone in *Drosophila*. Dev Biol. *80*: 379-387.

42. McCaman, M.W., R.E. McCaman, and G.J. Lees, 1972. Liquid cation exchange - a basis for sensitive radiometric assays for aromatic amino acid decarboxylases. Anal. Biochem. 45: 242-252.

43. Miksicek, R., Heber,H., Schmid,W., Danesch,U., Posseckert,G., Beato,M., and G.,Schutz, 1986. Glucocorticoid responsiveness of the transcriptional enhancer of Moloney murine sarcoma virus. Cell *46*: 283-290

44. Mullis, K.B., and Faloona, F.A., 1987. Specific Synthesis of DNA *in vitro* via a Polymerase-Catalysed Chain Reaction. Methods in enzymology 155: 335-350.

45. Nash, D. and J.B. Bell, 1968. Larval age and the pattern of DNA synthesis in polytene chromosomes. Can. J. Genet. Cytol. *10*: 82-92.

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

47. Pardue, M.L. and J.G. Gall, 1975. Nucleic acid hybridization to the DNA of cytological preparations. From "Methods in Cell Biology" *10*: 1-16. New York: Academic Press.

48. Rio, D.C., F.A. Laski and G.M. Rubin, 1986. Identification and immunochemical analysis of biologically active *Drosophila* P element transposase. Cell *44*: 21-32.

49. Rubin, G.M. and A.C. Spradling, 1982. Genetic transformation of *Drosophila* with transposable element vectors. Science *218*: 348-353.

50. Rubin, G.M., M.G. Kidwell and P.M. Bingham, 1982. The molecular basis of P-M hybrid dysgenesis: the nature of induced

mutations. Cell *29*: 987-994.

51. Scholnick, S.B., B.A. Morgan and J. Hirsh, 1983. The cloned dopa decarboxylase gene is developmentally regulated when reintroduced into the *Drosophila* genome. Cell *34*: 37-45.

52. Scholnick, S.B., B.A. Morgan, S.J. Bray, C.A. McCormick and J. Hirsh, 1986. CNS and Hypoderm Regulatory elements of the *Drosophila melanogaster* Dopa Decarboxylase Gene. Science 234: 998-1002.

53. Spector, T., 1978. Refinement of the Coomassie blue method of protein quantiation. Anal. Bioch. *86*: 142-146.

54. Spencer, C.A.,1987. Molecular genetic studies on the Dopa Decarboxylase gene in *Drosophila melanogaster*. *PhD Thesis, University of Alberta, Edmonton, Alberta.*

55. *Spradling, A.C. and G.M. Rubin, 1982. Transposition of cloned P elements into Drosophila* germline chromosomes. Science *218*:341-347.

56. Spradling, A.C. and G.M. Rubin, 1983. The effect of chromosomal position on the expression of the *Drosophila xanthine dehydrogenase* gene. Cell *34*: 47-57.

57. Shermoen, A., Jongens,J., Barnett,S.W., Flynn,S.K., and S.K.,Beckendorf, 1985 Developmental regulation of an enhancer from the Sgs-4 gene of *Drosophila*. EMBO J. *6*: 207-214.

58. Williams,J.A.,S.S. Pappu, and John B. Bell, 1988. Suppressible P-element alleles of the *vestigial* locus in *Drosophila melanogaster*. Mol. Gen. Genet. 212:370-374.

59. Wright, T.R.F., 1977. The genetics of dopa decarboxylase and α -methyl dopa sensitivity in *Drosophila melanogaster*. Amer. Zool. *17*: 707-721.

60. Wright, T.R.F., W. Beerman, J.L. Marsh, C.B. Bishop, R. Stewart, B.C. Black, A.D. Tomseth and E.Y. Wright, 1981. The genetics of dopa decarboxylase in *Drosophila melanogaster* IV. The genetics and cytology of the 37B10-37D1 region. Chromosoma (Berl) *83*: 45-58.

61. Wright, T.R.F., G.C. Bewley, and A.F. Sherald, 1976. The genetics of dopa decarboxylase in *Drosophila melanogaster* II. Isolation and characterization of dopa decarboxylase-deficient mutants and their relationship to the α-methyl dopa hypersensitive mutants. Genetics *84*: 287-310.

62. Zehring, W.A., D.A. Wheeler, P. Reddy, R.J. Konopka, C.P. Kyriacou, M. Rosbach and J.C. Hall, 1984. P-element transformation with *period* locus DNA restores rhythmicity to mutant, arrhythmic *Drosophila melanogaster*. Cell *39*: 369-376.