2nd Moult:		Pupariation:	
Ddc^{+}	100%	*Ddc*$^{+}$	100%
Ddc^{+4}	114% ±9%	*Ddc*$^{+4}$	51% ±8%
tDdc39	173% ±9%	tDdc39	124% ±8%
tDdc40	100%	tDdc40	100%

Table VIII. The normalized values of DDC activity of the two fusion gene constructs at two developmental stages. *Ddc*$^{+}$ activity levels are set to 100% and only statistically significant differences are shown. Note that Ddc39 contains upstream *Ddc*$^{+}$ sequences and downstream *Ddc*$^{+4}$ sequences; Ddc40 is just the reciprocal.

A potential complicating factor in this analysis is the presence of a *Ddc*$^{+4}$ version of element S. However, it does not appear to exert any effects in tDdc40 and in such manner mimics the inactivity of the *Ddc*$^{+4}$ version of element S in an entirely *Ddc*$^{+}$ background. This serves to map the sequences responsible (or perhaps the redundant sequences) for the inactivity of element S in *Ddc*$^{+}$ to the downstream portion of the gene.

These inferences can be tested by examining the DDC activity levels of the tDdc39 strains (upstream *Ddc*$^{+}$; downstream *Ddc*$^{+4}$). If the downstream portion of the gene alone is mediating the *Ddc*$^{+4}$ phenotype, tDdc39 should express at levels equivalent to those of *Ddc*$^{+4}$. This is not

the case at either stage (Table VIII). However, if the downstream region is also responsible for potentiating the silencer activity of element S, tDdc39 should overexpress (due to the presence of a *Ddc*$^{+}$ version of element S) at both stages at levels consistent with those determined previously (Table VII). At the second moult, the construct expresses at levels 1.5-fold higher than those of *Ddc*$^{+4}$ ([173-114]/114 + 1; see Table VIII). This is lower than the 1.9-fold increase expected (from Table VII), but quite consistent with the 1.5-fold increase seen if the erratically expressing strain 16 is ignored (column 3 of Table VII). The situation at pupariation is somewhat different. The level of DDC activity of tDdc39 (Table VIII) is increased to 2.4 times that of *Ddc*$^{+4}$, somewhat greater than the 1.6-fold increase expected from the results on transformants carrying a *Ddc*$^{+}$ element S in a *Ddc*$^{+4}$ context (Table VII). The unexpected magnitude of the increase suggests that novel regulatory interactions are occurring due to the juxtaposition of upstream *Ddc*$^{+}$ and downstream *Ddc*$^{+4}$ sequences.

The notion that downstream regulatory sequences play a role in *Ddc* regulation is in accordance with previously described as well as ongoing experiments. The deletion of all sequences upstream of -25 still permitted the induction of DDC activity in embryogenesis and at eclosion (Hirsh *et al.*, 1986). Also, several sequence elements conserved between *D. melanogaster* and *D. virilis* have been identified

in the first non-translated exon of *Ddc* (Bray and Hirsh, 1986). Both of the base differences between Ddc^{+} and Ddc^{+4} in this region are located within these conserved elements. Finally, recent experiments in the laboratory have detected *in vitro* binding to the first intron of Ddc^{+} by one of the products of the *broad* complementation group (R. Hodgetts, pers. comm.). The *broad* complementation group is part of the Broad Complex and mutants reduce the DDC activities of Ddc^{+} and Ddc^{+4} to equivalent levels at pupariation (M. Schouls, pers. comm.). Mutants in *broad* have no effect at the second moult nor an effect on neural levels of DDC, implying temporal and stage specificity of the interaction with the *Ddc* gene.

The results of this thesis lead to the working hypothesis that element S is silencing Ddc^{+4} expression. This effect appears to be potentiated by downstream sequence elements which differ between Ddc^{+} and Ddc^{+4}. To test this hypothesis, further investigation of the *broad* products is warranted. The model predicts that different binding affinities between the Ddc^{+} and Ddc^{+4} introns may be observed. This in turn could influence the function of element S, perhaps to silence *Ddc* expression both in tissues where it is not required as well as to fine tune *Ddc* expression in the epidermis.

Bibliography

Beall CJ; Hirsh J (1987): Regulation of the Drosophila dopa decarboxylase gene in neuronal and glial cells. Genes. Dev. 1:510-20.

Bray SJ; Hirsh J (1986): The *Drosophila virilis* dopa decarboxylase gene is developmentally regulated when integrated into *Drosophila melanogaster*. EMBO 5:2305-2311.

Bray SJ; Johnson WA; Hirsh J; Heberlein, U; Tjian, R (1988): A *cis*-acting element and associated binding factor required for CNS expression of the *Drosophila melanogaster* dopa decarboxylase gene. EMBO 7: 177-88.

Carter P (1987): Improved oligonucleotide-directed mutagenesis using M13 vectors. Meth. Enzymol. 154: 382-403.

Chen Z-Q (1986): M.Sc. Thesis, University of Alberta (Edmonton).

Crowley TE; Mathers PH; Meyerowitz EM (1984): A *trans*-acting regulatory product necessary for expression of the *Drosophila melanogaster* 68C glue gene cluster. Cell 39: 149-156.

Estelle MA; Hodgetts RB (1984): Genetic elements near the structural gene modulate the level of dopa decarboxylase during Drosophila development. Mol. Gen. Genet. 195: 434-441.

Estelle MA; Hodgetts RB (1984): Insertion polymorphisms may cause stage specific variation in mRNA levels for dopa decarboxylase in Drosophila. Mol. Gen. Genet. 195: 442-451.

Goldberg DA; Posakony JW; Maniatis T (1983): Correct developmental expression of a cloned alcohol dehydrogenase gene transduced into the Drosophila germ line. Cell 34: 59-70.

Hanahan D (1983): Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166: 557-580.

Hirsh J; Davidson N (1981): Isolation and characterization of the dopa decarboxylase gene of *Drosophila melanogaster*. Mol. Cell. Biol. 1: 475-485.

Hirsh J; Morgan BA; Scholnick SB (1986): Delimiting regulatory sequences of the *Drosophila melanogaster Ddc* gene. Mol. Cell. Biol. **6**:4548-4557.

Hodgetts RB; Clark WC; Gietz RD; Sage BA; O'Connor JD (1986): Stage-specific mechanisms regulate the expression of the dopa decarboxylase gene during Drosophila development. Arch. Insect Bioch. Phys.: 97-104.

Hofmann A; Garfinkel MD; Meyerowitz EM (1991): *cis*-acting sequences required for expression of the divergently transcribed *Drosophila melanogaster Sgs-7* and *Sgs-8* glue protein genes. Mol. Cell. Biol. **11**: 2971-2979.

Johnson WA; Hirsh J (1990): Binding of a Drosophila POU-domain protein to a sequence element regulating gene expression in specific dopaminergic neurons. Nature **343**: 467-470.

Johnson WA; McCormick CA; Bray SJ; Hirsh J (1989): A neuron-specific enhancer of the Drosophila dopa decarboxylase gene. Genes. Dev. **3**: 676-86.

Karlson P; Sekeris CE (1964): Biochemistry of insect metamorphosis. Vol.6. (Series Eds: Florkin,M; Mason,HS. Comparative Biochemistry) pp. 221-243. Academic Press, Orlando.

Kidwell MG; Kidwell JF; Sved JA (1977): Hybrid dysgenesis in *Drosophila melanogaster*: a syndrome of aberrant traits including mutation, sterility and male recombination. Genetics **86**: 813.

Klemenz R; Weber U; Gehring WJ (1987): The white gene as a marker in a new P element vector for gene transfer in Drosophila. N. A. R. **15**: 3947-3959.

Konrad KD; Marsh JL (1987): Developmental expression and spatial distribution of dopa decarboxylase in Drosophila. Dev. Biol. **122**: 172-185.

Kraminsky GP; Clark WC; Estelle MA; Gietz RD; Sage BA; O'Connor JD; Hodgetts RB (1980): Induction of translatable mRNA for dopa decarboxylase in Drosophila: An early response to ecdysterone. Proc. Natl. Acad. Sci. (U.S.A.) **77**: 4175-4179.

Maniatis T; Fritsch EF; Sambrook J (1982): Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Marsh JL; Gibbs PDL; Timmons PM (1985): Developmental control of transduced dopa decarboxylase genes in *D. melanogaster*. Mol. Gen. Genet. **198**: 393-403.

Mathers PH (1989): Ph.D. Thesis, California Institute of Technology (Pasadena).

Mazza A (1989): M.Sc. Thesis, University of Alberta (Edmonton).

McGinnis W; Shermoen A; Heemskerk J; Beckendorf SK (1983): DNA sequence changes in an upstream DNase I-hypersensitive region are correlated with reduced expression. Proc. Natl. Acad. Sci. (U.S.A.) **80**: 1063.

Mestril R; Schiller P; Amin J; Klapper H; Ananthan J; Voellmy R (1986): Heat shock and ecdysterone activation of the *Drosophila melanogaster hsp23* gene: a sequence element implied in developmental regulation. EMBO **5**: 1667-1673.

Nash DN; Bell JB (1968): Larval age and the pattern of DNA synthesis in polytene chromosomes. Can. J. Genet. Cytol. **10**: 82-92.

Rubin GM; Spradling AC (1982): Genetic transformation of Drosophila with transposable element vectors. Science **218**: 348-353.

Sanger F; Nicklen S; Coulson AR (1977): DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. (U.S.A.) **74**: 5463-5467.

Scholnick SB; Bray SJ; Morgan BA; McCormick CA; Hirsh J (1986): CNS and hypoderm regulatory elements of the *Drosophila melanogaster* dopa decarboxylase gene. Science **234**:998-1002.

Scholnick SB; Caruso PA; Klemencic J; Mastick GS; Mauro C; Rotenberg M (1991): Mutations within the *Ddc* promoter alter its neuron-specific pattern of expression. Dev. Biol. **146**: 423-437.

Scholnick SB; Morgan BA; Hirsh J (1983): The cloned dopa decarboxylase gene is developmentally regulated when reintegrated into the Drosophila genome. Cell **34**:37-45.

Sekeris CE (1991): The role of molting hormone in sclerotization in insects. Vol. I, part 3. (Series Ed: Gupta AP. Morphogenetic Hormones of Arthropods. Roles in Histogenesis, Organogenesis and Morphogenesis) pp. 152-212. Rutgers University Press, New Jersey.

Shermoen AW; Jongens J; Barnett SW; Flynn K; Beckendorf SK (1987): Developmental regulation by an enhancer from the *Sgs-4* gene of Drosophila. EMBO **6**: 207-214.

Spencer CA (1987): Ph.D. Thesis, University of Alberta (Edmonton).

Spradling AC; Rubin GM (1982): Transposition of cloned P elements into Drosophila germ line chromosomes. Science **218**: 341-347.

Spradling AC; Rubin GM (1983): The effect of chromosomal position on the expression of the Drosophila xanthine dehydrogenase gene. Cell **34**: 47-57.

Todo T; Roark M; Raghavan KV; Mayeda C; Meyerowitz EM (1990): Fine-structure mutational analysis of a stage- and tissue-specific promoter element of the Drosophila glue gene *Sgs-3*. Mol. Cell. Biol. **10**: 5991-6002.

Treacy MN; He X; Rosenfeld MG (1991): I-POU: a POU-domain protein that inhibits neuron-specific gene activation. Nature **350**: 577-584.

Vieira J; Messing J (1987): Production of single-stranded plasmid DNA. Meth. Enzymol. **153**: 3-11.

Walter MF; Black BC; Afshar G; Kermabon A-Y; Wright TRF; Biessmann H (1991): Temporal and spatial expression of the yellow gene in correlation with cuticle formation and dopa decarboxylase activity in Drosophila development. Dev. Biol. **147**: 32-45.

Weiher H; Konig M; Gruss P (1983): Multiple point mutations affecting the Simian Virus 40 enhancer. Science **219**: 626-631.

Wright TRF (1987): The genetic and molecular organization of the dense cluster of functionally related, vital genes in the dopa decarboxylase region of the *Drosophila melanogaster* genome. 14th ed. (Series Ed: Hennig W. Results and Problems in Cell Differentiation. Structure and Function of Eukaryotic Chromosomes) pp. 95-120. Springer-Verlag Berlin Heidelberg.

Yanisch-Perron C; Vieira J; Messing J (1985): Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33: 103-119.

oligonucleotide-directed *in vitro* mutagenesis to resemble the *Ddc*$^{+}$ version of element S. The reciprocal mutation in *Ddc*$^{+}$ was also induced, as well as an 18 bp deletion of element S. The two mutants and the deletion were transformed into the Drosophila germline using recombinant P elements. The results of DDC assays on the transformants indicate that element S serves little or no function in *Ddc*$^{+}$ at the times of development examined but that it acts as a silencer of gene expression in *Ddc*$^{+4}$.

Further experiments involved switching the regions of the *Ddc*$^{+}$ and *Ddc*$^{+4}$ alleles upstream of the transcription start site with one another. Two fusion genes were thus constructed, one with upstream *Ddc*$^{+}$ sequences spliced to downstream *Ddc*$^{+4}$ sequences and the other with just the opposite. Upon germline transformation, the results of DDC assays at different developmental stages implicated sequences downstream of the transcription start site as being responsible for the activity of element S in *Ddc*$^{+4}$ and its lack thereof in *Ddc*$^{+}$. The results indicate that two wild type alleles of *Ddc* have evolved different regulatory mechanisms involving the coordination of both upstream and downstream sequence elements.

Acknowledgements

I would like to thank Ross for all his guidance, his teaching and for his fine example of a scientist with a well balanced lifestyle. I would like to thank Bill Clark for his support, his dedication, his amazing fund of knowledge and for letting me win money from him at golf. Thank you, Chaim, for first sparking my interest in genetics. I would like to thank Timmy for all our late night conversations and the good times in baseball and football; Dina W. for the polo lessons; Michelle for being so good to me; Brian for his great sense of humour and of course, Mum, for all those lunches. A special thank you goes to Jana, Andrew and Andrew for their technical assistance in my project and in the preparation of this thesis. Finally, to all who were promised an acknowledgement in return for a small favour, I thank you:

Bill Brook
Adam Chen
Bill Clark
Raj Dhillon
Troy Harkness
Tim Heslip
Heather Konrad
Al Mazza
Sita Pappu
Jana Piorecky
Matthew Reece
Michelle Schouls
Andrew Simmonds
Brian Staveley
Andrew Swan
Dina Winkelman

Table of Contents

List of Tables

List of Figures

List of Abbreviations

bp	base pair(s)
dCGTTP	mixture of dCTP, dGTP and dTTP
ddH_2O	double distilled water
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
kb	kilobase pairs
PEG	Polyethylene glycol
SDS	Sodium dodecyl sulfate
TAE	40 mM Tris-acetate, 1 mM EDTA
TE	10 mM Tris-Cl (pH 8.0), 1 mM EDTA (ph 8.0)
u	units of activity as defined by the manufacturer

Introduction

The development from a fertilized ovum of a multicellular organism consisting of up to 10^{13} cooperating cells is one of the most fascinating aspects of biology. The information required to direct this complex chain of events is coded in a large set of genes, each of which is expressed in a timely and spatially correct manner. This temporal and spatial control of gene expression is the fundamental means by which ontogeny is effected. Elucidation of the mechanisms underlying the specification of these characteristics of gene expression is thus of central importance to understanding the process of development.

In order to study the regulation of gene expression through development, a number of model systems have been developed. Most share the property of being amenable to both genetic and molecular analyses which have allowed the definition of their spatial and temporal characteristics. Mutational analysis is often then used to delineate the important components of the regulatory network which controls their expression.

Our lab is studying a particularly well developed gene-enzyme system in *Drosophila melanogaster* as a model of steroid hormone action during development. The gene, *Ddc*,

encodes the enzyme dopa decarboxylase (DDC; EC 4.1.1.28), an essential enzyme in the catecholamine and quinone biosynthetic pathways (Sekeris, 1991). It functions in approximately 150 neurons and glial cells in the CNS to produce the neurotransmitters serotonin and dopamine (Beall and Hirsh, 1987; Konrad and Marsh, 1987). It also functions in the hypodermal epithelium to produce quinones, compounds involved in sclerotizing (hardening and pigmenting) the cuticle (Wright, 1987). The temporal distribution of the *Ddc* enzyme (DDC) is different for the two tissues. Levels in the CNS are fairly constant through development. They comprise about 10% of whole animal activity (Scholnick *et al.*, 1983). Levels in the hypoderm fluctuate greatly through ontogeny (Figure 1) and comprise 90% of whole animal activity.

Part or all of the stringent temporal regulation of *Ddc* in the hypoderm is mediated by the steroid hormone 20-OH-ecdysone (hereafter called ecdysone). The first evidence of this was obtained by Karlson and co-workers (see Sekeris, 1991 for a review) in the blow fly, Calliphora. This work, which began in the early 1960's, culminated with the demonstration that ecdysone caused increases in DDC activity when injected into hormonally deficient larval abdomens (Karlson and Sekeris, 1962). This work was extended to Drosophila by Kraminsky *et al.* (1980) who showed that *Ddc*-translatable mRNA accumulates at pupariation within two hours of a pulse of exogenous ecdysone administered to

Figure 1. DDC activity and ecdysone titer during the life cycle of the Canton S strain at 25°C. Enzyme activity is indicated by open triangles and ecdysone titer by open circles. This figure has been taken from Kraminsky *et al.* (1980).

Removed due to copyright restrictions

hormonally naive organisms. This might be expected from the data in Figure 1 which show that the DDC and ecdysone peaks are coincident at this stage. By contrast, in embryogenesis and larval life, the pulses of ecdysone precede the DDC peaks by 14 - 16 hrs (see Figure 1) suggesting that regulation by ecdysone, if present, is different in these three stages. The DDC peak at eclosion is preceded by an ecdysone peak in mid-pupal life, approximately 55 hrs earlier. This suggests that a third mode of regulation may be operating at this stage.

Ddc is present as a single copy gene with a single promoter (Hirsh and Davidson, 1981; Scholnick *et al.*, 1986). This promoter is thus subject to multiple regulatory inputs through which the temporal and tissue specific control of *Ddc*, discussed above, is effected. In order to gain insight into the mechanisms of this developmental regulation, the contributions of each of these inputs must be ascertained. This dissection of promoter function has been performed by the directed induction of mutation followed by a phenotypic analysis of germline transformants. A central role in this strategy has been occupied by P elements, the class of transposable elements responsible for hybrid dysgenesis in Drosophila (Kidwell *et al.*, 1977).

The P element is a transposon isolated from natural populations of Drosophila (Spradling and Rubin, 1982). It has been harnessed as a vector for the stable reintroduction of genes into the germ line (Rubin and Spradling, 1982).

Genes reintegrated in such a manner display roughly normal patterns of expression and are only partially subject to the effects of chromosomal position (Scholnick *et al.*, 1983; Spradling and Rubin, 1983; Goldberg *et al.*, 1983). Thus, recombinant P elements allow one to assess the contributions of *cis*-acting regulatory elements to levels of gene expression *in vivo*.

Initial studies using P elements demonstrated that all the genetic elements necessary for normal developmental expression of *Ddc* reside in a 7.55 kb *Pst*I fragment which includes 2.85 kb of 5' sequences, the 4 kb structural gene, and 0.7 kb of 3' sequences (Scholnick *et al.*, 1983). Further studies by Hirsh *et al.* (1986) involved the deletion of sequence from the 5' end of *Ddc* to endpoints of -800, -383, -209 and -25 relative to the transcription start site. These genes were cloned into recombinant P elements which contained the alcohol dehydrogenase gene (*Adh*) as a selectable marker for transformation. These vector constructs were then injected into *Adh*$^-$ Drosophila embryos and transformants selected on ethanol-containing media. DDC assays were done at various time points through embryogenesis, pupariation and eclosion. The conclusion was reached that no sequences upstream of -209 were required for normal developmental expression of *Ddc* in the hypoderm. However, the strains with no sequences upstream of -209 showed wide variability in expression levels and were in fact overproducers at the hatch and eclosion (see Figure 2

of Hirsh *et al.*, 1986). Also, significant regulatory interactions between *Adh* and the truncated *Ddc* genes were noted. Since this thesis attempts to characterize an element upstream of -209, these points will be returned to later. One final note of interest was that *Ddc* genes lacking all sequences upstream of -25 still showed induction of *Ddc* expression in embryogenesis (albeit delayed 4 hrs) and at eclosion. This implicates a role for downstream regulatory sequences in the control of *Ddc*.

The above deletion experiments indicated that most of the *Ddc* cis-acting regulatory elements were located between -25 and -209. A directed approach was undertaken to identify individual regulatory sequences in this region by searching for evolutionarily conserved sequences. The *Ddc* gene from the distantly related *D. virilis* was cloned and introduced into the *D. melanogaster* germ line, again using *Adh* as a marker (Bray and Hirsh, 1986). It displayed a similar pattern of expression to the *Ddc* gene from *D. melanogaster* indicating it was responsive to the same regulatory controls. The immediate 5' region of the *D. virilis* gene was sequenced and five conserved elements, 9 - 16 bp in length, were identified (Figure 2; only numbers I to IV are shown since element V has not been found to have detectable function; Scholnick *et al.*, 1991).

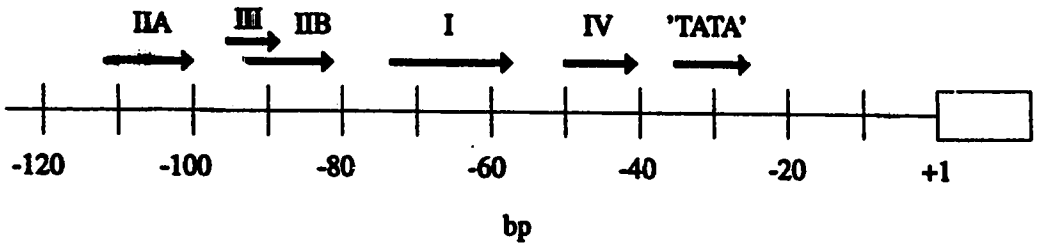


Figure 2. Schematic diagram of the *Ddc* promoter region from *D. melanogaster*. Four of the elements defined by their identity with *D. virilis* sequence are indicated by arrows and their designations shown. Elements IIA and IIB are identical. The empty rectangle denotes sequence from the first exon. Figure adapted from Scholnick *et al.*, 1986.

Deletion constructs were made in the *D. melanogaster Ddc* gene which resulted in the sequential removal of more and more DNA between -208 and -38, the TATA box identity (Scholnick *et al.*, 1986). The set of nested deletions removed one or more of the conserved elements in succession. Since the previous set of deletion constructs displayed considerable regulatory interaction with the *Adh* marker gene used, *Ddc* sequences upstream of -208 (2.65 kb) were retained to serve as a buffer. The various genes were transformed and developmental samples collected as before. The transformed strains exhibited a graded decrease in hypodermal expression at pupariation and eclosion as progressively larger deletions removed more of the promoter. The situation differed at embryogenesis where the major control element appeared to be element IV. Interestingly,

the severe deletion of all sequences between -208 and -38 still allowed properly timed induction of *Ddc* expression in embryogenesis, at pupariation and at eclosion, albeit to very low levels. This implicates a role for sequences downstream of -38 or one for redundant sequences upstream of -208 at these stages.

Element I was found to have an interesting tissue specificity. While its removal resulted in a mild decrease in hypodermal expression, its deletion resulted in a near abrogation of CNS *Ddc* expression. Subsequent experiments (Bray *et al.*, 1988; Johnson *et al.*, 1989; Johnson and Hirsh, 1990; Treacy *et al.*, 1991) showed this element to be the proximal part of a dual element, the distal portion of which is located between -1019 to -1623. Together, these regions specify the exact subset of neurons in which *Ddc* is to be expressed, as well as the precise level at which it is to be expressed.

The immediate upstream region of *Ddc* thus contains one CNS specific element (element I) and four hypodermal specific elements, each of which make different contributions depending on the developmental stage.

An alternate approach to the directed identification of *cis*-acting regulatory elements has been taken by R. Hodgetts and colleagues (Estelle and Hodgetts, 1984a). This involved the isolation and characterization of activity variants from natural populations. The notion was that changes in developmental expression might be correlated with DNA

sequence changes in regulatory elements, thus identifying the elements. DDC activity was measured at eclosion in 109 strains containing different wild type second chromosomes (the chromosome on which the *Ddc* gene resides) and compared to values from a standard laboratory strain, Canton S. An activity variant of *Ddc* was found and designated Ddc^{+4}. This activity variation was confined to the hypodermis as CNS levels were indistinguishable from those of Canton S. Relative to Canton S, Ddc^{+4} was a 40% overproducer at the hatch, a 50% overproducer at the larval moults, a 50% underproducer at pupariation and a 20% overproducer at eclosion.

It was hoped that an analysis of the variant might shed light on the different regulatory mechanisms hypothesised to operate during development. Pupariation is the only stage of development where DDC and ecdysone peaks are coincident and interestingly was the only stage where Ddc^{+4} was an underproducer. The levels of overproduction at the hatch and the second moult were similar, suggesting a second regulatory mechanism at these stages. And finally, the level of overproduction at eclosion seemed to represent a third, distinct phenotype. This partitioning of the Ddc^{+4} enzyme activity variation into three phenotypic classes seemed to correspond well with the three apparently different developmental situations inferred from the timing of DDC peaks relative to ecdysone peaks (see above and Figure 1).

Ddc^{+4} was cloned (Estelle and Hodgetts, 1984b) and restriction mapping indicated there were six RFLP's showing less than a 50 nucleotide increase or decrease in fragment length. Three were located in the 5' untranslated region, one in the first exon, one in the first intron and one in the second (epidermal) intron. *Ddc*$^{+4}$ sequence was obtained from -350 to +350 (Spencer, 1987) and showed a duplication, small deletions and insertions and a number of point mutations. Most of the changes were clustered 5' to the transcription start site (85%); 12% occurred in the first non-translated exon and only 4% occurred in the first 172 bp of the first intron.

None of the changes were located in the previously identified promoter elements (I - IV). In an attempt to uncover additional regulatory sequences, an identity search with other Drosophila genes was made using those sequences which differed between *Ddc*$^{+}$ and *Ddc*$^{+4}$. One such polymorphic element was found which showed identity to sequences found upstream of a number of other genes, all of them known to be regulated by ecdysone. This element consisted of two halves separated by a few bases. One half bore some resemblance to the SV40 canonical enhancer sequence (Figure 3).

Evidence for a functional role for the element came from three sources. First, investigation of a natural variant of the salivary gland secretion-4 (*Sgs-4*) gene associated with a 50% underproduction of mRNA has implicated a single base change at -344 in the element as the cause of

Gene	Sequence Element	Reference
Ddc^{+}	ATG**A**AAA-4-TGCCTTT^{-212}	Eveleth *et al.*, 1986
Ddc^{+4}	ATG**G**AAA-4-TGCCTTT^{-212}	Spencer, 1987
Sgs-3	ATCGAAA-7-TCCATTT^{-62}	Todo *et al.*, 1991
Sgs-4	ATGGAAA-3-TACCTTT^{-336}	Mestril *et al.*, 1986
Sgs-7/8	ACTGAAA-3-TCCATTG^{-55}	Todo *et al.*, 1991
Hsp 22	ATGGAAA-2-GACCTTC^{-286}	Mestril *et al.*, 1986
SV40 enhancer	(G)TTGGAAA(G)	Weiher *et al.*, 1983

Consensus: ATNGAAA-4-TNCCTTT

Figure 3. A common sequence element found upstream of a number of ecdysone-regulated genes. The single base difference between *Ddc*$^{+}$ and *Ddc*$^{+4}$ is indicated in bold. Note that the change in *Ddc*$^{+4}$ renders this more of a consensus element and more similar to the SV40 consensus than the *Ddc*$^{+}$ sequence.

the underproduction (McGinnis, 1983). In *Sgs-4*, the element is associated with a DNase I hypersensitive site which only appears in the third larval instar, the only time the glue genes are expressed. Second, further studies on *Sgs-4* by Schermoen and others (1987) indicated that a 0.41 kb restriction fragment from -158 to -568 was capable of directing the correct (*ie. Sgs-4* specific) tissue and temporal specific pattern of expression of an *Adh* reporter gene. The activities of this fragment, containing the

element, were shown to be independent of position and orientation. Third, studies on the heat shock protein 23 (*Hsp 23*) gene, which contains an isomer of the element (TTTCCAT-19-ATGGCAG^{-194}), have shown this element to be important for *Hsp 23* induction by ecdysone (Mestril *et al.*, 1986).

In order to assay the functionality of the *Ddc* element (designated element S), Charlotte Spencer cloned an oligonucleotide containing the *Ddc*$^{+}$ version of element S upstream of an *Adh* reporter gene (Spencer, 1987). The construct was introduced into the germ line of *Adh*$^{-}$ flies using P elements. *Adh* activity was measured at the second moult, compared with controls (*Adh* reporter genes lacking *Ddc* sequences) and an elevation of activity from 35% - 45% was seen. Element S was not found to confer any tissue specificity distinct from that of *Adh*. Since the reporter gene was only active at the second moult, the potential stage specificity of element S could not be assessed. A working hypothesis was therefore adopted in which element S was postulated to be an enhancer of *Ddc* gene expression. The single base change present in the *Ddc*$^{+4}$ version of the element, because it produced an element more like the consensus, was thought to be responsible for the overproduction of *Ddc*$^{+4}$ at the hatch and second moult.

The data of Spencer are in contrast to those of Hirsh and others (1986) who previously indicated that no sequences upstream of -209 were required for the normal developmental

expression of *Ddc*. However, in both experiments, gross disruption of the integrity of the *Ddc* gene was involved. The data from Hirsh's group were further complicated by regulatory interactions between the *Ddc* and *Adh* genes. To accurately determine the relevance of element S in the regulation of *Ddc* and in particular to test whether the polymorphism in element S contributed to the overproduction phenotype of Ddc^{+4}, it was felt that the integrity of the *Ddc* gene and flanking sequences should be maintained as far as possible. This was accomplished using *in vitro* mutagenesis to introduce a single base change into the 7.55 kb *Pst*I fragments containing either the Ddc^{+} or the Ddc^{+4} alleles. A Ddc^{+4} version of element S was thus introduced into a Ddc^{+} subclone and a Ddc^{+} version of element S was introduced into a Ddc^{+4} subclone. In this manner the significance of the single base difference in element S was determined following transformation. The reciprocal mutations were introduced into the germ line and DDC activities at the hatch, the second moult, pupariation and eclosion were determined. Surprisingly, the Ddc^{+4} change in the Ddc^{+} background *reduced* expression levels slightly at embryogenesis and had no effect at the other three stages. Equally unexpected, the Ddc^{+} change in the Ddc^{+4} background *increased* expression at all four stages.

The role of element S in the regulation of Ddc^{+} expression was also assessed unsing deletion analysis. Eighteen base pairs, including element S, were deleted from

-227 to -208 of *Ddc*$^{+}$. Upon transformation, no change in expression levels were noted, except for a slight increase in expression at eclosion. These results have led us to reformulate the role of element S in the regulation of *Ddc* to that of a silencer element. This role is very minor in *Ddc*$^{+}$ but is significant in *Ddc*$^{+4}$.

The controls used for the above experiments differed from that used in previous transformation experiments. The control used by Hirsh and others for their transformation experiments was the Canton S laboratory strain. This was felt to be inappropriate for two reasons. First, their constructs were injected into a *Ddc*^{ts2}; *Adh*$^{-}$ strain, which was derived from the Oregon R laboratory strain. Thus, the transformed *Ddc* genes were in a different genetic background than the control *Ddc* gene. Also, the transformed *Ddc* genes were located at different chromosomal locations and were thus subject to various position effects to which the control *Ddc* gene was not subject and *vice versa*. It was thus decided to use transformed versions of the *Ddc*$^{+}$ and *Ddc*$^{+4}$ alleles to serve as controls for all transformation experiments, as opposed to the original strains from which these alleles were cloned.

In addition to the studies directed towards assessing the functional significance of element S, a parallel and more general study was undertaken to roughly localize the regions responsible for the *Ddc*$^{+4}$ phenotypes to either the upstream or downstream (relative to the transcription start

site) portions of the gene. Previous studies had suggested that *Ddc* regulatory sequences may exist downstream of -25. Therefore, it was conceivable that some or all of the regulatory sequences responsible for the Ddc^{+4} phenotypes were located in this region. The sequencing of the *D. virilis Ddc* gene (Bray and Hirsh, 1986) provided additional evidence in support of this notion. The first exon (which is nontranslated in *D. melanogaster*) was found to contain six regions of identity (8 - 14 bp in size) with the first exon of the *D. melanogaster* gene. Both of the single base polymorphisms present in the first exon of Ddc^{+4} were located in these conserved regions. The experiments to construct the fusion genes were carried out by taking the upstream portion of Ddc^{+} and splicing it to the downstream portion of Ddc^{+4}. The reciprocal construct, with Ddc^{+4} upstream sequences and Ddc^{+} downstream sequences was also made. These constructs were transformed into an appropriate host and DDC activity determinations made at the second moult and at pupariation, two stages where the Ddc^{+4} phenotypes are opposed (that is, overproduction at the moult and underproduction at pupariation). The results were not clear-cut but did implicate a role for both upstream and downstream regulatory elements. No stage specificity of these elements were found as the different transformants displayed the same relative pattern of expression at the second moult and at pupariation.

Element IV (see Figure 2) had been previously implicated by Scholnick *et al.* (1986) to be a hypodermal specific positive regulatory element. In addition, it appeared to have differential effects at different stages of development, being the major contributor to maximal levels of gene expression at embryogenesis, and a minor contributor at pupariation and eclosion. This element was chosen for study to lend confidence to the above experiments as well as to determine if its effects were similar in embryogenesis and at the second moult. Point mutations were introduced into element IV by *in vitro* mutagenesis, the constructs transformed into an appropriate host strain and DDC activity measurements made at four stages of development. The results suggested that different base pairs in element IV were important at different stages of development and that each stage represented a distinct regulatory situation.

The results of the experiments presented in this thesis underline the very real difficulties of using P element transformation as an assay system for subtle *cis*-acting regulatory sequences. Some form of targeted transposition or, ideally, homologous recombination system is required to accurately assess the function of such elements. However, the variety of *in vitro* manipulations performed, including point mutation, deletion and rearrangement, have allowed the formulation of some preliminary conclusions while exposing the complexity *Ddc* developmental regulation. The data also serve to highlight the critical importance of genetic

context in analyzing the function of promoter elements.

Materials & Methods

Bacterial stocks

Strains:

JM 83 *ara* Δ(*lac-pro*) *rpsL thi* ϕ80d*lacZ*ΔM15. Used for routine subcloning, DNA preparations.

JM 103 Δ(*lac-pro*) *sup*E *thi str*A *sbc*B15 *end*A *hsd*R4 *F'tra*D36 *pro*AB *lac*I^{q}ZΔM15. Used as a host for phage M13KO7 growth.

DH5α *end*A1 *hsd*R17 *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 Δ(*arg*F-*lac*ZYA)U169 ϕ80d*lac*ZΔM15. Purchased as "subcloning efficiency" competent cells from BRL; used for DNA preparations.

XL-1 Blue *rec*A1 *end*A1 *gyr*A96 *thi hsd*R17 *sup*E44, *rel*A1, *lac*$^{-}$ *F' pro*AB *lac*i^{q}ZΔM15 Tn10. Used as a transformation host for mutagenesis reactions, to prepare single-stranded and double-stranded DNA. Purchased from Stratagene as frozen competent cells.

Growth media:

Media and antibiotics used were as in Maniatis *et al.* (1982) (except that 100 µg/ml ampicillin was always used) or according to manufacturer's specifications (in the case of frozen competent cells).

Storage:

All strains were streaked out for single colonies at least three times before making glycerol stocks. Glycerol stocks were prepared by adding 1 ml of a saturated overnight culture to 0.43 ml of 50% glycerol. Stocks were stored in duplicate at -70°C.

Drosophila stocks

Drosophila melanogaster chromosomes used in various strains:

Ddc^{ts2} cn	temperature sensitive allele of *Ddc* (lethal at 30°C) marked with *$cinnabar^{1}$* (an eye colour mutant).
CyO	second chromosome balancer marked with the dominant mutation *Curly*.
y w	first chromosome marked with *yellow* and *$white^{C6723(2)}$* (a deletion).
Sb	third chromosome marked with *Stubble*.
TM2	a third chromosome partial balancer (all except the tip of 3R and cytological positions 61C to 74) marked with the dominant *Ubx*.
TM6	third chromosome balancer marked with the dominant mutation *Ubx*.

Drosophila melanogaster strains:

Canton S	a standard laboratory strain from which the *Ddc*$^{+}$ allele was cloned.
y w	injection host strain.
y w; Ddc^{ts2}*cn/ CyO; Sb/Ubx*	balancer strain used to determine chromosomal location of transformants; used to recombine second chromosome inserts with *Ddc*^{ts2}.
Sb ry^{506} P[ry^{+}Δ2-3]99B /TM2 P[ry^{+}Δ2-3]99B	a strain carrying a stable, genomic source of transposase; used for mobilizing transposons.

Growth media:

All organisms were maintained on standard fly food (Nash and Bell, 1968) at 22°C. Infection was controlled with p-hydroxybenzoic acid methyl ester (Methyl paraben) from Sigma Chemical Co.

DNA preparation

Plasmid DNA:

Mini-preparations of plasmid DNA were carried out by the alkaline lysis protocol in Maniatis *et al.*(1982) with the only deviation being a 30 min, 37°C RNase A (final concentration - 75 µg/ml) step preceding the phenol/chloroform extraction.

Large scale preparation of DNA was performed by the alkaline lysis and sodium dodecyl sulfate (SDS) lysis

procedures of Maniatis *et al.*(1982). Although SDS lysis was used for larger plasmids, it was found that plasmids as large as 15.8 kb could be extracted without mishap using alkaline lysis. The following changes were made to the protocols outlined in Maniatis *et al.*(1982). Cell cultures of 250 ml were grown to saturation instead of amplifying; if 500 ml cultures were grown, the amount of all reagents used was doubled. After adding cesium chloride at 0.98 g/ml and ethidium bromide to 800 µg/ml to the DNA suspension, it was centrifuged at 15,000 RPM for 15 min and the supernatant centrifuged at 54,000 RPM for at least 6 hrs in a VTi 65 rotor. Following TE-saturated butanol extraction of the ethidium bromide, 2 volumes of TE were added to the DNA solution, 10 M ammonium acetate was added to a final concentration of 2 M and the DNA was precipitated with two volumes of 95% ethanol. After washing with 70% ethanol and desiccation, the pellet was resuspended in 400 µl TE and ethanol precipitated using sodium acetate or sodium chloride. Quantification and purity assessment were carried out by measuring the optical density at 260 nm and 280 nm and by agarose gel electrophoresis.

Single stranded DNA:

Single stranded DNA was prepared for sequencing as follows. A single colony, identified as a positive following *in vitro* mutagenesis (see below) was inoculated into 3 ml of 2X YT (Vieira and Messing, 1987) containing 100

µg/ml ampicillin. Then, 3 µl of M13KO7 helper phage (approximately 3 X 10^7 plaque forming units) were added and the culture shaken for 1 hr at 37°C. Kanamycin was added to 40 µg/ml and incubation continued for about 16 hrs. Three aliquots of 1 ml each were centrifuged in microfuge tubes and 800 µl of the supernatants were transferred to new tubes. Following addition of 200 µl 20% PEG-8000 in 2.5 M NaCl, the tubes were placed on ice for 30 min. They were then centrifuged 5 min, the supernatants removed, and following recentrifugation all traces of PEG were removed. The pellets of two of the tubes were resuspended in 100 µl TE each and these were pooled in the third tube and its pellet resuspended, followed by a 2 min centrifugation to remove any remaining bacterial cells. The supernatant was extracted with an equal volume of phenol, twice with equal volumes of phenol/chloroform and finally by two equal volume chloroform extractions. The DNA was ethanol precipitated, redissolved in 10 µl of TE and quantified on a mini agarose gel.

Genomic DNA:

Genomic DNA extraction was carried out as Mazza (1989). Eighty to 130 mg of flies were ground in 1.5 ml of 10 mM Tris-Cl (pH 7.5), 60 mM EDTA, 0.15 mM spermidine and 50 µg/ml RNase A in a tissue grinder. The mixture was then incubated for 30 min at 37°C. After addition of 1.5 ml of 0.2 M Tris-Cl (pH 9.0), 30 mM EDTA, 2% SDS, and 0.4 mg/ml

pronase E, the mixture was incubated for 90 min at 37°C. The suspension was then extracted twice with an equal volume of phenol, once with phenol/chloroform and once with chloroform. The DNA was precipitated by adding NaCl to the aqueous phase to 0.2 M and then adding 2 volumes cold 95% ethanol. The DNA was pelleted gently at 2000 RPM for 5 min in a clinical centrifuge and then washed with 70% ethanol and recentrifuged. After dissolving the DNA in 1 ml TE by shaking gently at 37°C, the DNA was spermine precipitated by adding KCl to 100 mM, spermine (pH 6.8) to 10 mM and incubating 30 min on ice. The DNA was collected by gentle centrifugation, washed with 10 ml of 75% ethanol, 300 mM sodium acetate, 10 mM magnesium acetate and left 2 - 4 hrs on ice. After repelleting, the DNA was washed in 10 ml of 70% ethanol, repelleted and dissolved in 1 ml TE. The DNA was stored at 4°C until use (up to 2 yrs. later).

Gel purification:

DNA fragments were purified by electrophoresis on a 0.7% agarose gel followed by excision of the desired band and purification of the DNA using the Geneclean I or II kit from Bio 101.

Phosphatase treatment:

Terminal phosphate groups were removed from DNA fragments by adding 1 u calf intestinal alkaline phosphatase (Boehringer Mannheim) to a restriction digest after it had

proceeded at least 3 hrs. The phosphatase reaction was allowed to continue for another 1 to 4 hrs. The DNA was isolated by gel purification.

DNA sequencing:

Single and double stranded sequencing were carried out using the method of Sanger *et al.* (1977). Generally, the Klenow fragment of DNA Pol I was used for single stranded sequencing and T7 DNA Polymerase supplied in a Pharmacia sequencing kit was used for double stranded sequencing. Radiolabelled [α-^{35}S]-dATP was used, gels were dried and autoradiographed with intensifying screens.

Oligonucleotide-directed *in vitro* mutagenesis

Most procedures were derived from the Amersham booklet of the above name published in 1986. The oligonucleotides were purchased from Dr. Ken Roy, Department of Microbiology, University of Alberta with one exception (see Figure 9 of Results section and corresponding text).

Single stranded DNA preparation:

Single stranded template was prepared by cloning the region to be mutagenized (either *Ddc*$^{+}$ or *Ddc*$^{+4}$) into the phagemid vector pVZ1. A single colony of the clone was used

to inoculate 100 ml of 2X YT containing 100 µg/ml ampicillin and about 1 X 10^8 plaque forming units(pfu) of the helper phage M13KO7 (Vieira & Messing, 1987) in a 500 ml Erlenmeyer flask. Following incubation at 37°C at 225 RPM for 1 hr, 4.8 µl of 25 mg/ml kanamycin was added to give a final concentration of 40 µg/ml. Incubation was continued under the same conditions for 15 - 18 hrs. The mixture was portioned into 3 SS34 rotor tubes and centrifuged at 10 000 RPM in an SS34 rotor at 4°C for 30 min. Approximately 80 ml of supernatant was transferred to a 150 ml Corex bottle and 0.2 volumes of 20% PEG-8000 in 2.5 M NaCl were added. Vigorous mixing followed by one hour on ice preceded a 20 min centrifugation in the GSA rotor at 4°C, 5000 RPM. Supernatant was discarded followed by another 5 min spin under the same conditions. Any remaining supernatant was removed using a drawn-out Pasteur pipette and the pellet resuspended in 500 µl TE and transferred to a 1.5 ml eppendorf tube. To remove any remaining cells, a 5 min microcentrifugation was performed followed by the addition of 200 µl 20% PEG-8000 in 2.5 M NaCl. The precipitation was mixed well, left to stand at room temp. for 15 min and centrifuged 5 min. The supernatant was discarded, the pellet recentrifuged for 2 min and all remaining PEG removed. Following addition of 200 µl phenol, the mixture was vortexed 20 sec, left to stand 15 min, vortexed for another 20 sec and centrifuged 2 min. The aqueous layer was then transferred to a fresh tube. The phenol extraction was

repeated, and followed by four diethyl ether extractions in which 500 µl ether was added and the top layer, along with any interphase, was discarded. This was followed by two 500 µl chloroform extractions. The resulting solution was made 0.3 M with respect to sodium acetate and then 2.5 volumes of 95% ethanol were added. Following incubation at -70°C for 20 min, the mixture was centrifuged for 15 min at 4°C and then washed with 1 ml cold 70% ethanol. The pellet was desiccated and redissolved in 75 µl TE. Following a 1000-fold dilution, the optical density was measured at 260nm and 280nm to determine concentration and purity. The DNA solution was finally diluted to 1 µg/µl. This procedure typically yielded 180 µg of single-stranded DNA.

Oligonucleotide design:

Four factors were taken into account in the design of oligonucleotides. First, palindromic sequences were avoided where possible; if impossible, the oligonucleotide was designed so that purine-purine match-ups would occur at the terminus of a self-annealed oligonucleotide in the hope that this would destabilize the hairpin structure. Second, G + C content was kept above 40% where possible and the size was kept at 19 - 21 bases. Third, the base(s) to be mutated were positioned at least 5 bases and 2 or more guanines or cytosines from either terminus. Finally, the oligonucleotide was designed with one or two guanines or cytosines at its 3' terminus to stabilize priming.

5'-phosphorylation of oligonucleotides:

In an Eppendorf tube were mixed: 4.8 µl of a 10 pmol/µl solution of oligonucleotide, 3 µl 10 mM ATP, 3 µl 100 mM DTT, 3 µl 10X kinase buffer (100 mM Tris-Cl (ph 7.5), 100 mM $MgCl_2$, 1 mg/ml BSA), 16 µl ddH_2O and 10 u T4 polynucleotide kinase. The mixture was incubated at 37°C for 30-45 min.

Annealing and extension:

Annealing between the mutant oligonucleotide and the single-stranded template was carried out in a 1.5 ml Eppendorf microfuge tube. The amounts were calculated to yield a ratio of 3 primer molecules per template molecule. Single-stranded template (10 µl at 1 µg/µl), 5 µl phosphorylated oligonucleotide (1.6 pmol/µl), 7 µl annealing buffer (250 mM NaCl, 200 mM Tris-Cl (pH 7.5), 50 mM $MgCl_2$) and 12 µl ddH_2O were mixed together and the capped tube placed in a 75°C temp blok for 3 min and then submerged in a 37°C water bath for 30 min. The mixture was quickly centrifuged and then placed on ice before setting up the extension reaction.

The synthesis reaction of the mutant DNA strand was set up on ice. To the annealing reaction were added: 10 µl 10 mM ATP, 2 µl 100 mM DTT, 10 µl 100 mM $MgCl_2$, 38 µl nucleotide mix (1.3 mM dCGTTP and 2.6 mM dATP) and 12 u Klenow (the large fragment of DNA Pol I). The reaction was incubated 5 min on ice, 10 min at room temperature and 30 min to 1 hr at 37°C. Finally, 4 u Klenow and 8 u T4 DNA

ligase were added and the reaction mixed well and submerged in a 16°C water bath overnight.

Transformation:

Transformation was carried out using XL-1 Blue competent cells. The extension reaction was diluted 1 µl in 999 µl SOC medium (see Stratagene XL-1 Blue Protocol), 2 µl of this dilution was transformed by the Stratagene protocol and 2 - 50 µl of the transformation was plated. Transformants were screened via filter colony hybridization.

Filter colony hybridization

Patching and lifting:

E. coli transformants (along with a positive and negative control where possible) were patched onto a 6 X 9 grid on two LB + amp plates - one to be lifted and one to serve as a master - which were incubated for 12 - 18 hrs at 37°C. The plate to be lifted was cooled to 4°C and a nitrocellulose filter (Millipore HATF 082 50) was placed on the plate and left for at least 10 min. The filter and plate were marked for later orientation and the filter was placed face up in a petri dish containing .5 M NaOH/1.5 M NaCl for 5 min. Taking care not to carry over too much liquid, the filter was transferred to a petri dish containing .5 M Tris-Cl (pH 7.5)/2.5 M NaCl and left for 5 -

15 min. The filter was then air dried on a piece of Whatman 3MM chromatography paper and incubated for at least an hour at 80°C under line vacuum.

Probe preparation and hybridization:

Probe preparation and hybridization were essentially as described by Carter (1987). End-labelled probes were synthesized by putting 1.5 µl of a 10 pmol/µl oligonucleotide solution in an eppendorf tube with 1 µl 100 mM DTT, 30 µCi [γ-^{32}P]ATP, 3 µl 10X kinase buffer (100 mM Tris-Cl (ph 7.5), 100 mM $MgCl_2$, 1 mg/ml BSA), ddH_2O to 30 µl and 10 u T4 polynucleotide kinase. The mixture was incubated 30 - 45 min at 37°C and then diluted with 4 ml 6X SSC in a petri dish.

The filter was prehybridized at 67°C in 30 ml of 10X Denhardt's Solution, 6X SSC and .2% SDS for at least 5 min. The filter was rinsed in 50 ml of 6X SSC and then placed face up in the probe solution. The petri dish containing the filter and probe was wrapped with two strips of Parafilm "M" and bound longitudinally with two strips of TimeTape 3/4" label tape which was found not to come off in the water bath. The petri dish was placed in a gently shaking 42°C water bath for 1 hr, followed by a similarly shaking 37°C water bath for 30 min and finally at room temperature for 30 min to 6 hrs. The filter was washed at room temperature in 100 ml 6X SSC 3 times for about 1 min with gentle swirling. The filter was finally washed in 100 ml 6X SSC just below

the empirically derived dissociation temperature of the oligonucleotide. This was found to correspond very closely (<1.5°C difference) to the theoretical dissociation temperature in 6X SSC calculated as follows: T_d(°C)= 4X(G + C) + 2X(A + T). The filter was wrapped in Saran Wrap and autoradiographed with the orientation marks of the filter being marked on the film.

Microinjection

Microinjections were performed as per Rubin and Spradling (1982) with some modifications.

Embryo collection:

Collections of the *y w* injection host strain were made by inserting a 35 mm petri plate of grape agar (1.5% agar, 1% Welch's purple grape juice concentrate, 1% sucrose; pH 7.4), smeared with fresh yeast paste, into a 2200 cm^3 population cage (Tyler Research) containing several thousand (8 - 10 bottles) 3 to 6 day old flies. The collections were made in the dark.

Embryo preparation:

Embryo collections, dechorionation and microinjection were all performed at 20°C. Embryos were dechorionated either mechanically or chemically. In either case, the embryos were initially washed twice with ddH_2O in a

Millipore filter apparatus onto a Whatman GF/C glass filter. Mechanical dechorionation was as previously described (Mazza, 1989). Chemical dechorionation was carried out by washing the embryos in a fresh 50% Javex bleach solution for 45 to 55 sec., followed immediately by several washes of ddH_2O. Dechorionation was carried out at 50% - 70% humidity. Embryos were subsequently desiccated for 11 - 19 min over Dri-rite H_2O absorbant in a sealed container.

Embryo injection:

A Leitz micromanipulator-microscope assembly was used for microinjecting the embryos. Any embryos older than four hours (*ie.* those with visible pole cell formation) were destroyed. The injection needles were made by pulling 25 µl Drummond micro cap pipettes with an electrode puller (Kopf Scientific). Injection solutions were prepared by co-precipitating the experimental construct and pπ25.7wc (a cloned P element incapable of transposition) DNA or pπ25.1 at a ratio of 1.2:1 molecules, respectively. The DNA was resuspended in injection buffer (5 mM KCl, .1 mM sodium phosphate pH 7.2) to a final DNA concentration of 350 µg/ml.

Injected embryos were covered with a minimum (1.2 mm to 1.4 mm) of Halocarbon oil (Series 700) and maintained in a humidified chamber at 18°C for 50 to 75 hours. All first instar larvae were transferred to 100 mm petri plates containing standard fly medium and incubated in a humidified chamber at 25°C. From 4 to 11 days later, any pupae found

were removed to vials at room temperature and adult flies (G_0) collected as they eclosed.

Eclosed G_0's were mated individually to 3 males or 4 females. Each fertile mating was transferred up to 3 times to additional vials and the G_1 progeny scored for the presence of any eye pigmentation. Male transformants always had a darker eye colour than female transformants, presumably due to dosage compensation of the *white*$^+$ gene carried on the P-element (Klemenz *et al.*, 1987). The eye colours obtained ranged from a very pale lemon to almost wild type and two strains displayed a variegated phenotype. No correlation was found between the degree of eye pigmentation and the DDC activity of a given strain.

A total of 15,622 embryos were injected. The average survival to the 1st instar larval stage was 42% with the daily figure varying between 14% and 64%. The average survival from the 1st instar to the pupal stage was 41% with a range of 18% to 63%. Sixty-seven percent of pupae eclosed (range: 53% to 83%) and of these, 71% were fertile (range: 54% to 93%). Transformed progeny were found in from 0.9% to 21% of G_0's with the average being 6.8%. A total of 49 transformants was recovered. See Figure 4 for a list of the transformants, their chromosomal location and the heterozygous eye colour phenotype. Although the eye colours varied greatly, this was not felt to warrant concern for two reasons. First, the *white* gene product is required in the mid-pupal stage, a time when *Ddc* is not expressed. Second,

the sensitivity of eye colour to the amount of *white* product was not known and thus the magnitude of the position effects could not be accurately assessed.

Figure 4. A list of the transformants generated, their strain designations, the chromosomal location of the recombinant P element(s), and the eye colour phenotype of heterozygous females. Note that strain 28-3 has been found to contain 3 inserts and strain 135-3 has been found to contain 4 inserts. All other strains contain one insert; asterisks indicate strains for which the number of inserts was not determined.

Injected plasmid	Strain	Chromosome	Heterozygous eye colour
pDdc29a	11*	II	light purple
	15*	II	orange
	28-1*	II	brownish orange
	28-3	III	light wild type
	31*	II	variegating orange
	35b*	II	very light orange
	38*	II	orange
	62*	II	orange
	71*	III	wild type
	104	I	light orange
	126	III	reddish orange
	135-1	I	orange
	135-3	III	light wild type
pDdc29b	43	III	light orange
pDdc30a	25*	II	orange
	25-3*	II	orange
	41-2a	III	reddish purple
	41-2b	III	orange
pDdc30b	12*	II	wild type
	16	II	cinnabar
pDdc31	1	II	light orange
	2b	I	light orange
	2b-1	I	dark orange
pDdc32	7	III	dark purple
	13*	II	light orange
	13-1*	III	light wild type
pDdc33	9	II	dark peach
	10	III	orange
pDdc36	14*	II	very light peach
	56	II	peachy-brown
pDdc39	8	III	light wild type
	53	III	deep orange
	87-1	III	rusty orange
	87-3	I	orange
pDdc40	3	III	peach
	3-2	III	wild type
	48	III	deep orange
	69-2*	II	orange
	134*	I	light orange
	146	III	dark orange
pDdc41	6	III	orange
	20-2	III	reddish purple
	20-3*	I	light orange
	66*	I	peach
	79*	III	light orange
	80*	II	pale lemon
pDdc42	24	I	purple
	44	I	orange
	44-1	III	wild type
	44-3	III	wild type

Strain establishment:

Up to four progeny from each transformed G_0 (the percent of transformed G_1's ranged from 0.2% to 20%) were mated individually to the *y w* injection host strain. The G_2 heterozygous progeny occasionally segregated two or three different eye colours and in these cases, representatives of each eye colour class were outcrossed to the *y w* host strain for at least two generations. On the other hand, if the eye colour was consistent and homogeneous, the G_2 heterozygous progeny of these sublines were mated in single pairs and homozygote G_3's scored by a marked darkening of eye colour. Two serial, single pair matings of homozygotes were set up and any instability of the inserts noted either by a change in the characteristic eye colour or by the presence of white-eyed flies. If no instability was found, a strain was thus established. A male was then mated to the multiple balancer strain *y w; Ddc^{ts2}cn/CyO; Sb/Ubx*. Subsequent crosses allowed the chromosomal location of the insert to be determined as well as the introduction of the insert into a Ddc^{ts2} background. This allowed DDC levels contributed by the exogenous gene to be measured against the minimal background enzymatic activity contributed by the endogenous locus.

Some inserts were recovered on chromosome 2. These were recombined onto the Ddc^{ts2} *cn* chromosome using *cn* as a marker. Since *Ddc* and *cn* are 10 map units apart, only inserts that were unlinked (or nearly so) to *cn* could be

recombined in this manner without running the risk of the *Ddc*^{ts2} and *cn* markers becoming separated. Thus, some transformed strains had to be maintained in a non-*Ddc*^{ts2} background.

Developmental staging:

Embryos were collected by taping a grape agar petri plate (60mm X 15mm; Fisher Scientific Co.) with a dab of live yeast paste on it to the top of a bottle and allowing the flies to lay for 1 hr. The plate was then placed at 26°C for 19.5 hours. All first instar larvae were removed from the plate at this time and 1 to 1.5 hours later all the hatched larvae were collected and placed immediately at -70°C. This process was repeated until 100 - 150 larvae were collected.

To collect the 2-3 moult stage, 3 to 6 day old adults were transferred to fresh bottles with a dab of live yeast paste and allowed to lay for 1½ to 2 hours. This was repeated once or twice and the bottles placed at 26°C for 76 hrs or alternatively at 27°C for 48 hrs and then room temperature for 24 hrs. Water was then swirled over the surface of the food and the larvae thus liberated were collected and washed on a Nitex screen. The larvae were placed in a drop of water on a petri plate and examined under a dissecting microscope. Based on the morphology of

the anterior spiracles and the degree of tanning of the posterior spiracles, all third instar larvae were removed. The remainder were placed in vials for 1½ hours at which time 5 - 10 third instars were removed and frozen at -70°C.

White prepupae were collected from the sides of bottles. They were examined under a dissecting microscope for a lack of tanning of the opercular ridges which are the first structures to become pigmented. In the case of X chromosome inserts, they were scored for sex as well and only females were chosen. Three white prepupae were used for each assay.

Newly eclosed flies were obtained from bottles 45 minutes after clearing and were aged for a half hour before freezing. Three adults were used for each assay (3 females in the case of X chromosome inserts and usually 2 females and 1 male in the case of autosome inserts).

Organisms from each stage were collected on at least two different occasions for each strain. Most samples were collected during the summer of 1991 and most DDC assays were performed during the summer of 1992. The whole organisms thus remained in the -70°C freezer for approximately one year before assays were performed. Based on comparisons between assays done on samples immediately after collection, and the results of assays done on year-old samples, an average deterioration of 22% was found. Samples could be stored in the freezer for up to 3 months before any significant deterioration became apparent. Therefore the

DDC activities of any samples which were assayed within three months of collection were reduced 22% in order to be comparable with the bulk of the data.

DDC and protein assays:

DDC assays were as per Walter *et al.* (1991). Newly hatched 1st instar larvae were ground at a concentration of 2 organisms per microlitre. Newly moulted 3rd instar larvae were ground in 15 µl per organism. White prepupae and adults were ground in 100 µl per organism. For each homogenate, three 5 µl aliquots were assayed with a typical standard deviation of 3%. One unit of DDC activity represents the amount of tritiated dopa (from NEN; determined by counting in Scinti Verse II scintillation cocktail (Fisher Scientific) in a Beckman LS 7500 scintillation counter) decarboxylated in 30 min at 30°C.

Protein determinations were made using the Coomassie Protein Assay Reagent kit from Pierce. Each homogenate was diluted 1:100 in duplicate and assayed. DDC activities were not corrected for protein concentration for reasons outlined in the discussion.

Southern analysis:

Southern hybridization was performed on the DNA of all strains in order to determined the copy number of the

inserts. DNA was digested and subjected to electrophoresis overnight on a 0.6% agarose gel made with TAE. A Southern transfer was set up using the nylon membrane GeneScreen*Plus* from Du Pont and all further preparation of and hybridization to the blot was as per the manual supplied with the membrane. For the hybridization, from 5 X 10^5 to 1 X 10^6 counts per ml of probe solution were added. For a representative autoradiograph, see Figure 10.

Results

1. Mutagenesis and deletion of element S

In order to render the *Ddc*$^{+}$ and *Ddc*$^{+4}$ cloned alleles more amenable to gene splicing techniques, they were first subcloned into pUC19 (Yanisch-Perron *et al.*, 1985). *Pst*I fragments containing the *Ddc*$^{+}$ and *Ddc*$^{+4}$ genes were liberated from the plasmids p10 and pZ (Chen, 1986), respectively. The fragments were gel purified and ligated to a *Pst*I digested, pUC19 vector from which the terminal phosphates had been removed to create pDdc23 (*Ddc*$^{+}$) and pDdc24 (*Ddc*$^{+4}$) (see Figure 5 for plasmid maps). The subclones were verified using restriction mapping with several enzymes, some of which are polymorphic between *Ddc*$^{+}$ and *Ddc*$^{+4}$ (see Figure 6 for the *Ddc* restriction map). In the course of verifying the subclones, two previously unnoted polymorphisms were found. One was an *Nco*I site at -228 only present in *Ddc*$^{+4}$, the other was a *Ddc*$^{+}$-specific *Sst*I site located in the third exon. DNA from the two subclones was prepared by the large scale isolation procedure in such quantity as to serve as a homogeneous source of DNA for the entire course of these studies.

To mutagenize element S, a program of *in vitro* mutagenesis was undertaken. To facilitate the isolation of

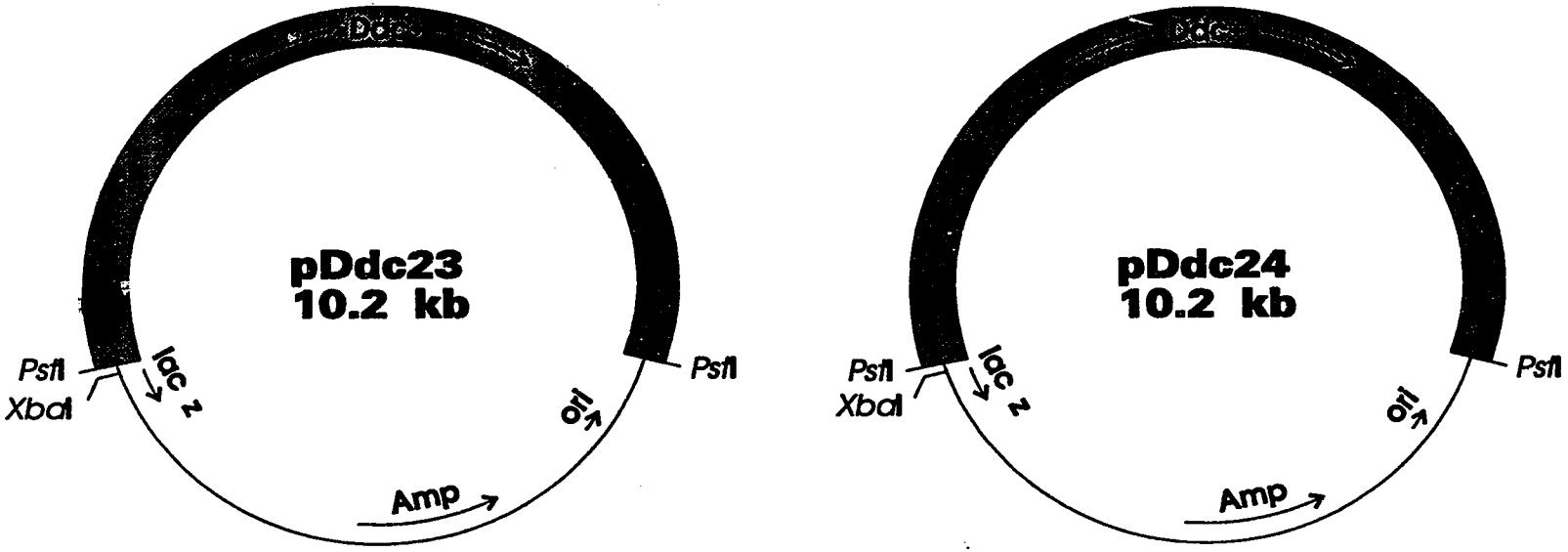
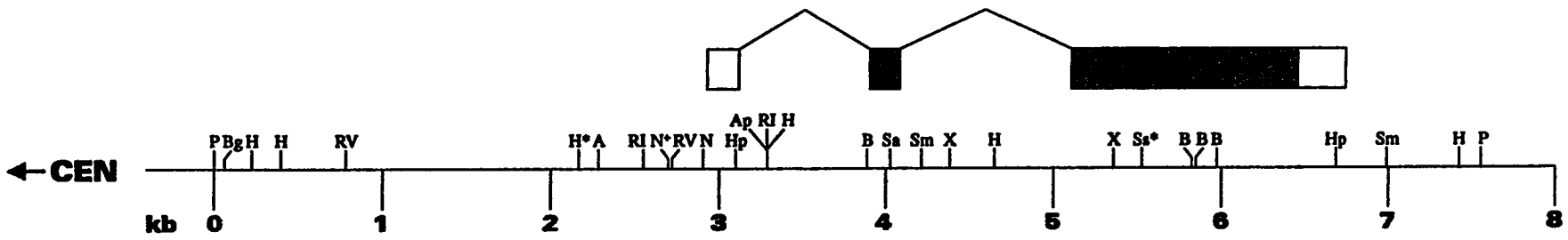


Figure 5. The subclones resulting from the introduction of the two *Ddc* alleles into pUC19 at the *Pst*I site. Boxed regions indicate *Ddc* DNA, thin lines indicate pUC19 sequences and thin arrows indicate direction of transcription or replication.



A - *Aat*II
Ap - *Apa*I
B - *Bam*HI
Bg - *Bgl*II
RI - *Eco*RI
RV - *Eco*RV
H - *Hin*dIII
Hp - *Hpa*I
N - *Nco*I
P - *Pst*I
Sa - *Sal*I
Sm - *Sma*I
Ss - *Sst*I, *Sac*I
X - *Xho*I
no sites for: *Kpn*I, *Not*I, *Xba*I
\+ - not present in Ddc^{+}
* - not present in Ddc^{+4}

Figure 6. Restriction map of the *Ddc* 7.55 kb *Pst*I clone. Symbols used are shown below the map; CEN indicates the direction of the centromere. Above the map is shown the epidermal *Ddc* transcript; empty boxes are non-translated exonic sequences; shaded boxes are translated exonic sequences; thin lines are intronic sequences.

single stranded DNA for use in oligonucleotide directed *in vitro* mutagenesis, the *Ddc* fragments from pDdc23 and 24 were subcloned into the phagemid vector pVZ1. pVZ1 is a derivative of the Stratagene vector pBS(+) modified by Steve Henikoff by the addition of a 33 bp linker into the *Eco*RI site. This addition introduces several unique restriction sites (*Not*I, *Nar*I, *Bde*I, *Bst*XI, *Nsi*I, *Apa*I). The *Ddc* fragments were liberated from pDdc23 and 24 by *Pst*I/*Bgl*II double digestion,purified from an agarose gel and ligated into pVZ1 digested with *Pst*I and *Bam*HI. These clones are designated pDdc25 (Ddc^{+}) and pDdc26 (Ddc^{+4}) and result in the template strand of *Ddc* being packaged under conditions of helper phage infection. Large scale preparations of single-stranded DNA from these clones were made and the DNA was utilized for all of the subsequent mutagenesis experiments.

In vitro mutagenesis was then performed (see Materials and Methods) to mutate the Ddc^{+} element to resemble the Ddc^{+4} sequence and *vice versa*. The two mutagenic oligonucleotides along with the wild type sequences are shown in Figure 7.

Saturated cultures were prepared from the clones identified as positives by filter colony hybridization and the DNA was isolated. It was characterized following cleavage with *Nco*I and *Hind*III followed by agarose gel electrophoresis to confirm their identity. The proposed mutagenesis of pDdc25 should create an *Nco*I site at -228

Ddc^{+} element S: $^{-234}$AATCCATGAAAAATAATGCCTTTGATATC^{-206}

mutagenic primer: CCATGGAAAATAATGCCTTTG

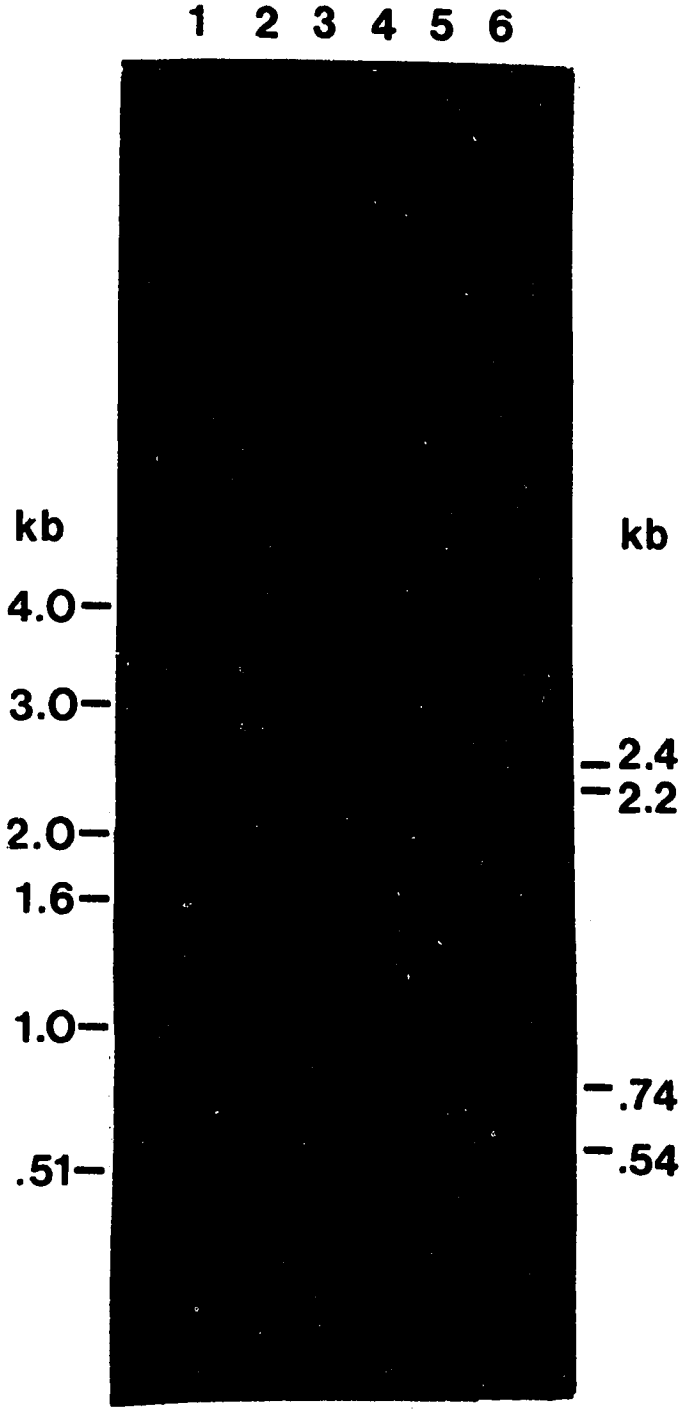
Ddc^{+4} element S: $^{-234}$AATCCATGGAAAATAATGCCTTTGATATC^{-206}

mutagenic primer: CCATGAAAAATAATGCCTTTG

Figure 7. The element S sequences from the *Ddc*$^{+}$ and *Ddc*$^{+4}$ alleles are shown, along with the oligonucleotides used to create the reciprocal transition mutations. Element S itself is denoted by underlined bases and mutations are indicated by bold bases.

and therefore a 0.74 kb band from an *NcoI/Hind*III digest of *Ddc*$^{+}$ DNA should disappear in the mutants and two new bands, of sizes 0.54 kb and 0.2 kb should appear in its place. In pDdc26, an *Nco*I site is eliminated and so a 2.2 kb and a 0.2 kb band should be replaced by a 2.4 kb band in the mutant. When electrophoresis was performed, all three bands were seen in both cases. Presumably this was due to mixed populations of plasmids within each cell and so the DNA from two separate isolates from each mutagenesis was retransformed at a limiting amount of 1 ng (Hanahan, 1983). Upon rescreening, several clones were isolated which demonstrated the desired restriction patterns (Figure 8). The plasmids pDdc27a and b are the wild-type alleles with the *Ddc*$^{+4}$ change introduced and pDdc28a and b are the *Ddc*$^{+4}$ alleles with the wild-type change introduced.

Figure 8. Agarose gel showing the RFLP's associated with the mutageneses. Lanes 1 and 6 contain DNA size markers (BRL); lane 2 contains pDdc25 DNA; lane 3 contains pDdc27a DNA; lane 4 contains pDdc26 DNA; lane 5 contains pDdc28a DNA. The DNA in lanes 2 - 5 was digested with *Nco*I and *Hind* III. Note the 0.74 kb band in lane 2 is lost in lane 3 to be replaced by a 0.54 kb band and a 0.2 kb band (not well visualized). The 2.2 kb band of lane 4 is lost in lane 5 and replaced by a 2.4 kb band.


1 2 3 4 5 6
kb
4.0
3.0
2.0
1.6
1.0
.51
kb
2.4
2.2
.74
.54

The products of the *in vitro* mutagenesis experiments were further confirmed by sequencing utilizing a 20-mer oligonucleotide which primes from -294 relative to the mRNA transcription start site . All four were then sequenced from -280 to +80. The plasmids pDdc27a and b were identical to the wild-type control (pDdc25) except for the intended transition. However, an insertion of two thymines 5' of position -264 was found in pDdc28a and b. This insertion differs from the previously determined Ddc^{+4} sequence (Spencer, 1986) and the wild-type sequence. This difference was also present in the control (pDdc26) and in pDdc24 as revealed by double stranded sequencing.

In order to introduce the various constructs into the germline of Drosophila, recombinant P elements were constructed. The pW8 vector of Klemenz *et al.* (1987) was utilized for this purpose (Figure 9A). The *Ddc* alleles were liberated from pVZ1 with a *Kpn*I/*Pst*I double digest and subcloned into pW8 cut with the same enzymes. The resulting constructs were designated pDdc29a and b (from pDdc27a and b) and pDdc30a and b(from pDdc28a and b). Their organization is indicated in Figure 9B.

The respective controls, Ddc^{+} and Ddc^{+4} were also subcloned into pW8. The fragments were liberated from pDdc23 and pDdc24 by *Pst*I digestion, purified from an agarose gel and ligated to *Pst*I digested pW8, treated with phosphatase. The results were pDdc31 and pDdc32, respectively. The Ddc^{+} allele was also recovered in pW8 in

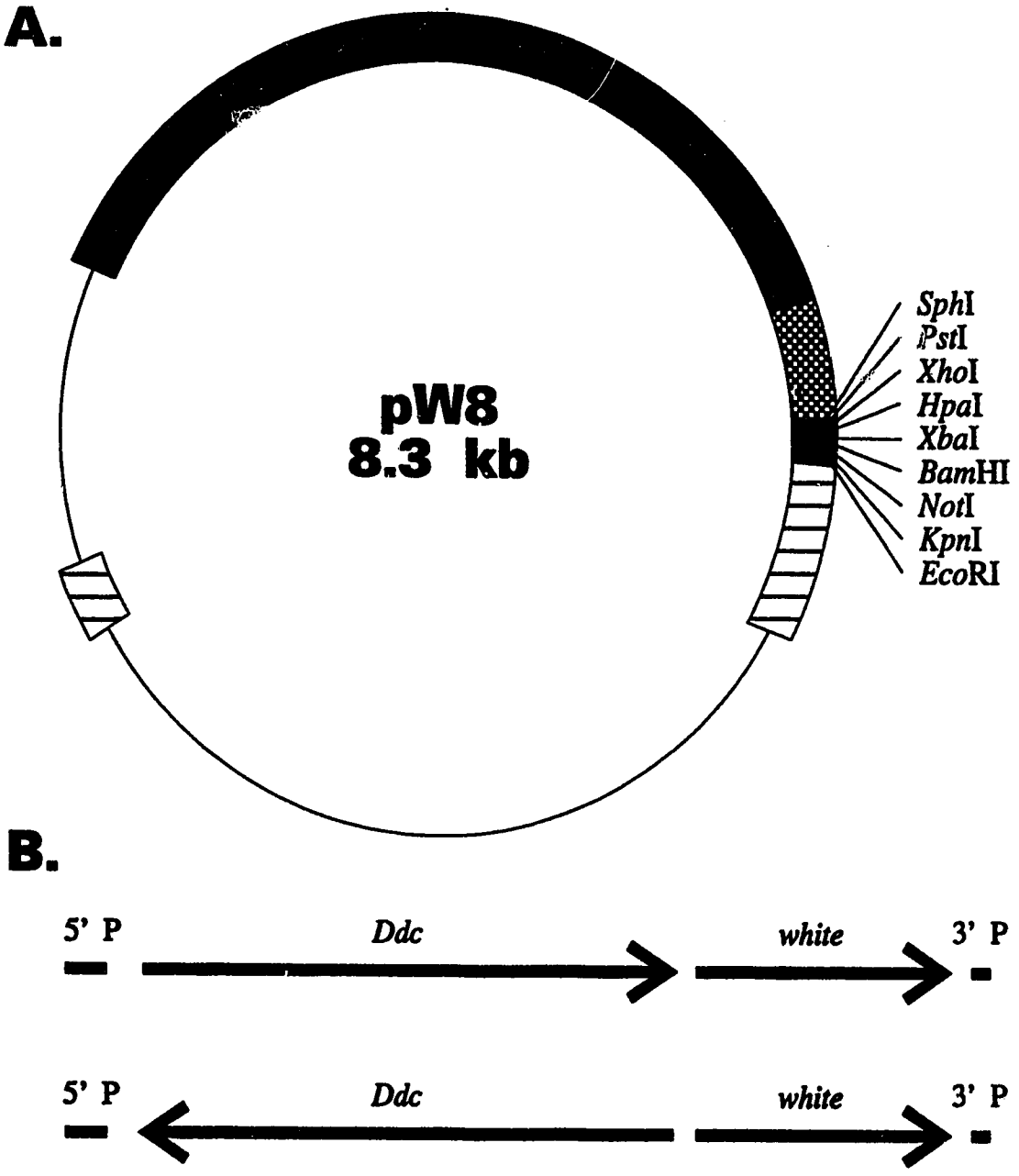


Figure 9. A) Plasmid map of pW8 derived from Klemenz *et al.* (1987). The transposable part of this vector comprises 4.6 kb. Icons used are as follows: thin lines, plasmid sequences; black box, pW8 polylinker; grey box, *white* DNA; checkered box, 388 bp *hsp 70* promoter/leader fragment; horizontally striped boxes, P-element sequences with the larger box being the 5' end.

B) Organisation of the recombinant P-elements showing the 5' end of the P-element, followed by the *Ddc* clone, followed by the *hsp 70/white* gene clone, followed by the 3' end of the P-element. Arrows indicate the the direction of transcription. The upper delineation indicates the organisation of all subclones discussed in thesis except pDdc33, shown in the lower delineation.

the opposite orientation (Figure 9B) and was designated pDdc33.

Upon microinjection of the recombinant P elements described above, several transformants of each construct were obtained and strains established. Following introduction of the inserts into a Ddc^{ts2} background, Southern analysis was performed to determine the copy number of the inserts. Figure 10 shows a representative autoradiograph. Samples were collected at the four developmental stages under study and DDC assays performed. The results of the mutation in element S in a Ddc^{+} background are reported in Table I. The results of the mutation in element S in a Ddc^{+4} background are reported in Table II.

As a further test of the functional importance of element S, a deletion of the Ddc^{+} element was constructed. The plasmid pDdc27a was utilized as the substrate as it contains two restriction sites immediately flanking element S - an *Eco*RV site located at -208 and the mutagenically engineered *Nco*I site at -228 (Figure 11). A double digest (in REact 4 from BRL) of *Nco*I followed by *Eco*RV was performed on pDdc27a and following agarose gel electrophoresis, the 1.9 kb fragment was gel purified. This fragment was end-filled using the Klenow fragment of DNA Polymerase I according to Maniatis *et al.* (1982), and was subcloned into 8.3 kb *Eco*RV fragmentfrom pDdc23 from which the terminal phosphates had been removed. This resulted in

Figure 10. Sample autoradiograph used to determine copy number of the P element inserts. Genomic DNA was digested with *Hpa*I, subjected to electrophoresis and transferred by Southern blotting. The probe used was a 1.9 kb *Eco*RV fragment from the upstream portion of *Ddc* (coordinates 0.8 to 2.7 on figure 6). The 5.0 kb band is from the endogenous locus, all other bands derive from exogenous copies of *Ddc*. Lane designations are as follows:

Lane 1 - Strain 6

Lane 2 - Strain 41-2a

Lane 3 - Strain 28-3

Lane 4 - *y w;Ddc^{ts2}cn/CyO;Sb/Ubx*

Lane 5 - *y w* (injection host strain)

Lane 6 - Strain 8

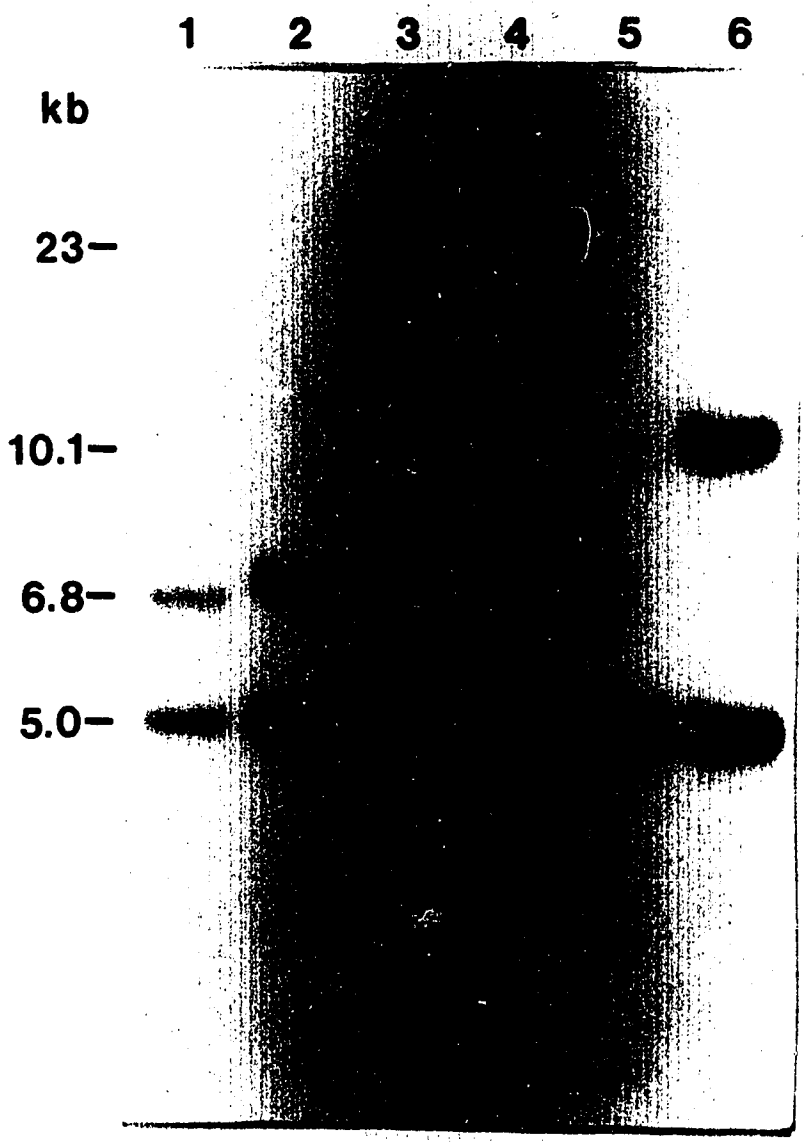

1
2
3
4
5
6
kb
23–
10.1–
6.8–
5.0–

Table I. Units of DDC activity in *Ddc*$^{+}$ (tDdc31 and tDdc33) strains and those *Ddc*$^{+}$ strains with a *Ddc*$^{+4}$ version of element S (tDdc29a and b) at four stages of development. The developmental stage is indicated in bold type, beneath which the nature of the insert is indicated. Note that strains transformed with pDdc33 are denoted tDdc33 to distinguish the transformants from the plasmids with which they were injected. The mean for each strain represents an average of the individual determinations made on that strain (Samples 1 - 4). The means for each strain were averaged to yield an overall mean for that construct type along with its standard error (SEM). The original values for strain 28-3 have been divided by 3 to correct for insert number.

Transformant	Sample 1	Sample 2	Sample 3	Sample 4	Mean	Mean ± SEM	
Hatch							
tDdc33 -Strain 9	357	304			331		
-Strain 10	321	332			327		
						329	14
tDdc29a -Strain 28-3	317	277			297		
tDdc29b -Strain 43	235	302			269		
						283	22
2nd Moult							
tDdc31 -Strain 2b	496	487			492		
tDdc33 -Strain 9	564	592			578		
-Strain 10	526	500			513		
						528	22
tDdc29a -Strain 28-3	584	481			533		
-Strain 104	521	556			539		
-Strain 126	601	525			563		
tDdc29b -Strain 43	577	394			486		
						530	32
Pupariation							
tDdc31 -Strain 2b	5099	5062			5081		
tDdc33 -Strain 9	4593	4658	4525		4592		
-Strain 10	3832	4980	4272	3452	4134		
						4602	312
tDdc29a -Strain 28-3	4339	4648			4494		
-Strain 126	5549	5007			5278		
tDdc29b -Strain 43	5536	5545			5541		
						5104	277
Eclosion							
tDdc31 -Strain 2b	3741				3741		
tDdc33 -Strain 9	4097	3376	4076		3850		
-Strain 10	3411	3298			3355		
						3648	189
tDdc29a -Strain 28-3	2920	2642			2781		
-Strain 126	4184	3803			3994		
tDdc29b -Strain 43	3081	3367			3224		
						3333	363

Table II. Units of DDC activity in the *Ddc*$^{+4}$ strain (tDdc32) and in those *Ddc*$^{+4}$ strains with a *Ddc*$^{+}$ version of element S (tDdc30a and b) at four stages of development. See Table I legend for further details.

Transformant	Sample 1	Sample 2	Sample 3	Mean	Mean ± SEM	
Hatch						
tDdc32 -Strain 7	398	367		-	383	11
tDdc30a -Strain 41-2a	449	537	593	526		
-Strain 41-2b	493	462		478		
tDdc30b -Strain 16	948	1054		1001		
					668	133
2nd moult						
tDdc32 -Strain 7	676	530		-	603	52
tDdc30a -Strain 41-2a	1001	1063		1032		
-Strain 41-2b	856	714		785		
tDdc30b -Strain 16	1660	1607		1634		
					1150	208
Pupariation						
tDdc32 -Strain 7	2607	2065		-	2336	192
tDdc30a -Strain 41-2a	3148	3496		3322		
-Strain 41-2b	2808	2995		2902		
tDdc30b -Strain 16	6883	3666		5275		
					3833	805
Eclosion						
tDdc32 -Strain 7	2527	2727		-	2627	71
tDdc30a -Strain 41-2a	4128	4066		4097		
-Strain 41-2b	4251	3814		4033		
tDdc30b -Strain 16	3502	3707		3605		
					3911	150

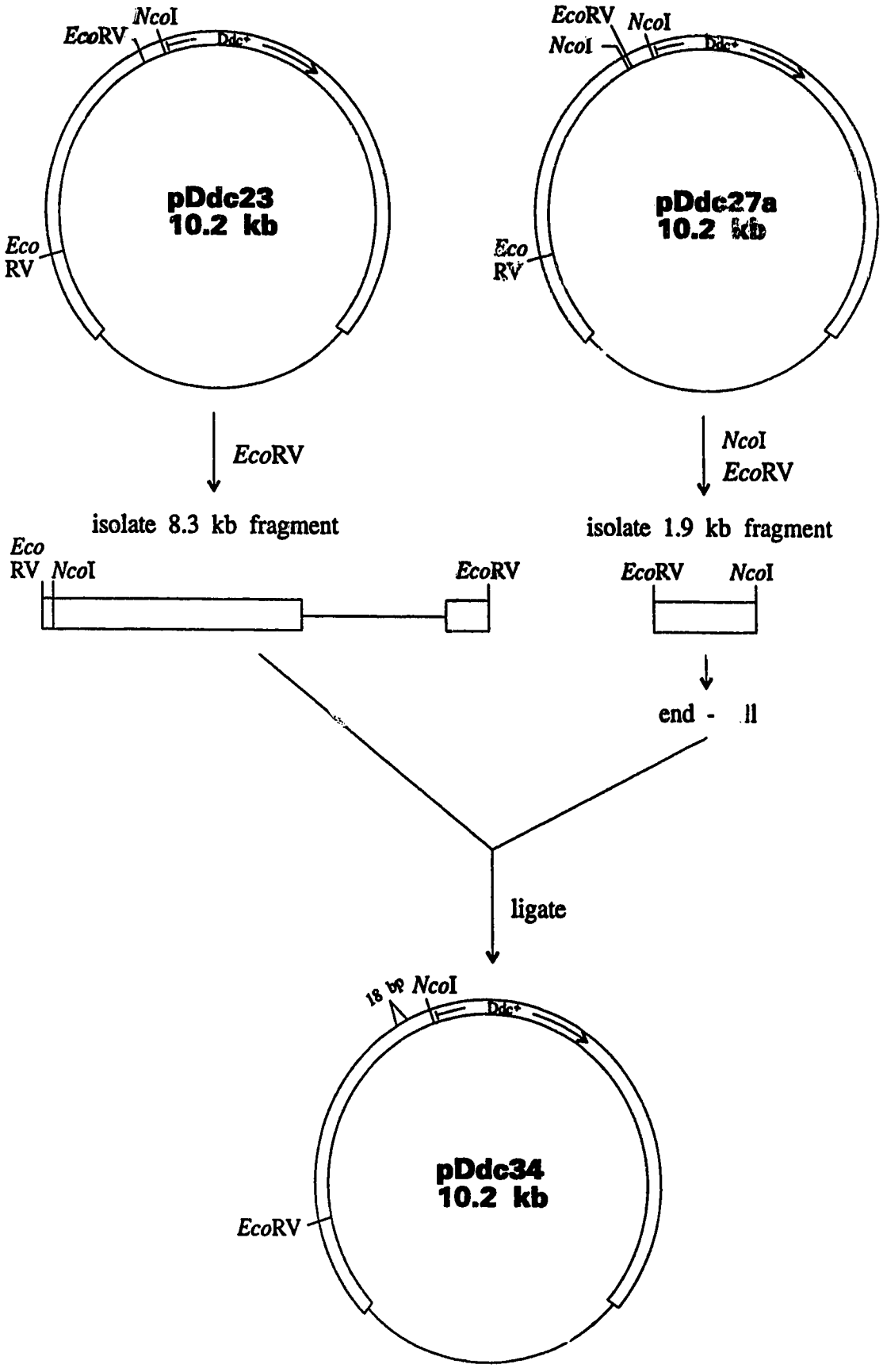


Figure 11. Deletion of Element S. See Figure 1 for iconography.

the deletion of 18 bp with the conversion of the sequence 5'-CCATG:GAAAATAATGCCTTTGAT:ATCC-3' to 5'-CCATG:ATCC-3' (element S bases shown underlined; the bases between the colons were deleted). The desired ligation product was obtained and designated pDdc34. The nature and size of the deletion was confirmed by restriction mapping and DNA sequencing.

The *Ddc* allele from pDdc34 was subcloned into pW8 using PstI digests and the resulting subclone designated pDdc36. Two transformants were obtained upon microinjection. Both were second chromosome inserts located close to the *cinnabar* locus. This made made their recovery on the Ddc^{ts2} *cn* chromosome problematic and it was therefore not attempted. For this reason, samples (and controls) in a non-Ddc^{ts2} background were collected from three developmental stages and DDC assays were performed. At this time, assays on only one of the two transformants have been completed and the results are reported in Table III.

Table III. Units of DDC activity in *Ddc*$^{+}$ strains (tDdc31 and tDdc33; containing *Ddc*$^{+}$ inserts in both orientations - see Figure 9B) and an element S deletion strain. The *y w* injection host strain, Canton S laboratory strain, Oregon R laboratory strain and *Ddc*^{ts2} *cn* strains are shown for comparison at two of the stages. See Table I for further details.

Transformant	Sample 1	Sample 2	Sample 3	Sample 4	Mean	Mean ± SEM	
2nd Moult							
tDdc31 -Strain 1	1075	1037			1056		
-Strain 2b	1330	1323			1327	1191	96
tDdc33 -Strain 9	1148	864	989		1000		
-Strain 10	989	882			936	968	72
					Avg. Ddc+:	1080	74
tDdc36 -Strain 56	1008	1128				1068	42
y w	658					658	
Ddc(ts2)	52	48				50	1
Pupariation							
tDdc31 -Strain 1	11527	11487	13102		12039		
-Strain 2b	13852	13833	14252		13979		
-Strain 2b-1	10766	10778	11110	10975	10907	12308	782
tDdc33 -Strain 9	11426	10776			11101		
-Strain 10	10354	9625			9990	10545	463
					Avg. Ddc+:	11603	677
tDdc36 -Strain 56	11449	13896	9379	12058		11696	806
y w	5768	5423				5596	122
Canton S	9500	9167				9334	118
Oregon R	6864					6864	
Ddc(ts2)	294	280				287	5
Eclosion							
tDdc31 -Strain 1	7950	8886	8395		8410		
-Strain 2b	8256	6559	6869		7228	7819	589
tDdc33 -Strain 9	8340				8340		
-Strain 10	7487	6120	8669		7425	7883	715
					Avg. Ddc+:	7851	265
tDdc36 -Strain 56	9215	8920	7480	8446		8515	329
y w	3959	3786	4023			3923	58
Canton S	7576	7398				7487	63
Oregon R	2844					2844	
Ddc(ts2)	325					325	

2. Construction of the *Ddc* fusion genes

Experiments were then undertaken to create the two fusion genes. One, pDdc37, would contain Ddc^{+} sequences 5' of -23 and Ddc^{+4} sequences 3' of -23. The second fusion construct, pDdc38, was just the reciprocal of pDdc37 (Figure 12). In order to separate the upstream (upstream being those sequence 5' of the transcription start site) and downstream portions of the gene, the *Nco*I site at -23 was utilized; this restriction site is located immediately 3' of the consensus TATA element. Thus, the downstream portions (of *Ddc*) plus vector were liberated from pDdc23 and 24 by an *Nco*I/*Xba*I digest. These 6.3 kb fragments were gel purified as was the 2.9 kb fragment representing the upstream region of pDdc23. Due to the *Nco*I site polymorphism at -228, a partial digest was performed on pDdc24 to obtain the entire 2.9 kb upstream fragment. The downstream 6.3 kb fragment of Ddc^{+4} was then ligated to the upstream 2.9 kb fragment of Ddc^{+} to produce pDdc37 and the reciprocal ligation was performed to generate pDdc38. The subclones were verified by restriction mapping.

The two fusion clones were liberated from their vectors by a *Pst*I/*Kpn*I double digest and subcloned into pW8, also cut with PstI and *Kpn*I. The results were pDdc39 (containing the *Ddc* fragment from pDdc37) and pDdc40 (containing the *Ddc* fragment from pDdc38). After microinjection, several transformants were obtained, strains were established and

Figure 12. Construction of the *Ddc* fusion genes, pDdc37 and pDdc38. Open boxes represent *Ddc*$^{+}$ sequence; shaded boxes, *Ddc*$^{+4}$ sequence; thin lines, plasmid sequences. See text for further explanation.

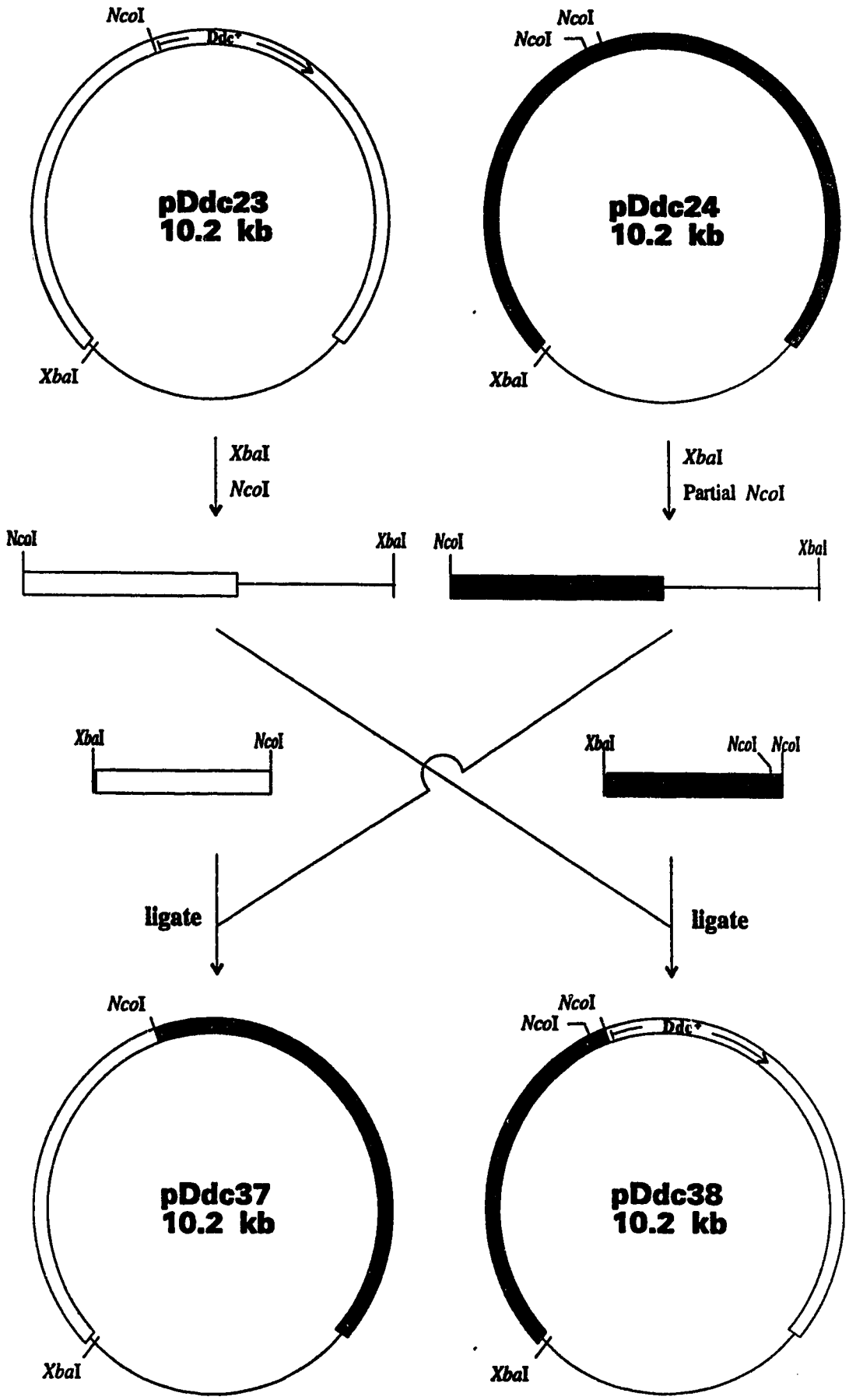

NcoI
Ddc+
pDdc23
10.2 kb
XbaI
NcoI
NcoI
pDdc24
10.2 kb
XbaI
XbaI
NcoI
XbaI
Partial NcoI
NcoI
XbaI
NcoI
XbaI
XbaI
NcoI
NcoI
XbaI
ligate
ligate
NcoI
pDdc37
10.2 kb
XbaI
NcoI
NcoI
Ddc+
pDdc38
10.2 kb
XbaI

Table IV. Units of DDC activity in *Ddc*$^{+}$, *Ddc*$^{+4}$ and the two fusion genes at two stages of development. See Table I for further details.

Transformant	Sample 1	Sample 2	Sample 3	Sample 4	Mean	Mean ± SEM	
2nd Moult							
tDdc31-Strain 2b	496	487			492		
tDdc33-Strain 9	564	592			578		
-Strain 10	526	500			513		
						528	22
tDdc32-Strain 7	676	530				603	52
tDdc39-Strain 8	755				755		
-Strain 53	1217				1217		
-Strain 87-1	765	820			793		
-Strain 87-3	817	960			889		
						913	82
tDdc40-Strain 3	504	418			461		
-Strain 3-2	445	480			463		
-Strain 48	527	588			558		
-Strain 146	562	624			593		
						519	33
Pupariation							
tDdc31-Strain 2b	5099	5062			5081		
tDdc33-Strain 9	4593	4658	4525		4592		
-Strain 10	3832	4980	4272	3452	4134		
						4602	312
tDdc32-Strain 7	2607	2065				2336	192
tDdc39-Strain 8	5157	5771			5464		
-Strain 53	6991	7188			7090		
-Strain 87-1	4893	5689			5291		
-Strain 87-3	4759	5206			4983		
						5707	432
tDdc40-Strain 3	3924	4593	3021		3846		
-Strain 3-2	3795	2775	4222		3597		
-Strain 48	4616	4406			4511		
-Strain 146	5353	4851			5102		
						4264	383

were crossed into a *Ddc*^{ts2} background. Four transformed strains for each construct were selected for study. Samples from the second moult and pupariation were collected and assayed. Table IV show the results of the DDC assays; the *Ddc*$^{+}$ control data is reproduced from Table I.

3. Mutagenesis of element IV

In vitro mutagenesis was performed on single stranded DNA isolated from pDdc25 (*Ddc*$^{+}$ in pVZ1). Two oligonucleotides were used, one purchased from Dr. Ken Roy (mutagen 1) and the other purchased from the Regional DNA Synthesis Laboratory at the University of Calgary (mutagen 2). The former oligonucleotide caused the deletion of a guanine at position -48 and its product is designated m1. The latter oligonucleotide was designed to delete the adenine at -49. However, after sequencing the mutagenesis products derived from this oligonucleotide, several unexpected changes were found (see Figure 13). In this figure, the wild type sequence and the sequence of mutagen 2 are shown along with the sequences of the three products recovered. As all the unexpected changes were located within the region specified by the oligonucleotide, it was assumed that the oligonucleotide used was partially degenerate. Bacterial mismatch repair of a palindromic sequence is an alternate explanation but is unlikely as all the changes make the sequence less palindromic. The product

wild type sequence: $^{-60}$GCGGAATTGGCAGCGCTGCTGGAC^{-37}

mutagenic primer: CGGAATTGGC GCGCTGCTG

mutagenesis products: CGGAATTGGC GCGCTGCTG

CGGAATTGGC GCGCTGCCG

m2: CGGAATTGGT GCGCTGCTG

Figure 13. Mutagenesis of element IV and its results. The wild type sequence is from the Canton S laboratory strain with element IV underlined. The mutagenic primer from the Regional DNA Synthesis Laboratory is shown below, followed by the three mutagenesis products. Gaps denote deleted bases and bold bases denote mutations. No other changes were found in the region sequenced (-265 to +15).

designated m2 in Figure 13 resulted in a C to T transition at -50 and the deletion of the adenine at -49 and is thus a more severe disruption of element IV than the m1 product is. The *Ddc* genes designated m1 and m2 were then subcloned into pW8 (to create pDdc41 and pDdc42, respectively) as described for previous mutageneses and microinjected. Two transformed strains were obtained for each mutant. They were crossed into a Ddc^{ts2} background and samples were collected at four developmental stages. The strain 24 insert (an 'm2' strain) created a recessive lethal so the DDC activities were obtained in heterozygotes and the numbers were doubled to yield the reported values. Samples for strain 24 were only collected at pupariation. Table 5 shows a list of

transformants and the DDC assay data.

Table V. Units of DDC activity in *Ddc*$^{+}$ and element IV mutant strains at four stages of development. Note that tDdc42 (m2) contains a more severe disruption of element IV than tDdc41 (m1) does. See Table I for further details.

Transformant	Sample 1	Sample 2	Sample 3	Sample 4	Mean	Mean ± SEM	
Hatch							
tDdc33 -Strain 9	357	304			331		
-Strain 10	321	332			327		
						329	14
tDdc41 -Strain 6	373	419			396		
-Strain 20-2	302	356			329		
						363	30
tDdc42 -Strain 44	251				-		
						251	
2nd Moult							
tDdc31 -Strain 2b	496	487			492		
tDdc33 -Strain 9	564	592			578		
-Strain 10	526	500			513		
						528	22
tDdc41 -Strain 6	791	911			851		
-Strain 20-2	1172	831			1002		
						926	105
tDdc42 -Strain 44	448	401			-		
						425	17
Pupariation							
tDdc31 -Strain 2b	5099	5062			5081		
tDdc33 -Strain 9	4593	4658	4525		4592		
-Strain 10	3832	4980	4272	3452	4134		
						4602	312
tDdc41 -Strain 6	3130	3164			3147		
-Strain 20-2	3446	2778			3112		
						3130	168
tDdc42 -Strain 24	3652	3576			3614		
-Strain 44	5610	4458	5336	5581	5246		
						4430	579
Eclosion							
tDdc31 -Strain 2b	3741				3741		
tDdc33 -Strain 9	4097	3376	4076		3850		
-Strain 10	3411	3298			3355		
						3648	189
tDdc41 -Strain 6	3385	3279			3332		
-Strain 20-2	2554	2333			2444		
						2888	320
tDdc42 -Strain 44	4026	3352	3416				
						3598	175

Discussion

1. Technical considerations

All previous studies on *Ddc* regulation have normalized DDC activity levels to the amount of protein present. This was not done in the current study for several reasons. First, the protein determinations which were performed tended to introduce more variation between duplicate samples than they resolved. More importantly, repeat determinations on the same homogenate varied widely despite relative consistency of the standard curves (data not shown). This problem has been encountered previously in our laboratory. Finally, the validity of comparing, on a per mg protein basis, qualitatively different organisms such as larvae and adults or males and females has been questioned (Marsh *et al.*,1985). We felt this was a reasonable concern given the amino acid specificity of most methods of protein determination and the predominance of certain types of protein at different stages of development(*eg.* larval serum proteins, yolk proteins).

Wet weight is an alternative method with which to standardize DDC activity levels. However, assays were done at pupariation and eclosion in which levels of DDC activity were shown not to correlate with wet weights in several duplicate samples (data not shown). It was thus decided to

present DDC activity levels on a per organism basis (or per 15 organisms in the case of newly hatched first instar larvae). Repeatability was maximized by collecting from generally uncrowded populations, by collecting similar ratios of males and females at the adult stage (see Materials and Methods) and by using the same tissue grinder and homogenization technique for all samples.

The controls used for many of the studies described herein were the tDdc31 and tDdc33 strains which contain the *Ddc*$^{+}$ allele subcloned into pW8 in opposite orientations. This was originally done to detect any position or orientation dependent interactions between the *Ddc* and the *white* promoters in the recombinant P elements. The data presented in Table III can be used to assess these interactions. The tDdc31 strains tend to express at levels somewhat higher than those of the tDdc33 strains at the second moult and at pupariation. However, these differences are largely attributable to the overexpression of the tDdc31 strain 2b, and at eclosion, where this strain does not overproduce, the differences are negligible. It was therefore concluded that the effects of orientation were not significant and that all *Ddc*$^{+}$ transformant strains would serve as equivalent controls.

Table III also indicates the levels of DDC activity contributed by the endogenous *Ddc*^{cs2} allele at the second moult, pupariation and eclosion. Since no direct comparisons were made between developmental stages, none of

the transformant DDC activity levels were corrected for these low endogenous levels.

2. Analysis of element IV

The role of element IV in the developmental control of gene expression has previously been investigated by deletion analysis involving gross disruptions of the promoter (Scholnick *et al.*, 1986). These studies showed element IV to contribute approximately 45% of DDC activity in embryogenesis and about 15% at pupariation and eclosion. The studies presented here provide a less homogeneous description of the activity of element IV. Table VI shows the normalized results and Table V shows the data from which they were derived.

The less severe m1 mutation reduced expression by about 25% at pupariation and eclosion and paradoxically increased expression by 75% at the second moult. A possible explanation for this is that the mutation fortuitously increases the affinity of binding of a positive regulatory factor at this stage. The possibility of a second site mutation causing the effect cannot be eliminated. The more severe m2 mutation caused an approximately 20% decrease in gene expression levels at the hatch and second moult and had no effects at pupariation or eclosion. The tissue specificity of element IV cannot be commented upon as CNS specific DDC activities were not determined.

Hatch	m1:	100%
	m2:	(76%)
2nd Moult	m1:	175% ± 11%
	m2:	80% ± 4%
Pupariation	m1:	68% ± 5%
	m2:	100%
Eclosion	m1:	81% ± 11%
	m2:	100%

Table VI. The effects of mutations in element IV at four stages of development. The values are normalized such that *Ddc*$^{+}$ expression is equivalent to 100% and only statistically significant[1] differences are shown. The m1 mutant consists of deletion of a guanine residue and the m2 mutant consists of a cytosine to thymine transition and deletion of an adenine. The bracketed value is derived from a single determination.

These data seem to indicate that different base pairs within element IV are critical for regulatory factor binding at different stages of development. Furthermore, the very different results for m1 at the hatch and second moult argues against the suggestion (Hodgetts *et al.*, 1986) that *Ddc* regulation at these stages may involve similar

[1]A statistically significant difference is arbitrarily defined here as one where the difference between two means is greater than the sum of their standard errors

mechanisms. Further experiments to confirm stage specific roles for element IV would involve *in vitro* footprinting experiments using epidermal protein extracts from one or more stages of development. A correlation between the observed gene expression levels and the *in vitro* binding affinity of each of the mutants would be the goal of such studies.

3. Analysis of element S

Previous studies on the *Ddc*$^{+4}$ strain (Estelle and Hodgetts, 1984a) indicated that the activity ratios of *Ddc*$^{+4}$ to *Ddc*$^{+}$ were 1.41 at the hatch, 1.50 at the second moult, 0.50 at pupariation and 1.18 at eclosion. These findings were not entirely reproduced in the transformants recovered in this study. The data in Tables I and II show the ratios to be 1.16 at the hatch, 1.14 at the second moult, 0.51 at pupariation and 0.72 at eclosion. The latter finding is the most startling considering *Ddc*$^{+4}$ was selected as an activity variant based on its overproduction at eclosion. The observed underproduction may be due to the different genetic backgrounds of the endogenous and transformed alleles. Alternatively, the single *Ddc*$^{+4}$ control transformant strain may have an insert integrated at a chromosomal position which is repressed at eclosion. This possibility exposes a significant weakness in this study and that is the absence of more than one *Ddc*$^{+4}$ control strain. This has been

rectified by mobilizing a *Ddc*$^{+4}$ insert to generate an additional control strain. DDC analyses on this strain await collection of appropriately staged organisms.

Previous experiments by Spencer (1987) indicated that element S conferred an approximately 40% enhancement of gene expression on a heterologous promoter. This finding has been substantiated by the work of Todo *et al.* (1990) who were investigating the *cis*-acting regulatory sequences of the glue gene, *Sgs-3*. They fused *Sgs-3* sequence from -980 to +12 to an *Adh* reporter gene and the region between -106 and -52 was deleted piece by piece by a series of non-overlapping 7 or 8 bp deletions. These deletion constructs were then assayed for *Adh* expression by histochemical staining in a transient assay expression system (see Roark *et al.*,1990 for a description of this system).

Only two regions were found to have functional importance, one of which was the proximal half of element S ($^{-68}$**TCCATTT**$^{-62}$; see Figure 3). A deletion of sequences from -74 to -66 resulted in a loss of staining. A fine structure analysis was then undertaken by introducing point mutations at every site from -103 to +3. The only two bases of element S found to have functional activity by this method were the thymine and the cytosine at positions -68 and -67, respectively (shown in bold type above). Because histochemical staining is not a useful quantitative assay, the -74/-66 deletion and the two point mutant constructs were introduced into the germ line to determine their

quantitative effects. The deletion showed a 60% loss of *Adh* activity relative to the control. Mutation of the thymine only reduced activity levels by 10% whereas mutation of the cytosine reduced levels by 60%, the same amount as the deletion. This is surprising since the thymine is conserved in almost all the variants of element S whereas the cytosine is in a non-conserved position (Figure 3). A possible explanation for this finding may be that an element S-binding structural motif is shared by a group of *trans*-acting regulatory proteins. The promoter specificity of each of these regulatory proteins would be determined by the nonconserved bases of element S. In fact, the critical regions of element S appear to differ, depending on the promoter context in which it occurs. The suspected functional change described in *Sgs-4* underproducers is located in the middle of the element and the change in Ddc^{+4} is located in the distal half of element S.

The hypothesis of a functional role for the proximal half of the *Sgs-3* element S was strengthened by *in vivo* footprinting experiments (Mathers, 1989). These demonstrated that the hemi-element is bound by protein *in vivo* only at the times and in the tissues in which the *Sgs-3* gene is expressed. The experiments were also performed in $npr\text{-}1^{3}$ mutants, an allele of a complementation group of the Broad Complex whose function is required for *Sgs-3* mRNA accumulation in mid-third instar (Crowley *et al.*, 1984). In the absence of the *npr-1* product, *in vivo* footprinting

failed to detect any binding to the proximal half of element S. Interestingly, *Ddc* also requires the function supplied by the *npr* complementation group to achieve maximum expression levels at pupariation, but not at the second moult (M. Schouls, pers. comm.).

Recently, an 86 bp fragment from *Sgs-7/8* which contains an element S identity has also been shown to be capable of conferring the stage and tissue specific pattern of expression of those genes on a heterologous promoter in a position and orientation independent manner (Hofmann *et al.*, 1991).

The experiments just described on the glue genes paint a picture of element S as an enhancer of gene expression. This differs from its apparent function in the *Ddc* promoter. The mutation induced in Ddc^{+} which produced a more consensus-like (*ie.* more enhancer-like) element S was expected to result in overproduction. Instead, statistically significant underproduction was observed at embryogenesis (Table I). The change was of the order, but not the direction, expected (14%±8% decrease) from the phenotype of the Ddc^{+4} control transformant. No significant changes were found at the other three stages. These results indicate that element S serves as a silencer element in Ddc^{+} at the hatch and is either inactive or redundant at the other three stages in Ddc^{+}. The effects observed are quite weak and this may explain why Hirsh *et al.* (1986) concluded that no sequences upstream of -209 were required for normal

developmental expression of *Ddc*. An alternate explanation is that the induction of *Ddc* expression was delayed in embryogenesis by the mutation, resulting in lower DDC activities at the hatch.

The hypothesis of element S being a negative promoter element at the hatch cannot be confirmed at present since the transformant containing the deletion of element S was not studied at the hatch (Table III). The deletion transformant does confirm the inactivity (or redundancy) of element S at the second moult and pupariation, as no changes in DDC activity were detected at these stages. It also suggests that element S serves as a very weak negative regulatory element at eclosion since tDdc36 (element S deletion) expressed at (statistically significant) levels 8% (±4%) greater than those of controls. This overexpression was also observed by Hirsh *et al*. (1986, Figure 2) at eclosion in the transformants lacking sequences upstream of -209. It should be noted that similar overexpression was observed at the hatch by these workers leading to the prediction that tDdc36 should overexpress at the hatch. The slight increase in expression levels at eclosion resulting from the deletion of element S may explain why no effect was seen at eclosion with the mutation in element S. Since the effects of mutation and deletion of element S were so minor, it can be said to have essentially no effect in a *Ddc*$^{+}$ background.

In contrast, the guanine to adenine transition induced in the *Ddc*$^{+4}$ version of element S resulted in significant overexpression in a *Ddc*$^{+4}$ background, with the absolute level of increase varying two-fold (Table VII; see Table II for original data).

Hatch	175% ±20%	131% ± 3%
Second Moult	191% ±18%	151% ±10%
Pupariation	164% ±21%	133% ± 5%
Eclosion	149% ± 4%	155% ± 1%
	Average:170%±15%	Average:143%±11%

Table VII. The effects of a mutation in element S in a *Ddc*$^{+4}$ background. The values are normalized such that expression of the *Ddc*$^{+4}$ transformant control strain is equivalent to 100% and only statistically significant differences are shown. The third column shows the expression levels if the outlier strain 16 values are neglected.

The data show clear overexpression of DDC activity at these four stages, and this is still true if the contribution of strain 16 is disregarded. There are several explanations for the observed overexpression. It may be due to an unintended mutation introduced during *in vitro* mutagenesis. The fact that two separate isolates from the mutagenesis procedure both overexpress argues against this

notion. The overexpression could also be due to a mutation introduced during subcloning prior to *in vitro* mutagenesis. This possibility cannot be entirely eliminated since constructs and controls were only sequenced from -280 to +80.

The mutation under study produced a version of element S with less similarity to the consensus element S. Under the working hypothesis that element S is an enhancer-like element, this was expected to result in underexpression. That the engineered change resulted in overexpression implies that element S is serving a negative regulatory role in Ddc^{+4}. Whether this role is being played in the epidermis or in other tissues where *Ddc* is not normally expressed cannot be determined from these data. However, the location of the overexpression (as well as its prevalence during development) is currently under study in our laboratory. The magnitude of this negative regulatory role varies considerably between stages. However, the average increase (70%) is akin to the decrease detected for the deletion of element S in Todo *et al.*, 1990 (60%). It is somewhat higher than that detected by Spencer, 1987 (40%), using the isolated element S of Ddc^{+} to drive the expression of a reporter gene. It appears that, in the context of the Ddc^{+4} allele, the Ddc^{+4} version of element S has effects of like magnitude, but of opposite character, to those detected previously for other versions of element S.

At pupariation, the degree of overexpression in transformants carrying the *Ddc*$^{+}$ version of element S in a *Ddc*$^{+4}$ context was 64%. At this stage, *Ddc*$^{+4}$ is a 50% underproducer and thus the single base pair change in element S between *Ddc*$^{+}$ and *Ddc*$^{+4}$ is a good candidate for the source of this underproduction. The *Ddc*$^{+4}$ transformant was found to be a 27% underproducer at eclosion and so the role of element S in this phenotype as well as in the overproducer phenotypes of the hatch and second moult is less clear. Characterization of the second *Ddc*$^{+4}$ transformant strain, generated by mobilization, will be required before further analysis is possible.

Why the reciprocal change had such minor effects in *Ddc*$^{+}$ is unclear. Element S may be a largely redundant sequence element in *Ddc*$^{+}$. Alternatively, as suggested above, element S may require interaction with a distant element(s) in order to achieve its effects. The distant element may be inactive in *Ddc*$^{+}$ with element S therefore being rendered inactive. This concept of element S being one part of a molecular interaction involving (at least) two separate DNA sequences is not without support. The version of element S present in the *Sgs-3* gene is contained in one of two regions, each of which is sufficient for correctly regulated but low-level expression. Together, the regions act synergistically to enhance levels of gene expression 20-fold. The dual element notion may explain the discrepancy between the data presented here, showing element S to have

silencer activity, and those of Mestril *et al.* (1986), Spencer (1987) and Todo *et al.* (1990), who showed element S to have enhancer-like activity. In this model, the distant element would mediate the character of the interaction of element S with the promoter. When adjacent to *Hsp 23* and the *Adh* reporter genes, this would be a positive interaction, resulting in enhancer-like properties for element S. In Ddc^{+}, this would be a non-functional interaction. However, in Ddc^{+4} this would be a negative interaction, resulting in silencer properties for element S.

4. Analysis of the fusion genes.

The fusion genes were constructed in an attempt to map the control elements responsible for the differences between Ddc^{+} and Ddc^{+4} to positions either upstream or downstream of the transcription start site. The data acquired from the fusion genes seem to map the site of some of these differences to the downstream region of the gene. The original data can be found in Table IV and the normalized values are presented in Table VIII. The activities of tDdc40 (upstream Ddc^{+4}, downstream Ddc^{+}) at the second moult and pupariation are indistinguishable from those of Ddc^{+} at these stages. This would imply that the portion of the gene downstream of -25 is mediating the underexpression of Ddc^{+} relative to Ddc^{+4} at the second moult as well as mediating the overexpression of Ddc^{+} relative to Ddc^{+4} at pupariation.