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THE UNIVERSITY OF ALBERTA

MOLECULAR GENETIC STUDIES ON THE DOPA DECARBOXYLASE GENE IN *DROSOPHILA MELANOGASTER*

BY

Charlotte Anne Spencer

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Doctor of Philosophy.

DEPARTMENT OF GENETICS

EDMONTON, ALBERTA

FALL, 1987.

ISBN 0 315-41004 3

London WC2R 3LF
Telephone 01-836 6633
Telex 262024

Invoice N°: 0235

Date: 21.01.87

Attn: C A Spencer
University of Alberta
Dept. of Genetics
G216 Biological Sciences Cen.
Edmonton
CANADA T6G 2E9

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(Address) Department of Genetics

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.....
Dr. R.B. Hodgetts (supervisor)

.....
Dr. W.R. Addison

.....
Dr. J.M. Tyler

.....
Dr. R.C. Bleackley

.....
Dr. J.W. Fristrom

Date: Jan. 18/85....

To Gregor Mendel - even if he didn't do the experiments.

ABSTRACT

Three separate studies constitute the text of this thesis. The first study (Chapter 1) was a biochemical characterization of a novel enzyme discovered in a clone of the Drosophila Kc cell line. Although the cell line enzyme resembled dopa decarboxylase (DDC) from whole organisms in its substrate specificity, cofactor requirements and inhibitor sensitivities, many differences were apparent. The cell line enzyme was antigenically distinct from DDC from whole flies. Its molecular weight and charge differed from in vivo DDC and peptide maps of the two purified enzymes were distinct. Also, dopamine was not detectable as an endproduct of decarboxylation of dopa in cell line extracts. These results suggest the production, in the 7E10 subline, of a different enzyme hitherto undetected in whole organisms.

The second study (Chapters 2 and 3) involved the sequencing, mapping and characterization of a transcription unit which overlaps that of the Ddc gene. The 3' termini of the two convergent transcription units overlap by 88 base pairs. Temporal and spatial distribution studies of the two transcripts within the organism showed that high levels of both transcripts were not concordant. However, within adult testes, where the 3' transcript was maximally expressed, low levels of Ddc transcript were detected. These results raise the possibility that a sense-antisense hybrid molecule involving the two transcripts may occur in vivo or that transcriptional interference may occur, with concomitant regulatory implications.

The final study (Chapter 4) was a functional analysis of an enhancer-like sequence element which occurs upstream of the Ddc gene. Through a sequence comparison of wild-type Ddc DNA with that of a naturally-occurring activity variant, an element was identified that bore homology to the SV40 enhancer core sequence and to elements upstream of two other ecdysterone-regulated Drosophila genes. An oligonucleotide was synthesized corresponding to this short (37 bp) element, it was cloned upstream from a reporter gene, alcohol dehydrogenase, and the hybrid gene introduced via P element germ line transformation into an Adh^- host strain. Preliminary characterization of Adh levels and histochemical staining reveal that an expression level effect is associated with the presence of the Ddc element, but that the element does not appear to contribute to tissue specificity.

ACKNOWLEDGEMENTS

Thank you, Ross, for all the guidance, enthusiasm, support, devil's advocacy, good ideas and a thousand intangibles. A boatload of thanks to Bill Clark - for technical advice, for keeping the pillaging hoards in line, and for making our lab the best-organized this side of the Sierra Madre. Thanks to fellow lab-mates through the years: Mark Estelle - for temperate and philosophical advice, and Ddc^{+4}; Dan Gietz - for technical tricks and a unique vocabulary; Keith Salmon - for good humored patience and friendship; Adam Chen - for a cheerful demonstration of the use of meditation on frigid auto trips; Laura Querengesser - for an unrelenting good nature and advice on the perils of science and moving; Al Mazza - for continuing Ddc^{+4} after I'm gone; and Diane Winkelman - for continuing the tradition of unique and interesting females from the RBH lab. Thanks also go to the Alberta Heritage Foundation for Medical Research - and Mom - for making the whole venture financially feasible, and to Colin who demonstrated that love and perseverence win in the end.

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LIST OF ABBREVIATIONS

1-2 Molt	molt occuring between the first and second instar larval stages.
2-3 Molt	molt occuring between the second and third instar larval stages.
AChE	acetylcholinesterase
ACR	Adh-fn^{b}, cn, ry^{506}
Adh	alcohol dehydrogenase gene locus
ADH	alcohol dehydrogenase protein/enzyme
bp	base pairs
BSA	bovine serum albumin
ConA-Sepharose	concanavalin A bound to Sepharose 4B
cn	cinnabar gene locus
CNS	central nervous system
CRM	cross reacting material
CS	Canton-S laboratory strain of Drosophila
Ddc	dopa decarboxylase gene locus
DDC	dopa decarboxylase protein/enzyme
DEAE	diethylamino ethyl cellulose
DEHPA	diethylhexylphosphoric acid
DHFR	dihydrofolate reductase
DQID	dopa quinone imine decarboxylase
DTT	dithiothreitol
ecd^{1}	Drosophila ecdysone-less mutant
EDTA	ethylenediaminetetraacetic acid
EPPS	N-(2-hydroxyethyl)piperazine-N'-3-propanesulfonic acid
Exo III	exonuclease III

G^0 generation	surviving embryo after injection of P-element vector
G^1 generation	progeny of a surviving G^0 adult
Gart	glycinamide ribotide transformylase
IVS	intervening sequence (intron)
kb	kilobase
MMLV	Moloney murine leukemia virus
MMTV	Mouse mammary tumor virus
NAD^+	β-nicotinamide adenine dinucleotide
oligo-dT	oligodeoxythymidylic acid
ORF	open reading frame
PBA	phenyl boronate agarose
pr	purple locus of Drosophila
PTU	phenylthiourea
Pu	purine
Py	pyrimidine
RF	replicative form of single stranded DNA virus
ry	rosy locus (xanthine dehydrogenase) of Drosophila
SDS	sodium dodecylsulfate
SSC	0.15M NaCl; 0.015M Na citrate
TK	thymidine kinase
TLC	thin layer chromatography
Xdh	xanthine dehydrogenase (rosy) locus of Drosophila
XDH	xanthine dehydrogenase protein/enzyme

INTRODUCTION

"Biology has been fortunate in discovering within the span of one hundred years two great and seminal ideas. One was Darwin's and Wallace's theory of evolution by natural selection. The other was the discovery, by our own contemporaries, of how to express the cycles of nature in a chemical form that links them with nature as a whole."

Jacob Bronowski, The Ascent of Man, 1973, Little, Brown & Co., Toronto. P.317.

We biologists living in the second half of the twentieth century have been the fortunate benefactors of an explosion of information triggered by these two important insights. Much of this progress has occurred in the fields of molecular biology and biochemistry, within the last thirty years. The traditional sciences of genetics, biochemistry and cell biology are fusing their technologies and approaches towards the common goal of "expressing the cycles of nature in a chemical form" and the boundaries between these disciplines are becoming blurred. Structure and function in molecular biology have now become two sides of the same coin.

Our ability to interpret the function of an organism through the structure of its constituent molecules has inspired a certain optimism that we have the biological world in our intellectual grasp.

"We have complete confidence that further research of the intensity given to genetics will eventually provide man with the ability to describe with completeness the essential features that constitute life."

J.D. Watson, Molecular Biology of the Gene, 1975, 3rd edition, Benjamin/Cummins Publishing Co., Menlo Park, CA.

Perhaps fortunately, Dr. Watson's optimism is probably excessive. The history of science teaches us that there is no absolute knowledge. Therefore, we must treat current "knowledge" with some humility. The history of science, and human endeavor in general, teaches us that just when we think we have a reliable dogma or consistent viewpoint, our human creativity points to a new way to see the world and our beliefs are turned upside down. Science, like all human knowledge, is in constant flux.

"Science is not a mere collection of discoveries, an album of facts and theories that have been established once and for all. Science is the process of discovery itself, a living process. It is not what scientists know that matters to them, but what they do not know; and what drives them is the urge to know more. In short, knowledge is a form of experience for the scientist - as, indeed, it is for all of us.

Jacob Bronowski, A Sense of the Future, 1977, The MIT Press, Cambridge, Mass. P. 198.

This compulsion to imagine, symbolise, take the world apart and then recreate it with our own interpretation, is what makes us uniquely human. It is reassuring, then, to realize that nature will not give us the final word on biology - or any science.

"We are here face to face with the crucial paradox of knowledge. Year by year we devise more precise instruments with which to observe nature with more fineness. And when we look at the observations, we are discomfited to see that they are still fuzzy, and we feel that they are as uncertain as ever. We seem to be running after a goal which lurches away from us to infinity every time we come within sight of it."

Jacob Bronowski, The Ascent of Man, P. 356.

Our experience in molecular biology reflects this observation. The goal threatens to elude us once more, as gene regulation now involves a truly molecular study of protein-protein interactions at active sites. As we get even closer to the essense of biological function, we are approaching the physics of the behaviour of atoms. The mystery remains.

OUTLINE OF THIS THESIS

The progress in research on the dopa decarboxylase (Ddc) gene of Drosophila parallels and reflects, to a great degree, the ideas just mentioned. The study has advanced from traditional genetics and biochemistry to a more recent molecular biological approach. Using germ line transformation, DNA sequences are now being correlated with several aspects of the gene's regulation. Also, complex tissue and developmental specific splicing patterns are emerging for Ddc mRNA processing. Like other studies in molecular biology, Ddc research has yielded rewarding answers to many questions, but at the same time, has expanded the complexity of the remaining problems.

The three research projects documented in this thesis also represent this progression. Initially, a study was made of the biochemical characteristics of a presumed dopa decarboxylating enzyme present in cell lines. This was followed by an examination of aspects of Ddc genetic structure, which involved sequence analysis and molecular mapping of transcripts within the Ddc region. The final inquiry involved a functional study of a putative Ddc regulatory sequence element.

The knowledge gained from these studies has been rewarding. However, more complex questions are raised by the discoveries made during the course of this research. What is the identity and nature of a dopa decarboxylating enzymatic process hitherto undetected in whole Drosophila? What are the implications for gene regulation of the presence of anti-sense overlapping transcripts in eucaryotes? What are the mechanisms by which a change in a DNA sequence can effect a change in a gene's expression?

If, as Dr. Bronowski states, the process of discovery is its own reward, inspiring us to know more, then research into the Ddc system has indeed provided a profitable basis for continued inspiration.

LEVELS OF GENE CONTROL

Studies on the regulation of the dopa decarboxylase (Ddc) gene in Drosophila have revealed a complex of regulatory mechanisms operating on this single gene in different tissues and at different stages in the fly's development. These will be described in the next section. In order to place these studies of Ddc regulation into a larger context, this section will outline the mechanisms of control that are presently believed to operate on eucaryotic cellular genes.

I. Transcription Initiation

Control of the rate and site of transcript initiation is probably the most important and frequent mode of regulating gene expression in both procaryotes and eucaryotes. Often multigene families of closely related sequences encode a series of protein isoforms whose distributions may be temporally or spatially regulated during development. Selection for expression of any individual members of a family can be considered a form of transcription start site selection. Two examples of differential expression within multigene families are presented in Table 1. Several examples also exist of regulation through choice of different start sites within the same gene. Control of α-amylase levels in mouse liver and salivary gland is a classic example. See Table 2 for examples of transcriptional control by start site selection.

Nuclear runoff assays from isolated nuclei are used as a measure of the number of RNA polymerase molecules present on any selected region of DNA and hence transcription rate (53). Such assays applied to a number

TABLE 1 - Transcriptional Control by Start Site Selection - Gene Families

GENE	ORGANISM	DESCRIPTION	REFERENCE
Actin genes	Drosophila	Two of Drosophila's six actin genes are expressed at all times in undifferentiated cells. Two others are expressed in larval, pupal and adult intersegmental muscles. One is expressed in adult thoracic and leg muscles.	22.
Globin genes	Human	Switching from fetal to adult globin gene expression is at least partially controlled via selection of promoter sequences . Selection involves interaction of promoter elements with trans-acting factors.	36.

TABLE 2 - Transcriptional Control by Start Site Selection - Single Genes

GENE	ORGANISM	DESCRIPTION	REFERENCE
Alpha-amylase	Mouse	One promoter initiates RNA at a low level in both salivary gland and liver. The other promoter, 2.8 kb. away, is 30-fold stronger and operates in salivary gland only. It produces a transcript with a salivary gland-specific leader.	71.
Myosin light chain	Chicken	Two proteins in chicken skeletal muscle are produced from a single gene by differential transcription initiation. The two promoters are 9.5 kb. apart and transcription is followed by different modes of splicing.	58.
Adh	Drosophila	Larval and adult promoters are about 700 bp. apart. The two RNAs are spliced differently in the 5' untranslated leader and the concentrations of the two mRNAs differ in tissue and stage-specific ways.	4.
SUC2	Yeast	Two invertase RNAs (1.8 and 1.9 kb.) are transcribed from the same gene using two different transcription start sites. The 1.8 kb. RNA is produced constitutively and is translated into intracellular invertase. The 1.9 kb. transcript is glucose-regulated and produces secreted invertase.	8.

of selected genes from several tissues have demonstrated that the predominant level of control for most genes is via differential rate of mRNA synthesis (16,66). Many examples of transcriptional ratecontrol in response to external stimuli (hormones, growth factors, environmental stimuli) can be cited. (See Table 3 for several examples.)

Studies of the mechanisms by which RNA polymerase II is able to vary transcription sites and rates provide subject matter for the most active investigations in molecular biology. A large array of DNA sequences that influence transcription in temporal and tissue specific ways have been identified. These elements are considered to operate in cis, and in conjunction with trans-acting factors, to affect DNA topology, to interact with other DNA elements, promoters and other factors and to produce conformations which govern the access of RNA polymerase to the DNA template. Several excellent reviews of our current understanding of this topic exist (65,84). Chapter 4 will deal with control sequences in more depth.

2. Transcription Termination

Control of gene expression can occur at the transcription termination step by two known mechanisms. The first involves the choice of alternate termination sites, yielding qualitatively different transcripts. The second involves an attenuation-like mechanism where the option of premature termination or read-through provides an on-off switch regulating a gene's expression. For a recent review, see Reference 64.

TABLE 3 - Transcriptional Control by Modulating Rate of mRNA Synthesis

GENE	ORGANISM	DESCRIPTION	REFERENCE
Ovalbumin Conalbumin	Chick oviduct	The transcription rate of conalbumin mRNA increases 3-fold within 30 minutes of treatment with estrogen or progesterone. Ovalbumin mRNA transcription rate increases 20-fold. Transcript stability is also involved in regulation. See Table 6.	53.
Heat shock	All organisms	Heat and other types of stress induce HS mRNAs 10 to 1000-fold. The response is primarily transcriptional, but selective translation also operates in some organisms. See Table 7.	47.
Metallothionein	Mouse liver	Dexamethasone and heavy metals result in a 25 to 100-fold increase in MT-1 transcription rate.	19. 30.
MMTV	Mouse	Glucocorticoids stimulate MMTV mRNA transcription rate via interactions of hormone-receptor complex with DNA control sequences.	9.
Phosphoenol-pyruvate carboxykinase	Rat liver	cAMP causes a 7-fold increase in transcription rate within 20 minutes.	43.

Although factor-dependent or factor-independent attenuation in procaryotes is well documented, premature transcription termination in eucaryotes is less well characterized. One of the problems inherent in defining eucaryotic termination sites is that termination per se is far removed - up to 1-4 kb.- from the site of the mature 3' end, which is generated by post-transcriptional processing and poly(A) addition. Several examples and some features of control through transcription termination in eucaryotes are given in Table 4.

3. Transcript Processing

Selective RNA processing is a major factor in the control of a number of eucaryotic cellular genes. Table 5 summarizes many examples of differential processing - both selection of poly(A) cleavage sites and intron-exon splicing sites. (For a recent review, see Reference 26.) The mechanisms of differential splicing are not understood at present, but alternate conformations of RNA at or near splice junctions may play a role.

4. Transcript Stability

Most of the cases of differential stabilization of transcripts in eucaryotes involve hormonal effects on tissue-specific mRNAs. However, some other examples exist - for instance that of histone transcript stabilization during the cell cycle. This type of control is usually accompanied by transcriptional control as well. The mechanisms of differential RNA stability are poorly understood at present. See Table 6 for examples of the use of transcript stability in gene control.

TABLE 4 - Transcription Termination Control

GENE	ORGANISM	DESCRIPTION	REFERENCE
Early and late transcription units	Adenovirus-2	In early infection, transcripts from the major late promoter terminate at map position 60-70. In late infection, they read-through to position 99.	61.
Immunoglobulin heavy chain	Human	During early stages of B-lymphocyte maturation, transcription occurs throughout the 25 kb μ-δ locus. In mature IgM secretors, transcription terminates between μ and δ loci, eliminataing δ mRNA production. Hybridoma fusions of cell lines at two developmental stages result in expression changes, suggesting a role for trans-acting factors in termination site selection.	52.
16S mRNA	SV-40	The 16S leader sequence contributes to an attenuation-like mechanism and can be folded into two alternative secondary structures, followed by a poly-U stretch. In late infection, a 94-base attenuated RNA appears but no full-length transcripts are present. Transcription truncation requires the presence of agnoprotein, itself a product of an ORF within the 16S leader.	31.
c-myc	Mouse Human	The 90% down-regulation of c-myc RNA that occurs on differentiation of cell lines is due to a block in transcription elongation occurring near the exon 1 - intron 1 boundary. This region contains alternate secondary structures followed by oligo-dT stretches. Labile negative regulatory factors appear to be involved in the premature termination.	3. 60.

TABLE 5 - Transcription Processing Control

GENE	ORGANISM	DESCRIPTION	REFERENCE
1) Poly(A) site choice:			
Calcitonin/CGRP	Rat	Different poly(A) selection from the same primary transcript in thyroid or brain is followed by two different splicing variants. Two functionally distinct proteins with different COOH termini - calcitonin hormone or neuropeptide CGRP - result.	2.
Kininogen	Bovine	Poly(A) site selections result in two bradykinins that differ in protease susceptibility.	39.
Immunoglobulin heavy chain	Human Mouse	Membrane and secreted forms of μ chain mRNA are generated from a longer primary transcript by differential choice of poly(A) cleavage sites.	20a).
Myosin heavy chain	Drosophila	Two different muscle proteins differ in embryos and adults; a third form is found in pupae and adults. The three mature mRNAs are transcribed from a single gene and differ by both poly(A) site selection and 3' intron splicing.	68.
GAR transformylase	Drosophila	Two transcripts of 4.7 kb. (GART) and 1.7 kb. arise from the same primary transcript. The latter transcript results from use of a poly(A) sequence in an intron. This transcript codes for a second protein.	32.
Vimentin	Chicken	Two forms of mRNA are expressed in a tissue-specific manner. The two species differ in the lengths of their 3' untranslated regions.	7b.
Actin 5C	Drosophila	The relative levels of three mRNAs (2.2 kb., 1.95 kb. and 1.7 kb.) vary during the life cycle. They are produced from a primary transcript through differential choice of three poly(A) sites.	6.

TABLE 5 - (contd.)

2) Splicing Variation:

Troponin T	Rat	Two tissue-specific, developmentally regulated mRNAs are transcribed from one gene. They differ by two alternative splicing events that incorporate 1 of 2 possible internal mini-exons.	54.
Myosin light chain	Rat Chicken	Differential sites of transcript initiation accompany differential splicing at the 5' end, to yield two distinct light chains.	58.
Preprotachykinin	Bovine	The neuropeptides, substances K and P, arise from two different mRNAs transcribed from the same gene in a tissue-specific manner. Their mRNAs share 5' and 3' sequences, differing only in the insertion or deletion of an exon encoding substance K.	59.
P-element	Drosophila	Lack of transposition in somatic tissue is caused by a failure to splice a transposase mRNA intron in somatic tissues. Therefore functional transposase is only produced in germ line cells.	44.
Fibrinogen	Rat	Two forms of mRNA from the same gene encode two different proteins.	15.
Fibronectin	Rat	The single fibronectin gene encodes three different mRNAs from a single primary transcript. These three mRNAs arise through utilization of 1 of 3 splice sites, 1 at the beginning of and 2 within a single exon. These mRNAs may encode some of the forms of fibronectin seen in different tissues.	77.
Alpha-A-crystallin	Mouse	Two polypeptides differ as a result of inclusion of an extra small exon in one mRNA species.	37.

TABLE 6 - Transcript Stability Control

GENE	ORGANISM	DESCRIPTION	REFERENCE
Casein	Rat	Presence of prolactin causes a 100-fold increase in steady-state levels of casein mRNA, but only a 3-fold increase in transcription rate. Transcript half-life increases from 5 hrs. to 92 hr. in presence of hormone.	29.
Vitellogenin	Frog liver Rooster	Estrogen creates a several hundred-fold increase in RNA steady-state levels. mRNA half-life contributes an 8-fold (roosters) or 30-fold (frog) increase to the total.	73.
Ovalbumin Conalbumin	Hen oviduct	Estrogen and progesterone increase mRNA half-life from 2 - 5 hrs. to greater than 24 hrs.	53.
Thymidine kinase	Chicken	Actively dividing cells contain 5 to 10-fold more TK mRNA than stationary cells but nuclear runoff assays demonstrate constitutive transcription rate at all mitotic stages.	28.
Dihydrofolate reductase	Mouse cells	The rate of DHFR transcription and cytoplasmic half-life do not change during mitotic stimulation. However, levels of DHFR transcripts are 3-fold higher after growth stimulation, suggesting increased nuclear stability.	46.
Tubulin	CHO cells	Colchicine treated cells have reduced steady-state levels of mRNA but transcription rate is unchanged.	12.
Histone H4	Human	mRNA levels decrease 120 to 180-fold in G1-arrested cells but transcription rate decreases only 3-fold. Instability is dependent on protein synthesis.	25.
Histone H3	Human	Transcription rate is unchanged, but steady-state levels of mRNA decrease by 90% when DNA synthesis is inhibited by various drugs. Sequences in the 5' leader confer the cell-cycle dependent instability.	57.

TABLE 6 - (contd.)

c-fos	Mouse cells	Transcription rate changes are less than mRNA steady-state changes after mitogen stimulation.	27.
c-myc	Hamster fibroblasts	Transcription rates do not vary in quiescent and mitogen-stimulated cells, but steady-state levels increase 100-fold transiently.	17.
Various genes	Dictyostelium	Transcripts specific for the aggregation phase accumulate with a long half-life. When no longer required, these RNAs are rapidly and selectively degraded.	51.

5. Differential Translation

Differential translation also affects gene expression (35). This level of control was first dicovered with "maternal RNAs" - mRNAs that are stored but not translated in oocyte cytoplasm. After fertilization, these RNAs are "unmasked" and translated rapidly. Another well-characterized translational control operates on the heat shock RNAs of Drosophila and other organisms. Table 7 gives examples of several cases of gene expression at the level of qualitative and quantitative translation.

6. Post-translational Control

Several types of post-translational controls exist which affect either the quantity or type of proteins in a cell type. Differential protein turnover or prosthetic group additions contribute to this control level, as do proteolytic cleavages of overlapping or tandem peptides within a single precursor protein. Table 8 lists many examples of post-translational cleavages for eucaryotic cellular polyproteins. It is interesting that these examples involve the generation of small (40 amino acids or less) peptides and most of these peptides function as neuroendocrine hormones. It is hypothesized that this mechanism evolved due to difficulties in translation of such small peptides individually. Also, post-translational cleavage allows a very rapid response to a stimulus, while transcriptional levels may be maintained constitutively. (For recent review, see Reference 18.)

TABLE 7 - Translational Control

GENE	ORGANISM	DESCRIPTION	REFERENCE
Various genes	Spisula	After fertilization, translation stops for certain specific mRNAs while others become associated with polysomes and are translated.	67.
Ribosomal RNA	Mouse cells	After growth arrest, rRNAs dissociate from polysomes and translation stops. The process is reversible after return of cells to growth medium.	23.
Heat shock	Drosophila	At high temperature, non-heat shock mRNAs are translated poorly, whereas heat shock mRNA is translated well at both normal and elevated temperatures. Signals for this selective translation lie within the HSP 70 non-translated leader.	40.
GCN4	Yeast	The level of GCN4 mRNA is constant regardless of nutritional state, whereas translation of this message is increased 100-fold during amino acid starvation. The long (577 b) leader containing 4 unused AUG codons is thought to contribute to translational control.	79.
Fibronectin	Xenopus	Maternal mRNAs are untranslated until mid-blastula stage (6 - 7 hrs. after fertilization).	45.
Various genes	Volvox	Translation patterns change dramatically in response to light changes.	38.
Various genes	Dictyostelium	When cells shift from vegetative to developmental phases, 30% of mRNAs dissociate from polysomes, and newly-synthesized mRNAs are selectively translated.	62.
Collagen	Human	A peptide fragment derived from Type 1 Collagen specifically inhibits translation of procollagen mRNA.	34.

TABLE 8 - Post-translational Control

GENE	ORGANISM	DESCRIPTION	REFERENCE
Alpha-mating factor	Yeast	The carboxy-terminal half of the 165 amino acid precursor contains four tandem copies of the mature alpha-factor peptide. Each copy is separated by 6 - 8 amino acids and proteolytic processing signals.	42.
Egg Laying Hormone (ELH)	Aplysia	A 357 amino acid precursor can yield peptides α,β,γ,δ - bag cell factors and ELH, depending upon cell type.	
Preproenkephalin	Human Bovine	The 263 amino acid precursor contains 6 copies of Met-enkephalin and 1 copy of Leu-enkephalin.	13.
Preprosomatostatin	Human Rat	Two forms of somatostatin (14 amino acids and 28 amino acids) are derived from a 92 amino acid precursor which itself is derived from a 116 amino acid precursor.	49.
Prolactin/ Growth hormone	Rat Bovine	Proteolytic cleavage within the disulfide loop yields an active amino-terminal fragment. The carboxy-terminal fragment and intact protein are inactive.	55.
Proopiomelanocortin (POMC)	Human Bovine	The 265 amino acid primary translation product can yield, after tissue-specific proteolytic cleavage, 9 different bioactive peptides- α,β,γ-MSH, ACTH, β and γLPH, β-endorphin, Met-enkephalin, and CLIP.	14.

Polyprotein precursors are known for many other bioactive peptides: (see Reference 18 for review)

vasopressin	CGRP
oxytocin	beta-nerve growth factor
corticotropin RF	epidermal growth factor
Growth hormone RF	bradykinin
glucagon	angiotensin
calcitonin	vasoactive intestinal peptide
gastrin	caerulein

7. Gene Rearrangement and Amplification

Rearrangements of genomic DNA usually occur in a relatively random manner. Recombination, duplications, deletions, and transpositions generally do not directly contribute to gene regulation and often have deleterious effects. However, there are a number of chromosomal rearrangements that appear purposeful and have probably evolved to contribute to diversity in gene expression within some types of somatic cells. Table 9 lists examples of several types of functional genetic rearrangements.

THE DOPA DECARBOXYLASE GENE

The dopa decarboxylase (Ddc) gene in Drosophila melanogaster is of interest to students of gene regulation for several reasons. It is a well-characterized genetic locus which provides an excellent example of exquisite genetic economy. The single Ddc gene encodes an enzyme with at least two isoforms which display multiple enzyme functions. Ddc's expression shows strict tissue and temporal specific regulation and is hormonally regulated at one stage. DNA sequencing has revealed splicing variants and transformation studies suggest a rather complex regulatory region containing multiple and interacting control elements.

DDC Protein - Structures and Functions

The Ddc gene is the sole structural gene for the enzyme dopa decarboxylase (DDC) in the Drosophila melangoster genome (24,33,82). DDC catalyzes the decarboxylation of dopa to dopamine (50) and

TABLE 9 - Gene Rearrangements and Amplification

GENE	ORGANISM	DESCRIPTION	REFERENCE
MAT locus	Yeast	Switching of mating phenotype is controlled at the MAT locus. Gene conversion and recombination between MAT and 1 of 2 silent copies of mating type information result in displacement of MAT sequences but no change in the silent copies.	76.
Variable surface glycoproteins	Trypanosomes	Silent copies of surface antigen protein genes are activated via gene conversion events which enter the information into an activation locus, similar to the yeast mating type situation.	7a.
Immunoglobulin light chain	Vertebrates	Formation of a complete variable region involves recombination of one of several hundred V units with any one of 5 Joining regions in somatic B-cell DNA. This process either deletes or inverts intervening DNA. The joining process is imprecise, which generates even more diversity.	68.
Immunoglobulin heavy chain	Vertebrates	In the first rearrangement, one of about 20 D segments recombine with one of 4 J segments, deleting intervening DNA. In the second stage, one of several hundred V segments join with the DJ unit, creating the VDJ heavy chain variable region. Imprecise joining, deletion and insertion of a few new nucleotides contribute to diversity during rearrangement.	69.
Immunoglobulin heavy chain	Vertebrates	Recombination events bring the VDJ unit close to a new C region when B cells switch from IgM and IgD to IgG, IgE and IgA classes.	14.
Ribosomal RNA	Amphibians	Gene amplification (2000-fold) of rDNA in frog eggs occurs via rolling circle DNA synthesis.	75.
Chorion genes	Drosophila	A 100 kb. region of genomic DNA containing the chorion genes is amplified 50-fold in ovarian follicle cells.	74.
DHFR	Mammalian cells	Gene amplification occurs in cells treated with methotrexate, leading to drug resistance.	1.

5-OH-tryptophan to serotonin, but not tyrosine to tyramine (48). The enzyme itself is a homodimer of 52,000 daltons per monomer (11) and is found in two tissues where it performs two distinct functions:

1) Over 90% of DDC activity is located in the epidermis where it is necessary for pigmentation and hardening of the newly-deposited cuticle. Figure 1 depicts the tyrosine pathway involving DDC.

2) Approximately 5% of DDC activity is located in the central nervous system where it is required for production of neurotransmitters dopamine and serotonin (48). There is now evidence that a distinct DDC isoform, differing from epidermal DDC by 33-35 amino acids at the amino terminus, occurs in the central nervous system and its differential expression occurs as a result of differential mRNA splicing (21,56).

DDC Developmental Profile

The DDC activity profile in neural and epidermal tissues reflects its uses. Levels of enzyme activity in brain and nervous system remain relatively constant throughout the life cycle, reflecting the presumably constitutive requirement for neurotransmitters (72). However, DDC levels in epidermis show sharp peaks each time a new cuticle is sclerotized. Enzyme activity peaks five times during development: hatch, 1st to 2nd instar molt, 2nd to 3rd instar molt, pupariation and adult eclosion. Figure 2, reprinted from Kraminsky et al (41), is a summary of epidermal DDC enzyme levels throughout development. Temperature sensitive Ddc mutations which reduce expression to levels

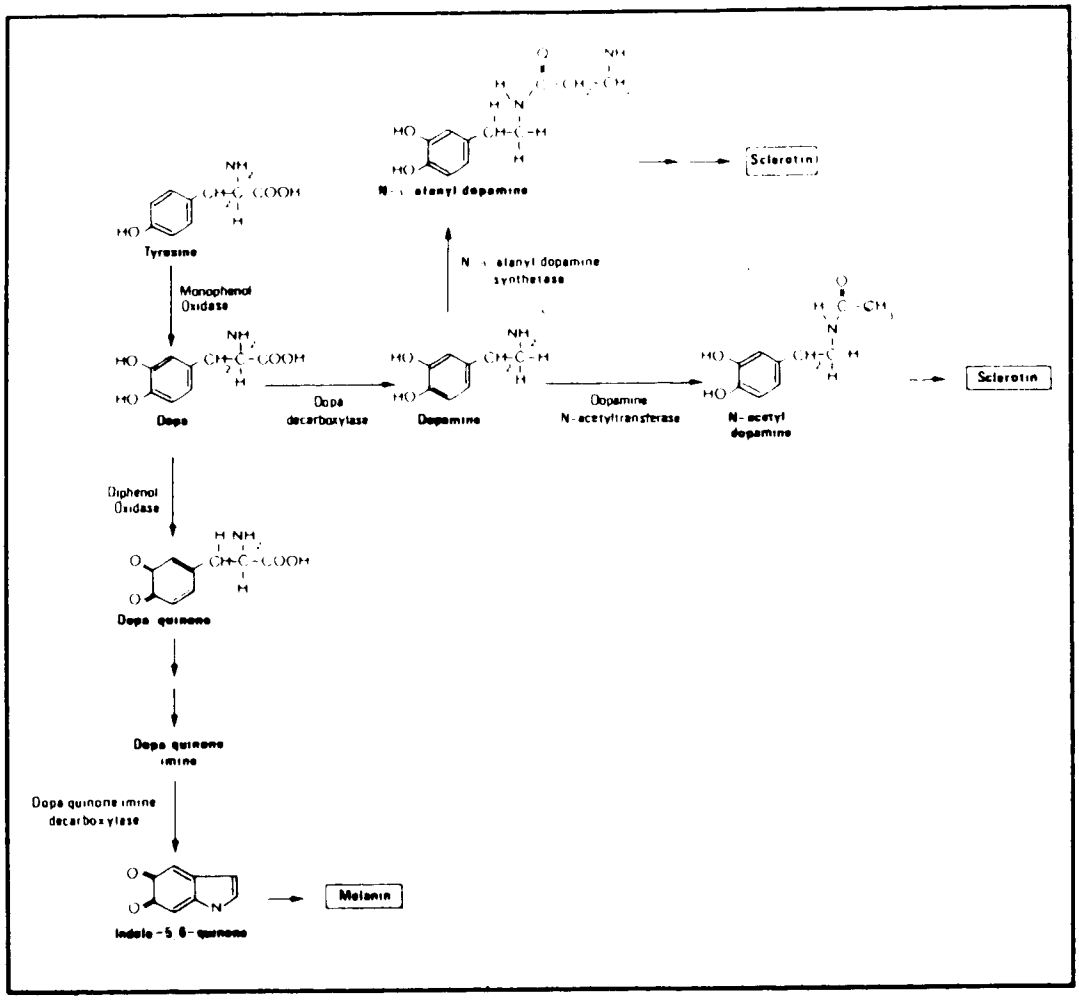

Figure 1 Metabolic Pathways Involving Dopa and Dopa Decarboxylase in *Drosophila melanogaster*.

Figure adapted from Wright (82b).

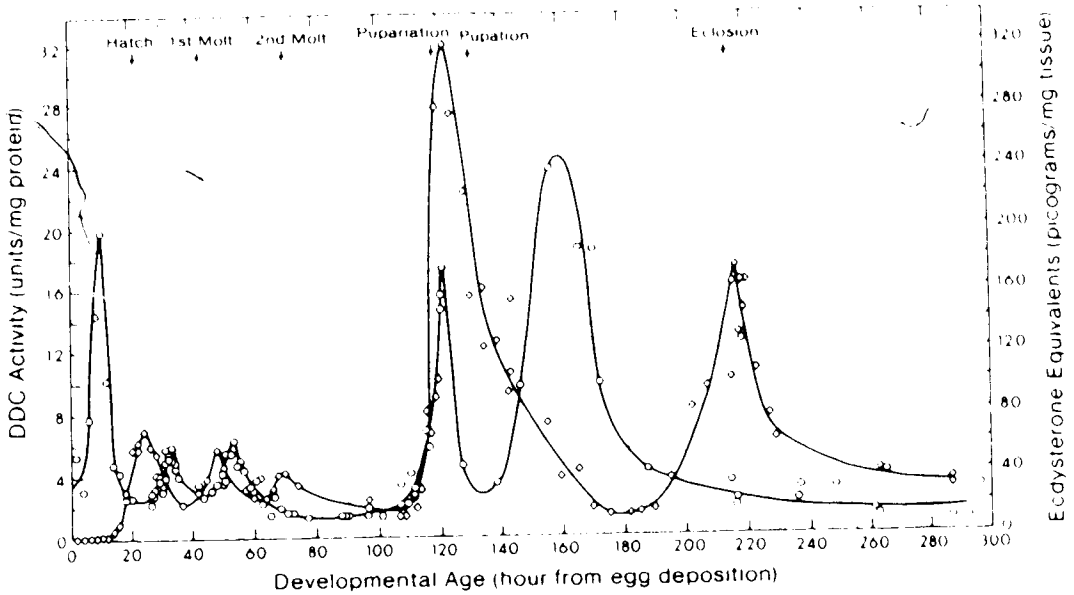

Figure 2 DDC Activity (△) and Ecdysteroid Titer (o) during the Life Cycle of Canton-S Strain of *Drosophila melanogaster* at 25°C.

Figure from Kraminsky et al. (41).

less than 10% of wild-type result in lethality at each of these 5 stages (33), demonstrating that epidermal DDC activity is essential. Also, these mutants, at restrictive temperatures, show learning disabililties (but memory retention and behavioural patterns are unaffected), suggesting the levels at which these neurotransmitters are essential in the Drosophila central nervous system (78).

Ddc mRNAs and Gene Structure

Ddc mRNA levels at embryogenesis (24), pupariation (41), and in imaginal discs (10) reflect temporally and quantitatively the enzyme activity and/or CRM in each of these situations. This suggests that the major control is at the transcript level. Levels of Ddc regulation will be discussed in the next section. This section will summarize what is known about the complex RNA structures encoded by the Ddc gene. Two recent papers (21,56) present the Ddc genomic and cDNA sequences.

The Ddc transcribed region covers about 4 kb of DNA. (See Figure 3 for composite diagram of Ddc genomic and RNA structures.) The RNA start site has been mapped by primer extension (56) and the junctions of exons A,B,C, and D were mapped by comparison of cDNA and genomic sequence, S1 mapping, primer extensions and by comparison of sequences with consensus splice junction sequences. Differential splicing of exons B and C gives rise to two different mRNAs in either the central nervous system (CNS) or epidermis. Two different isoforms of DDC are translated from these alternatively-spliced messages, as described in the next section. Exons C plus D contain a single ORF of 476 amino acids, which is sufficient to code for a 53,500 dalton protein, consistent with the subunit size of 52,000 daltons for 3rd instar epidermal DDC (11). An amino acid region

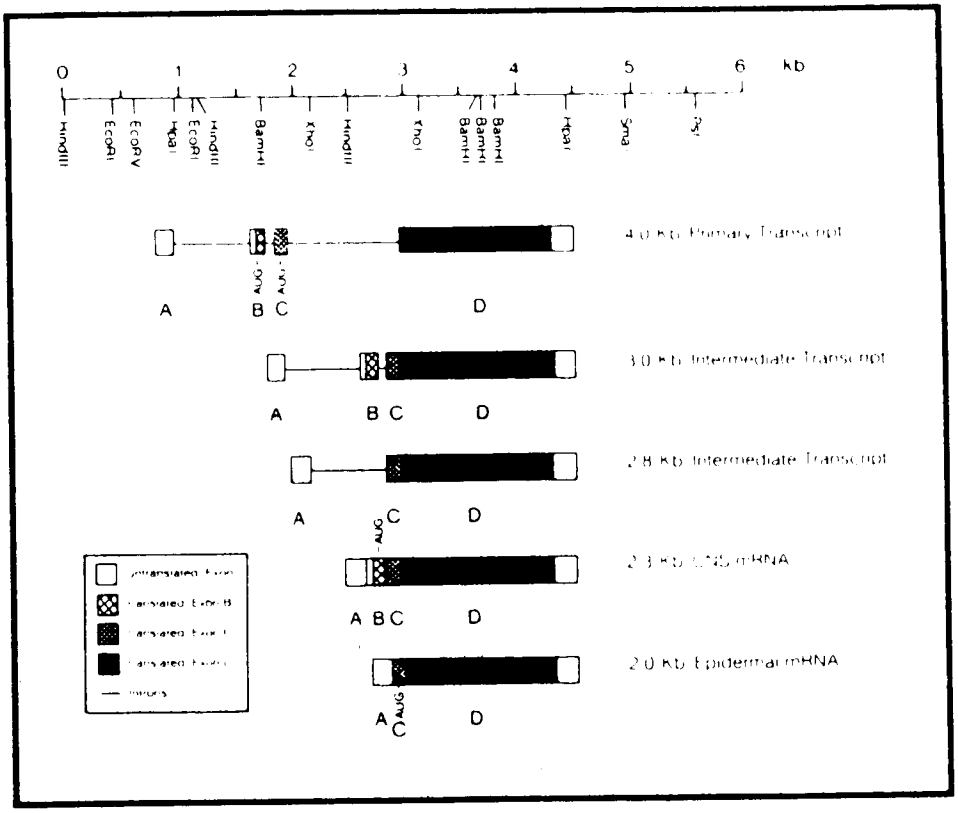

Figure 3 Ddc Genomic DNA and RNA Structures.

of homology to the porcine DDC pyridoxal phosphate binding site is located midway through exon D. The use of the only large ORF in the genomic sequence has been confirmed by an expression vector test (21). Table 10 summarizes the complex developmental patterns of mRNA splicing that occur for Ddc.

Ddc Gene Regulation

1. CNS-Specific Transcriptional and Post-transcriptional Controls.

The tissue-specific regulation of Ddc has been elucidated by some recent work on CNS Ddc expression (56,72). Primer extensions show that the 2.3 kb mRNA is the only mature Ddc mRNA found in the CNS and is the same 2.3 kb species found in embryos and presumably in adults (21). The 2.3 kb CNS mRNA, because of an additional AUG codon in its exon B, encodes a 57,000 dalton protein, differing from epidermal DDC by 33-35 amino acids at the amino terminus. The two isoforms from isolated fly heads can be separated on DEAE ion-exchange columns. In transformed strains lacking CNS control sequences, one DDC protein peak is eliminated as well as the 2.3 kb mRNA. However, in another transformant containing a gene lacking intron 1 and exon B (ie. it cannot produce the 2.3 kb transcript), normal levels of DDC were found in epidermis and even slightly higher levels in the CNS. This indicates that the tissue-specificity of the CNS transcript species is not due to specific degradation of the epidermal 2.0 kb transcript in the CNS. Although these experiments show that the absence of the 2.3 kb species and the presence of the 2.0 kb species with its smaller protein isoform in the CNS is not lethal, studies of learning disabilities were not done in these experiments. It is not clear yet what physiological function is

TABLE 10 - Tissue and Developmental Distribution of Ddc Transcript Species

SPECIES	TISSUE		DEVELOPMENTAL STAGE				TRANSLATED[1]	COMMENTS
	CNS	Epidermis	14 hr. Embryos	Evaginating Discs	Late 3rd Instar	Newly-eclosed Adults		
4.0 kb.	+	+	+	+	+	+	-	The primary transcript.
3.0 kb.	+	+	+	+	+	+	-	Product of first splicing step. Lacks 3'-intron.
2.8 kb.	?	?	trace	trace	+	trace	-	May be splicing intermediate between 3.0 and 2.0 kb. transcripts, lacking Exon B, mini-intron and the 3'-intron.
2.3 kb.	+	-	+	+	-	-	+	Mature CNS transcript. Contains Exons A,B,C,D.
2.0 kb.	-	+	+	+	+	+	+	Mature epidermal transcript. Contains Exons A,C,D.

[1] - present on polysomes.

served by the presence of a CNS isoform, however, post-translational modifications resulting from a different amino terminus may contribute to substrate specificity (dopa as well as 5-OH-tryptophan) which occurs in the CNS.

Transformation experiments involving deletion constructs downstream of -208 from the transcription start site indicate that a CNS control element necessary for expression in nervous tissue is located between -83 and -59 relative to the start site (72).

2. Epidermal-Specific Controls

One of the interests in Ddc gene expression has been its proposed regulation, at least at one developmental stage, by the steroid molting hormone, 20-OH-ecdysone. At pupariation, the hormone maximum is coincident with both Ddc mRNA and enzyme activity maxima (see Figure 2). Continuous feeding of 20-OH-ecdysone to the ecdysone-less temperature sensitive mutant, ecd^1, results in detectable translatable Ddc mRNA within 2 hours of feeding, reaching a maximum at 8 hours. DDC enzyme activity parallels that of mRNA levels. More recent experiments by Clark et al (10) using protein synthesis inhibitors in similar feeding experiments, show that protein synthesis is required for 6-fold of the 36-fold transcript accumulation, but that another 6-fold accumulation is unaffected by protein synthesis inhibition. This suggests that at least part of the response to hormone is a primary effect - perhaps at the level of transcript initiation. Newly-synthesized proteins may contribute to the transcript accumulation by affecting mRNA stability, the transcription rate itself, hormone-receptor stability, or mRNA transport.

This primary and rapid response of the Ddc gene to hormone in late 3rd instar epidermis is in striking contrast to its response at other times in the life cycle. As Figure 2 shows, the DDC maxima at hatching, the larval molts and adult eclosion are not accompanied by hormone maxima. Lags of between 8 and 60 hours occur between hormone and DDC activity peaks. In fact, the presence of hormone and enzyme activity appear almost mutually exclusive. To clarify these quite different responses of the Ddc gene, an in vitro "feeding" study was performed on 3rd instar imaginal discs, considered progenitors of adult epidermis (10). The results show that:

1) continuous presence of 20-OH-ecdysone prevents the appearance of both Ddc mRNA and enzyme activity in cultured imaginal discs, and

2) when a pulse of hormone is given to the discs, there is a 6 hour lag between removal of hormone and the first appearance of transcript or activity.

These results suggest that a falling ecdysterone titre may trigger a cascade that eventually leads to Ddc mRNA accumulation. A direct inhibitory effect of hormone-receptor complex over the 6 hours is unlikely, as the complex has a half life of approximately 30 minutes (85).

These experiments of Clark et al (10) demonstrate the very different regulatory mechanisms that act on the epidermal Ddc gene at different stages of development:

1. A direct and rapid transcriptional effect of steroid hormone - in 3rd instar epidermis.

2. A secondary rapid effect requiring protein synthesis - in 3rd instar epidermis.

3. An indirect, cascade effect, with a several hour delay - in imaginal discs.

Transformation experiments from Dr. J.Hirsh's laboratory suggest that the regulatory sequences necessary for epidermal Ddc control lie within 208 bp upstream of the transcription start site (72). A gradual loss of expression occurs in the epidermis as sequences are deleted through this region, perhaps due to the presence of several regulatory elements which act cooperatively. However, transformants lacking all sequences between -208 and -24 still express from 10-50% of wild-type levels of activity at the proper developmental stages, suggesting that other regulatory elements may exist within or upstream of the gene.

A further discussion of Ddc control sequences will be presented in Chapter 4.

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CHAPTER 1

Analysis of a Novel Enzyme produced in *Drosophila* Cells in Response to 20-hydroxyecdysone.

A version of this chapter has been published:
Spencer, C.A., Stevens, B., O'Connor, J.D. and R.B. Hodgetts, 1983. A Novel Form of Dopa Decarboxylase Produced in *Drosophila* Cells in Response to 20-hydroxyecdysone. Canadian Journal of Biochemistry and Cell Biology 61: 818-825.

INTRODUCTION

Dopa decarboxylase (DDC) is found in epidermal and neural tissues of Drosophila. In the epidermis, the conversion of dopa to dopamine leads to the production of sclerotin and melanin, compounds necessary for hardening and coloring the insect cuticle after each molt (44). In neural tissue, DDC is necessary for production of neurotransmitters dopamine and serotonin (42).

When DDC activity in whole organisms is measured throughout development, five maxima in the titre of the enzyme are noted (24). These maxima reflect changes in epidermal levels of DDC since the contributions to total activity from neural tissue is less than 5% (34). Five maxima in the titre of 20-hydroxyecdysone, the insect steroid molting hormone, are also noted during ontogeny (24). In mature larvae, the hormone and enzyme maxima are virtually coincident. At the other stages, however, the hormone maxima precede the DDC peaks by times ranging from 8 to 60 hours.

Our interest in epidermal DDC originated with the observation of Karlson and his co-workers whose early work on the blowfly Calliphora suggested that DDC activity in the mature larval epidermis was induced by 20-hydroxyecdysone (23). Subsequently, it was shown that a correlation existed between the high ecdysteroid levels in mature larvae and increased levels of translatable mRNA for DDC. The isolation of the temperature-sensitive mutant ecd^{1} in Drosophila (17) allowed us to subject mature larval epidermis to a controlled dose of 20-hydroxyecdysone (24). This mutant, when raised to the nonpermissive temperature during third instar, fails to exhibit either the hormone or

the DDC maxima which normally occur at pupariation. At nonpermissive temperatures we were able to demonstrate that a rapid increase in translatable DDC mRNA in the epidermis of mature larvae occurs following the administration of 20-hydroxyecdysone. More recently (9) we have confirmed that this increase in translatable messenger occurs as a result of an increase in the size of the pool of mRNA transcripts, as measured by hybridization to a genomic DNA clone of the dopa decarboxylase (Ddc) gene isolated by Hirsh and Davidson (21). Six-fold of the 36-fold increase in the pool of Ddc mRNA occurs in hormone-stimulated organisms in the virtual absence of protein synthesis. A further 6-fold increase in Ddc mRNA requires the presence of newly-synthesized proteins. Thus we believe that at least a portion of DDC in the mature larval epidermis appears as a result of a "primary" response to the steroid, perhaps at the level of transcription initiation.

We are also interested in exploring the mechanisms which effect the rapid increases in DDC activity seen in the epidermis at other stages of development. In contrast to the situation just described, at these stages an appreciable lag occurs between elevated hormone levels and the appearance of DDC.

Clark et al (9), in a recent study of cultured imaginal discs, showed that neither DDC activity nor transcript appeared when discs were cultured in the continuous presence of 20-hydroxyecdysone. However, when discs were given a 6 hr. pulse of hormone, DDC activity and mRNA began to accumulate after a further 6 hr lag.

In this paper, we report our analyses of cloned derivatives of the Kc cell line (39) which we hoped would provide an homogeneous tissue source, responsive to 20-OH ecdysone. The intention was to use these

cell lines as experimental models for those situations in vivo where a lag occurs.

The suggestion has been made that the Kc cell line, which was derived from embryonic tissues, is ectodermal in origin (13). This is supported by the observation that these cells undergo some remarkable morphological changes in the presence of 20-hydroxyecdysone, including the extension of nervelike processes (1,12). Furthermore, Kc cells possess an ecdysteroid-binding protein in the cytoplasm (27) whose characteristics resemble those of the ecdysteroid receptor found in imaginal discs (45). Finally, it was shown some years ago that these cells produce two insect nervous system enzymes, acetylcholinesterase (1b) and β-galactosidase (3), following exposure to 20-hydroxyecdysone. While these data are by no means proof of the ectodermal origin of the Kc line, we were interested to discover whether induction of DDC, an enzyme principally confined to tissues of ectodermal origin, could be demonstrated. In this paper, we analyse the hormone-dependent appearance of DDC enzyme activity and mRNA in cloned derivatives of the Kc cell line. We also present a biochemical characterization of this enzyme, since we have discovered that the protein from cell lines is immunologically distinct from DDC produced in vivo.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The Kc cell line was originally obtained from Echalier and Ohanessian (14). It was maintained at 22°C as a suspension in serum-free and antibiotic-free D-20 medium (15) made up in Milli-Q purified water. The Kc subclones 6D10, 7E10 and 7C4 were obtained by limiting dilutions of Kc cells onto irradiated (12,000 - 24,000 rads; 1 rad = 10mGy) feeder layers (38). Cells were maintained in spinner flasks at a density of 2×10^4 - 8×10^6 cells/ml. To initiate experiments, exponentially growing cells were transferred to roller bottles and allowed to grow for about 12 hrs to a density of 2×10^6 cells/ml before 20-hydroxyecdysone was added.

Drosophila Stocks

Wild-type Drosophila melanogaster flies (Canton-S strain) were maintained at room temperature (22°C) on synthetic medium (31). Newly-eclosed adult flies were selected by clearing bottles and collecting white eclosed adults at 2 hr intervals. Wandering third instar larvae were collected by washing mature larvae from the sides of culture bottles with distilled water.

Hormone Treatments

Stock solutions of 20-hydroxyecdysone (Sigma) were prepared at 1 or 2 mg/ml in 4% ethanol and stored at -70°C. The hormone was added to exponential cultures in roller bottles at densities of 3×10^6 cells/ml to a final concentration of 2×10^{-7}M. Control cultures

were treated with an equivalent amount of ethanol. Cell growth was monitored under phase contrast using a Hawksley counting chamber.

Aliquots of cells to be harvested for enzyme determinations were removed at appropriate time intervals, centrifuged at 735 × g for 5 min in an SS-34 Sorvall rotor, washed in fresh D-20 medium and centrifuged again. The supernatant was removed and the pellets were frozen in liquid nitrogen and stored at -70°C. Hormone-withdrawal experiments were performed by centrifuging the culture after 6 hr in the presence of hormone at 1000 × g in a Sorvall GSA rotor at room temperature. The culture medium was removed and an equivalent amount of fresh D-20 medium was added. The culture was then divided into two parts; one was returned to 2×10^{-7}M 20-hydroxyecdysone while the other served as the withdrawn culture.

Enzyme Assays

1. $^{14}CO_2$-dopa Microdiffusion Assay - DDC

Frozen pellets were resuspended in 0.05M Tris-HCl (pH 7.3) containing 1.2×10^{-4}M phenylthiourea and homogenized with 50 strokes of a Bellco conical sintered glass tissue grinder. The extract was centrifuged at 12,800 × g for 5 min and the supernatant was recovered. Extracts of adult flies were prepared by homogenizing newly-eclosed adult organisms as described for the frozen pellets. DDC assays were carried out as described previously (10). In this assay, radioactive CO_2 is liberated from ^{14}C-dopa (dihydroxyphenylalanine DL-3,4,-[alanine-1-^{14}C]) and is trapped by Oxifluor-CO_2(NEN). One unit of activity corresponds to the decarboxylation of 1 nmol L-dopa/hr at 30°C.

2. ^{3}H-Ring-Labelled Dopa Assay - DDC Extracts were prepared as above, in homogenization buffer (0.1M sodium phosphate pH7.1, 0.3M sucrose, 0.2mM phenylthiourea). Assays were performed as described by McCaman et al (29) with minor changes. In this assay, radioactive dopamine, liberated from the decarboxylation of uniformly ring-labelled ^{3}H-dopa (dihydrophenylalanine L-3,4[3H(G)]) is trapped in chloroform: diethylhexylphosphoric acid. An aliquot of the organic phase is dissolved in fluor and radioactivity determined. Heat denatured blanks for this and the above assay were prepared by heating aliquots of extract for 5 min at 100°C and assaying as usual.

3. Acetylcholinesterase Assay

Acetylcholinesterase assays were performed according to Cherbas et al (8). One unit of activity corresponds to an increase of 1 OD unit/hr measured at 412 nm.

4. Protein Determinations

Protein concentrations were determined using the Bradford Assay (5), modified by Spector (36).

Drosophila Brain Dissections

Fifty adult Drosophila brains were dissected into D-20 tissue culture medium and collected into a drop of D-20 held on ice. Brains were then frozen in liquid nitrogen and stored at -40°C. Extracts were prepared by making volumes up to 300µl with Tris/PTU (0.05M Tris-HCl pH7.3/1.2 $\times 10^{-4}$M phenylthiourea, grinding in a glass conical tissue grinder and centrifuging. Supernatants were immunoprecipitated and assayed as described below.

Immunoprecipitations

Monospecific antiserum was raised against DDC purifed from mature larvae (10) and IgG-containing fractions were prepared. Immunoprecipitations using heat-killed Staphylococcus aureus as a solid phase immunoabsorbent of antigen-antibody complexes were carried out as follows. Two-hundred microlitres of crude homogenate were incubated at 0°C for 3 hrs in the presence of 2 - 10 µl of an appropriate dilution of purifed IgG. Ten microlitres of a suspension (10% v/v) of immunoabsorbent cells was added and, after 5 min at 0°C, the cells were centrifuged at 27,000 × g for 10 min in an SS-34 rotor. The supernatant was then assayed for residual DDC activity.

DE-32 Column Chromatography

Chromatography was carried out on DE-32 cellulose (Whatman Biochemicals, Ltd.). Crude homogenates were loaded onto a column (1.0 × 8.5 cm) equilibrated with 0.05M Tris-HCl (pH 7.3) containing 10% glycerol and 1 mM dithiothreitol. A 40 ml sodium chloride gradient (0-0.25M) in the same buffer was applied to the column at a flow rate of 16 ml/hr. Fractions of 1 ml were collected and DDC activity was measured on 100 µl aliquots.

Concanavalin-A Sepharose Binding

ConA-Sepharose (Pharmacia Fine Chemicals) is concanavalin-A bound to Sepharose 4B, and is used to separate and purify many glycoproteins, polysaccharides and carbohydrates. ConA-Sepharose interacts with enzymes provided they contain a sugar (α-D-mannopyranosyl, α-D-glucopyranosyl) bearing C-3, C-4 or C-5 hydroxyl groups (19).

A 1 ml column of ConA-Sepharose was poured, washed with 10 volumes of Start Buffer (0.05M Iris pH 7.4, 0.5M NaCl), and loaded with 20,000 cpm activity of partially-purified cell line DDC. The column was then washed with 8 ml Start Buffer, and eluted with 10 ml of 0.5M α-D-Methylglucoside in Start Buffer. Fractions of 1 ml each were collected and assayed by the CO_2-microdiffusion DDC assay.

Thin Layer Chromatography - Dopa and Dopamine

Plates used were 20 × 20 cm, 0.1 mm thick, plastic TLC plates from Merck (BDH Chemicals, Toronto). The solvent was butanol:acetic acid:water (4:1:1) (22). Samples were 1) Third instar larval crude extract. Activity in the CO_2-microdiffusion assay was 19,000 cpm/50μl. 2) Partially-purified 7E10 cell line DDC extract from a G-200 column (an approximately 20-fold purification). Activity in the CO_2-microdiffusion assay was either 15,500 cpm/50μl or 4,000 cpm/50μl. 3) Mixtures of the above 2 extracts. 4) Heat-denatured aliquots of the above extracts. Samples were mixed with the reaction mixture of the ^{3}H-ring-labelled dopa assay as described above and reactions incubated 37°C for 60 minutes. Aliquots were spotted directly onto TLC plates and dried. Plates were then placed in a chromatography tank and the solvent allowed to run in an ascending direction for approximately 6 hrs. Plates were dried, sprayed with "En^{3}hance" (NEN) and autoradiographed.

Molecular Weight Determinations

1. G-200 Gel Filtration

A Sephadex G-200 column (Pharmacia) was prepared as described in "Enzyme Purifications". The column was calibrated with 6 mg Blue

Dextran, 15 mg bovine serum albumin (m.wt. 68,000), 15 mg bovine liver catalase (m.wt. 240,000) and 400,000 cpm partially-purified larval DDC (m.wt. 112,000). The cell line DDC sample was a partially-purified (G-200 column stage) preparation. Elution profiles were determined by Abs_{280} (Blue Dextran), Bradford protein assays (catalase and BSA) or DDC assays (larval and cell line DDCs). The partial coefficients of each standard as well as cell line DDC were calculated as in References 7 and 25. The partial coefficients of the standard proteins were plotted against the corresponding molecular weights on a semi-logarithmic scale and a straight line was obtained. The K_{av} of cell line DDC was then compared to the standard curve.

2. Sucrose Gradients

Molecular weight estimates were obtained following the methods of Pass (33) and Martin and Ames (28) by sedimentation through a 5-20% linear gradient of sucrose in 0.05M Tris-HCl pH 7.3, 10% glycerol, 1 mM DTT. Centrifugation was at 30,000 rpm for 20 to 36 hrs at 4°C in an SW-40 rotor. Gradient fractions (250μl) were collected through a 21-guage needle puncture at the base of the tube. Protein standards were 1 mg bovine serum albumin (m.wt. 68,000), 2 mg bovine liver catalase (m.wt. 240,000) and larval DDC (m.wt. 112,000). The cell line DDC sample was partially purified to the G-200 column stage. Assays were carried out as in G-200 Gel Filtration, above. Molecular weights were determined by comparison of the sedimentation of cell line DDC with that of the standards, as described in References 28 and 33.

3. Variable Porosity Non-denaturing Polyacrylamide Gel Electrophoresis

Native polyacrylamide gels of 8.5% and 10.5% were prepared as slabs and run at 8-12 ma for 3 hrs, according to protocols in Pass (33). Molecular weight standards were: chicken egg ovalbumin (m.wt. 43,000), bovine serum albumin (m.wt. 68,000), yeast alcohol dehydrogenase (m.wt. 150,000), bovine liver catalase (m.wt. 240,000) and larval DDC (m.wt. 112,000). Gels were silver stained, using the Bio-Rad Silver Stain Kit according to manufacturers instructions (Bio-Rad Technical Bulletin 1089). Distances migrated by proteins were noted, Rm's and slopes calculated and data plotted according to Hedrick and Smith (20) and Pass (33). Plots of molecular weights vs. slopes of standard proteins formed a straight line. These standard curves were then used to estimate the size of the purified native cell line enzyme.

4. Denaturing SDS Gel Electrophoresis

To determine subunit molecular weights, 10%/4.5% SDS slab gels were used following protocols in Pass (33) and Weber and Osborn (42b). Gels were run at 100 V for 2.5 hrs and stained with the Bio-Rad silver stain. Migration distance vs. molecular weights of standard proteins (ovalbumin, catalase, BSA and larval DDC) were plotted and the standard curve used to estimate subunit size of the purified cell line enzyme.

Peptide Maps

One-dimensional peptide maps of purified larval and cell line enzymes were made after digestion of these proteins with S. aureus V8 protease (Miles Laboratories) which cleaves proteins at aspartic and glutamic acid residues, and bovine pancreatic trypsin (Sigma) which

cleaves at the carboxy-terminus of arginine and lysine residues. The protocol was an adaptation of the methods of Cleveland et al (11). Approximately 20 ng of each purified enzyme was dissolved in 0.1M Tris-HCl pH 7.5, 0.1% SDS, 10% glycerol, to a volume of 68 µl. This reaction mix was heated to 65°C for 10 min, cooled to 37°C and 50 ng of either V8 protease or trypsin added. After a 2 hr incubation at 37°C, a further 50 ng of each protease was added and digestion proceded for a total of 24 hrs. Reactions were reduced by adding 3µl β-mercaptoethanol and heated to 65°C for 10 min, then loaded onto 10% SDS polyacrylamide gels and run for 6 hrs at 200V. The gels were then silver stained following the method of Oakley et al (32).

Protein Purifications

Thirty-two separate purifications of the cell line enzyme were made - each from two 800 ml batches of 7E10 cells, hormone-induced for 5 days. Usually, 2 combined batches contained between 600-900 mg protein and a total of approximately 5×10^6 cpm DDC activity. All purification steps were performed at 4°C. A crude supernatant was prepared by resuspending cell pellets in sufficient Tris/Glycerol/DTT buffer (0.05M Tris-HCl pH 7.3/ 10% glycerol/ 1mM DTT) to bring protein concentrations to 5 mg/ml, homogenizing in a sintered glass tissue grinder and centrifuging at 4000 rpm for 20 min to remove debris. The extract was then subjected to the following purification steps:

1. Ammonium Sulfate Precipitations

Ammonium sulfate precipitations were performed as in Clark et al, 1978 (10). The 50-70% precipitate was collected, resuspended in Tris/Glycerol/DTT and dialysed against 1 liter of Tris/Glycerol/DTT.

2. DEAE Column Chromatography

A DEAE (Sigma) column was prepared and used as described in Clark et al (10) and Chen (7) except that proteins were eluted with a 0 - 0.25M NaCl gradient in Tris/Glycerol/DTT buffer. Fractions with DDC activity were pooled, concentrated to 10 ml using an Amicon Ultrafiltration Unit and YM 10 membrane (Amicon Corp.), and loaded onto a Sephadex G-200 column.

3. G-200 Sephadex Gel Filtration

G-200 column chromatography was performed following Clark et al (10) and manufacturers specifications (Pharmacia), except that the column was run in a descending direction and pump speed was maintained at 22 ml/hr.

4. Preparative Electrophoresis

Non-denaturing polyacrylamide tube gels (7.5%) were polymerized with riboflavin and light, following the protocols of Pass (33). The gels were placed in a preparative electrophoresis apparatus (Tyler Research Corp.) and pre-run for 30 min. Cell line DDC (500 μl of G-200 concentrate) was loaded onto the gel and run in Tris/Asparagine buffer (33) with the addition of 10% glycerol, 1mM DTT and 1.2×10^{-4} pyridoxal 5'-phosphate at 300 V (1 ma) for 12 hrs. Fractions were collected at 8 ml/hr following elution of tracking dye. The protein concentration of eluted fractions was monitored during the run with a UV monitor-recorder set at 280 nm. Fractions (200 μl) were assayed for DDC.

RNA Extractions

Spinner cultures of exponentially-growing 7E10 cells were divided into 4 roller bottles, diluted with D-20 medium and allowed to grow to 2 × 10^6 cells/ml. Hormone was added to 3 of the 4 roller bottles as described under "Hormone Treatments". Cells were harvested by centrifugation at 48 hr, 96 hr and 120 hr after hormone addition. The roller culture containing no 20-hydroxyecdysone was harvested after 24 hr. Pellets were frozen in liquid nitrogen and stored at -40°C until use.

Pellets were resuspended in 50 ml RNA Extraction Buffer (see Table I-1), SDS added to 0.5% and EDTA to 25mM. The slurry was homogenized in a Bellco sintered glass tissue grinder on ice and the debris removed by centrifugation. The supernatant was extracted twice with phenol:chloroform:isoamyl-alcohol (25:24:1) equilibrated with RNA Extraction Buffer, and twice with chloroform:isoamyl-alcohol (24:1). Nucleic acids were precipitated with 2 volumes ethanol and 0.1 volume sodium acetate pH 7.0.

Poly(A^+) RNA was purified from these extractions using oligo-dT chromatography following the protocol of Gietz and Hodgetts (18). Typical recovery was approximately 300μg poly(A^+) RNA per gram of cell pellet extracted.

TABLE I-1 - RNA Extraction Buffer

This recipe was modified from Spradling et al, 1975 (37).

30 mM Tris-HCl, pH 8.3
100 mM NaCl
5 mM KCl
10mM $MgSO_4$
25 μg/ml polyvinyl sulfate
35 μg/ml spermine
0.5% NP-40
5 mM dithiothreitol
0.5% β-mercaptoethanol
4 mM EGTA
5 mM N-ethylmaleimide
1% diethylpyrocarbonate
(optional - add just prior to use)

The final pH should be pH 7.5. Extraction buffer is prepared in DEP-treated double distilled water. This buffer contains multiple RNase inhibitors which are necessary for the integrity of RNAs from both cell lines and Drosophila embryos, both of which contain high levels of nucleases (18,37).

Northern Analysis

Agarose formaldehyde gels (1.5%) were run of poly(A^+) RNAs and ribosomal RNA markers as in Spencer et al (36) and Gietz and Hodgetts (18). Gels were blotted to nitrocellulose, baked, prehybridized and hybridized under conditions of high stringency (42°C for 24 hr) to a nick-translated BamHI 2.0 kb internal Ddc DNA probe (Probe 2 of Figure II-1 of this thesis). The probe was used at a concentration of 3.5 x 10^7 cpm/ml of hybridization mix.

RESULTS

Physiological Responses of Cell Lines to 20-hydroxyecdysone

1. Hormone-dependent Appearance of DDC in Clone 7C4

When cells of the clone 7C4 were exposed to 20-hydroxyecdysone at a concentration of 2×10^{-7}M, cell division arrest occurred (Figure I-1a) as reported elsewhere (39). Release from division arrest occurred 144 hr after initial exposure to the hormone. The morphological changes which have been described by others (1,12) began to appear at 48 hrs, but cell morphology was normal by the time of release from division arrest. It has been determined (39) that the resumption of cell division is not due to metabolism of hormone. Two-week old culture supernatants contain high levels of 20-hydroxyecdysone as determined by radioimmunoassay and are able to induce G2 arrest in previously untreated cells. Dopa decarboxylase activity appeared after an appreciable lag of 48 hr following the addition of 20-hydroxyecdysone and increased to its maximum at 144 hr (Figure I-1b). Activity was never observed in control cultures grown in the absence of 20-hydroxyecdysone. To investigate the possibility that the induction of DDC activity in the cell lines was a response to division arrest, cells were exposed to either 2×10^{-7}M 20-hydroxyecdysone or 10^{-7}M colchicine. Although colchicine treatment resulted in division arrest, enzyme activity did not appear when followed for 173 hrs. DDC activity did appear in the hormone-treated cultures. Further support for the view that the appearance of enzyme activity is not merely a response to division arrest is found by analysing data from untreated cultures. Figures I-1 and I-2 show that control cultures were maintained in

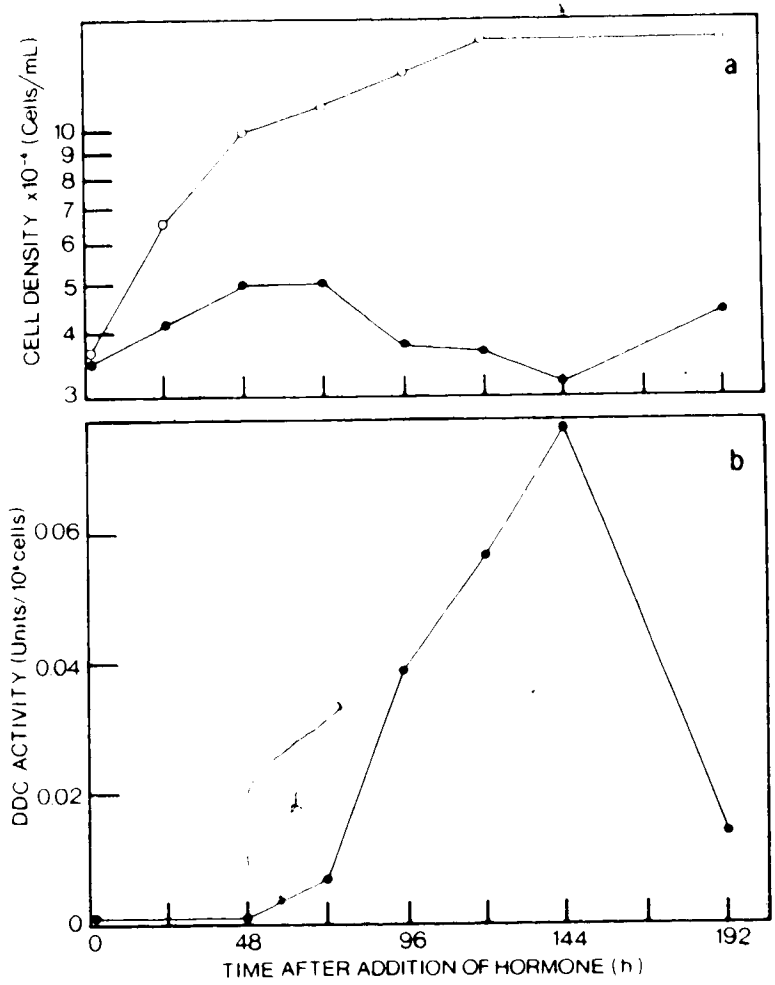


Figure I-1 Response of the Line 7C4 to the Continuous Presence of 20-hydroxyecdysone.

Cells were cultured in the presence (●) or absence (o) of 2×10^{-7}M 20-hydroxyecdysone.

a) Growth curves.
b) DDC activity.

Each enzyme determination in Figures I-1 to I-5 represents an average of three replicate assays on a single extract.

stationary phase for 3 to 6 days. No appearance of enzyme activity appeared in these stationary phase cultures. Previous work (39) showed that such stationary phase cells are synchronously arrested in G1.

2. Induction of DDC and AChE in Clone 7E10

As DDC activity began to decrease with the resumption of cell division in 7C4, we were interested in the response in 7E10, a line in which the cells do not release from division arrest. As the data in Figure I-2 show, the DDC activity per cell increased continuously in the presence of the steroid. The lag in the appearance of DDC activity following hormone administration contrasts with the induction of acetylcholinesterase (AChE)in derivatives of the Kc cell line. Shown in Figure I-2 is the hormone-dependent induction of AChE in clone 7E10. In contrast to DDC, virtually no lag is observed in the induction profile of this enzyme and, further, activity per cell declines continuously from its early maximum.

During the course of development, the ecdysteroid titre in Drosophila exhibits sharp maxima (24). To test the possibility that the cell lines would respond to a relatively brief exposure to the hormone, 7E10 was cultured in 20-hydroxyecdysone for 6 hr. The hormone was then washed out as described in Materials and Methods and DDC activity was monitored. As shown in Figure I-3, the cells released from divtsion arrest under this regime of hormone treatment. Dopa decarboxylase activity began to increase between 36 and 48 hr in the withdrawn cells (Figure I-3b) and reached a maximum at 96 hr. Thus in the instance of hormone withdrawal, the 7E10 line was behaving similarly to 7C4 continuously cultured in 20-hydroxyecdysone (Figure I-1), although the kinetics of DDC induction were more rapid.

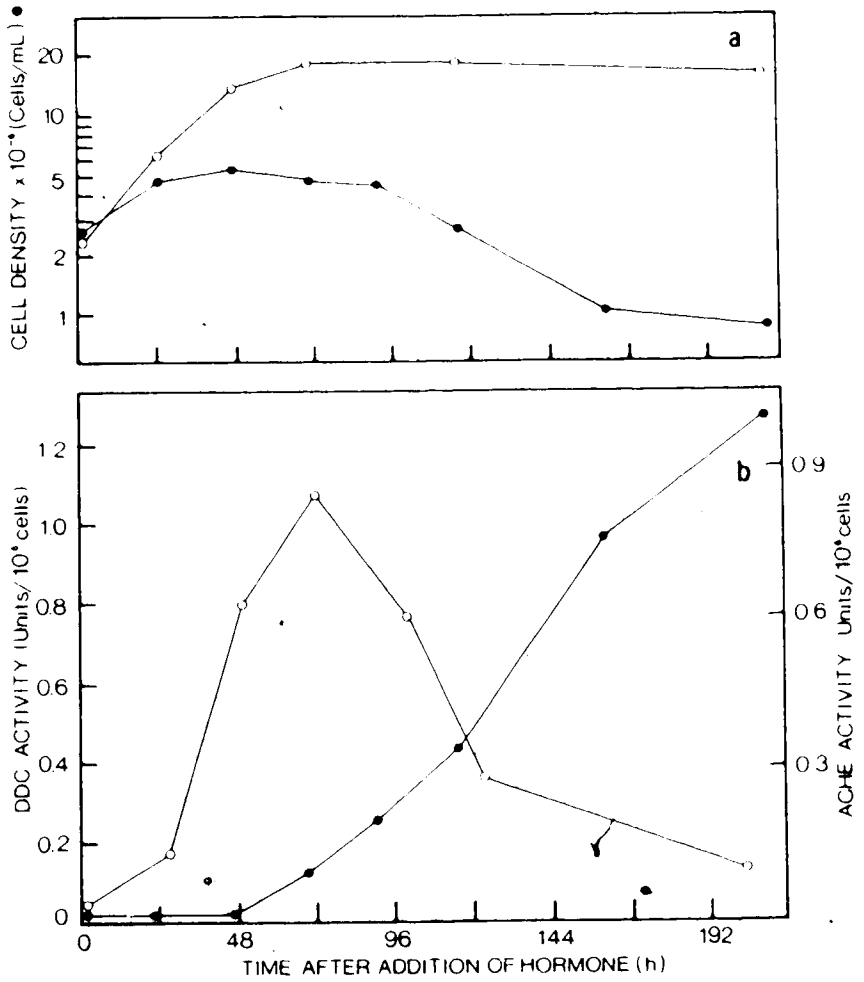


Figure I-2 Response of Line 7E10 to the Continuous Presence of 20-hydroxyecdysone.

Cells were cultured in the presence of 2×10^{-7}M 20-hydroxyecdysone.
a) Growth curves of cells cultured with (●) or without (o) hormone.
b) DDC activity (●) and AChE activity (o).
Acetylcholinesterase data were obtained in a separate experiment in which the growth kinetics were similar to those in a).

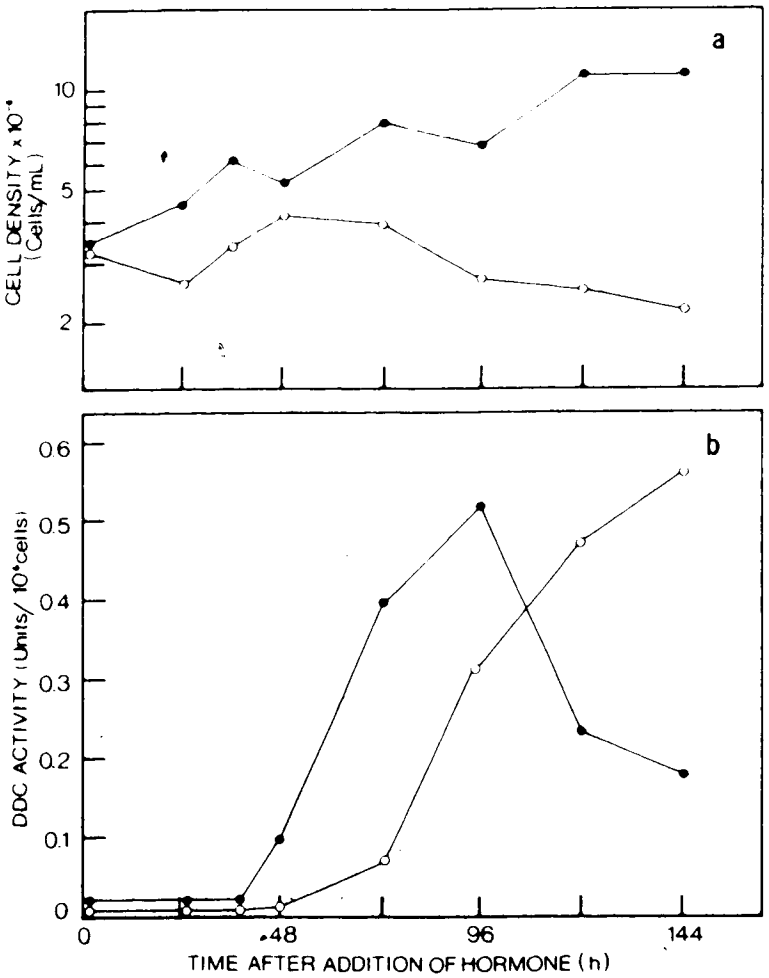


Figure I-3 Response of Line 7E10 to a 6 hr Exposure to 20-hydroxyecdysone.

Cells were cultured continuously in 2 x 10^{-7}M 20-hydroxyecdysone (o) or were removed from the hormone after 6 hr. (●) as described in Materials and Methods.

a) Growth curves.
b) DDC activity.

Immunological Characterization of the Cell Line Enzyme

1. Antigenic Dissimilarity between DDC from Cells and Whole Organisms

Antibody directed against DDC isolated from the epidermis of mature larvae (10) was tested for its ability to recognize the DDC produced by the cloned cell lines. As the data in Table I-2 show, the antibody raised against larval DDC reacted with the enzyme present in crude homogenates of the adult. This result is corroborated by Oucterlony immunodiffusion studies (10) and by thermolability studies (18) which show similarity between larval, embryonic and adult DDC enzymes. In nine separate experiments, DDC antiserum was mixed with fly extracts at enzyme concentrations between 1.3 and 4.1 units/50 µl. In all cases immunoprecipitation occurred and it was possible, with sufficient concentrations of serum, to precipitate virtually all the enzyme. On the other hand, in the same experiments, DDC from two different clones, 7E10 and 6D10, and from the parent line Kcw^{+}, was not recognized by the DDC-reactive serum. In these experiments, cell enzyme concentrations between 0.2 and 1.1 units/50 µl were mixed with DDC-specific antiserum, and in no case did immunoprecipitation occur. Several trivial explanations for this result have been ruled out. First, as seen in Table I-2, no inhibitor of the antigen-antibody recognition event existed in cell extracts, since mixing cell and whole adult homogenates together did not inhibit the ability of the antibody to inactivate the adult DDC present. Secondly, pyridoxal 5'-phosphate is a cofactor of DDC and is probably bound to the enzyme in the intact organism. Thus, the antibody might not recognize DDC from cells if the cells are unable to produce pyridoxal 5'-phosphate to bind with the enzyme. However, adding this compound to cell extracts did not result

TABLE I-2 - Immunoprecipitation of DDC Activity from Cells, Adult Flies and Mixed Extracts

Sample	Initial Activity (units)	Final Activity (units)	Activity Remaining ( )
Cell extract + Tris buffer (1:1)	0.2	0.2	100
Fly extract + Tris buffer (1:1)	1.2	0.1	8
Cell extract + fly extract (1:1)	1.2	0.2	17

Note: Newly-eclosed adults were homogenized at a concentration of 17 mg/ml and 7E10 cells at a concentration of 4.6 × 10⁷ cells/ml. Cells were harvested after 120 hrs in the continuous presence of 20-hydroxy-ecdysone. Extracts were either mixed or diluted with Tris-HCl pH 7.2 to the same volume in each sample. Identical volumes of antisera were added to each sample. Immunoprecipitations were performed as described in Materials and Methods and residual DDC activity was measured in the supernatants.

in recognition of the cell DDC by the antibody. Thirdly, specificity differences between antibody batches was excluded since sera raised in three rabbits failed to recognize the cell enzyme.

2. Antigenic Similarity between DDC from Adult Neural and Epidermal Tissue

Since the cells assume a nerve-like cellular morphology and produce acetylcholinesterase upon hormone treatment, the possibility that they are undifferentiated neural cells exists. We therefore compared the antigenic properties of DDC found in adult brains with that found in the larval epidermis. The results, shown in Table I-3, clearly demonstrated that the neural and epidermal DDC molecules are antigenically similar. Thus, the failure of the antibody to recognize the DDC from cells cannot be explained by assuming the cells are nerve cells and invoking antigenic differences between DDC molecules from brain and epidermis.

Biochemical Characterization of the Cell Line Enzyme - from Crude or Partially-Purified Extracts

1. Substrate Competitions and pH Optima

The possibility that we were measuring the residual dopa decarboxylating activity of some other amino acid decarboxylase was next investigated. This was done by including in the enzyme reaction an excess of the nonradioactaive substrate to be tested as a possible competitor of the $^{14}CO_2$-dopa. Adding a 2- to 10-fold excess of tyrosine, phenylalanine, tryptophan, histidine, or a mixture of amino acids glycine, serine, proline, OH-L-proline, alanine, valine, leucine and isoleucine to the assay for DDC had no effect on the amount of DDC activity measured in extracts of either cells or adults. In contrast,

TABLE I-3 - Immunoprecipitation of DDC Activity from Adult Flies and Dissected Brains

Sample	Initial Activity (units ×100)	Final Activity (units ×100)	Activity Remaining (%)
Dissected brains	13.4	0.5	3.7
Adult Flies	130.6	10.3	7.8

Note: Brain and fly extracts were prepared and immunoprecipitations performed as described in Materials and Methods. The amount of antiserum and volume of liquid in each sample was identical. One unit of DDC activity corresponds to the decarboxylation of 1 nm of L-dopa per hour.

the addition of non-radioactive L-dopa to the reaction mixture effectively competed with $^{14}CO_2$-dopa in the extracts of both flies and cells (Figure I-4). The figure also shows a competition experiment with 5-hydroxytryptophan. Because the decarboxylation of both L-dopa and 5-hydroxytryptophan in neural tissue leads to the production of neurtransmitters, we investigated the possibility that the cell enzyme might exhibit specificity for one of the substrates. The decarboxylating activity in extracts of both cells and adults is somewhat reduced in the presence of increasing amounts of 5-hydroxytryptophan. Taken as a whole, these data serve more to establish a similarity between the two enzymes rather than any pronounced differences. Further studies emphasized this point in some detail.

Both N-acetyl dopamine (10) and α-methyl dopa are competitive inhibitors of the enzyme obtained from mature larvae. The data in Fig. 4 indicate that N-acetyl dopamine inhibited the DDC activity from cells to virtually the same extent as that from adults. Alpha-methyl dopa at 10^{-3}M inhibited the fly enzyme to 52% of control activity and the cell enzyme to 23%. Zinc ions, which are strong inhibitors of the larval enzyme at 10^{-4}M, completely eliminated DDC activity in cell extracts. N-ethyl maleimide, another strong inhibitor of DDC activity from whole organisms (10), reduced DDC activity in cell extracts to 30% when present at a concentration of 10^{-2}M. In addition, the dependence of DDC activity upon the addition of pyridoxal 5'-phosphate to the reaction mixture was common to extracts of both cells and adults. Finally, pH optima tests on both purified DDC from mature larvae and partially purified enzyme from 7E10 revealed similar activity optima. As seen in Figure I-5, both showed maximum activity between pH 7.2 and 7.6. Thus,

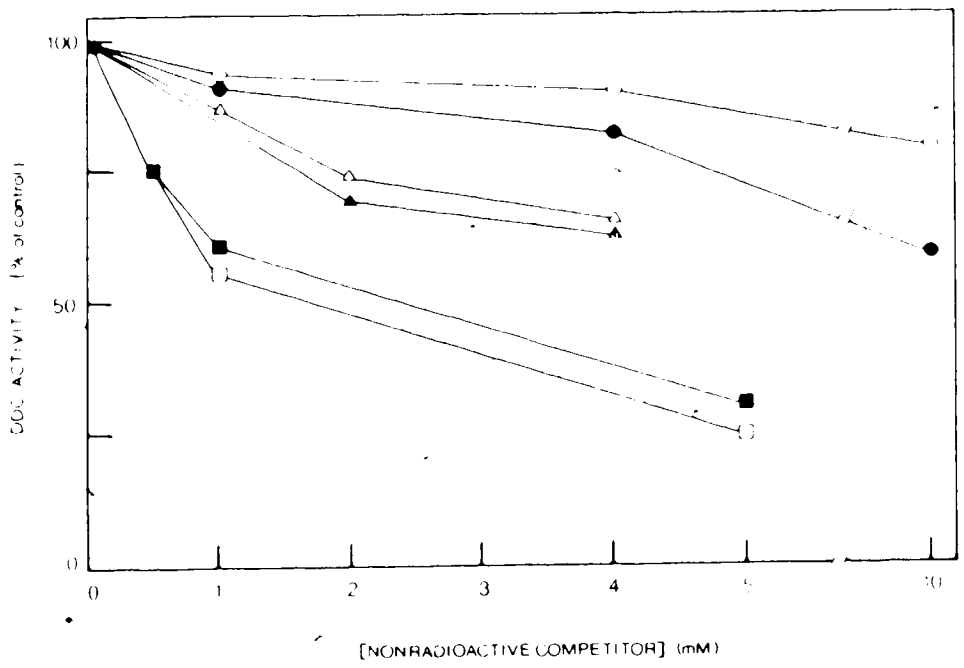


Figure I-4 Effect of the Addition of Nonradioactive 5-hydroxy-tryptophan, L-dopa or N-acetyl Dopamine on DDC Activity Measured Radiometrically.

DDC activity as a percent of the control value was measured in the presence of increasing amounts of:
5-OH tryptophan (●, fly extract; o, 7E10 extract).
L-dopa (■, fly extract; □, 7E10 extract).
N-acetyl dopamine (▲, fly extract; △, 7E10 extract).
The concentration of the radioactive substrate in the reactions was 1 mM.

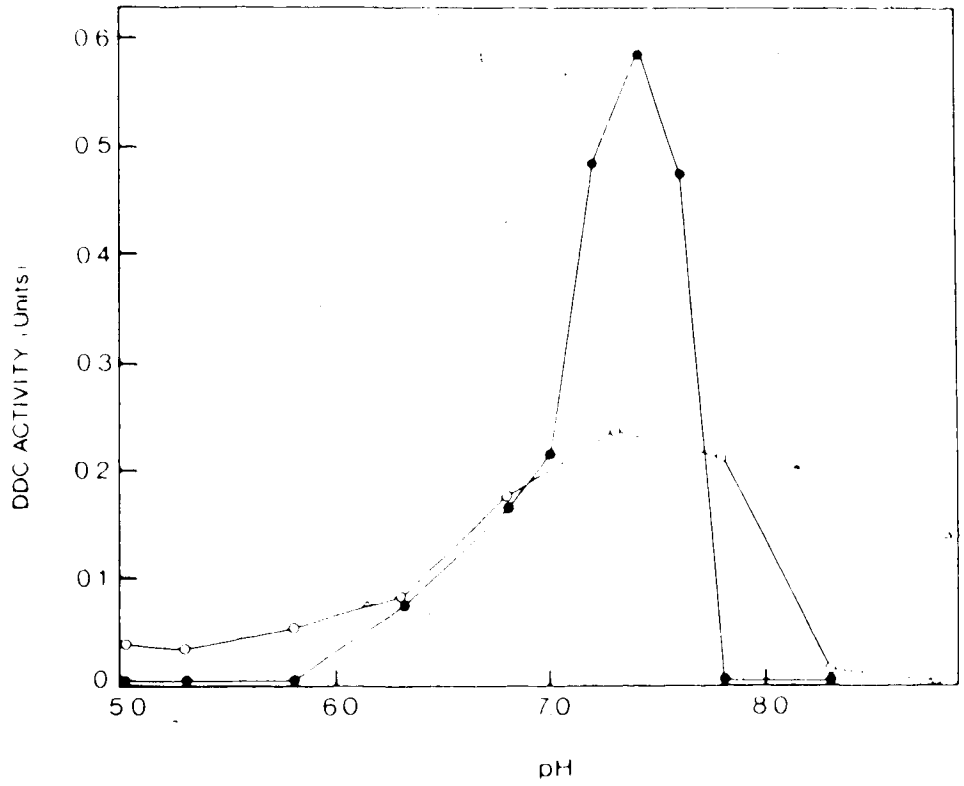


Figure I-5 Optimal pH for DDC Activity from Flies and Cells.

Dopa decarboxylase was purified to near homogeneity from mature larvae by affinity chromatography (33). Cell enzyme from clone 7E10 was partially purified from a crude homogenate by ammonium sulfate precipitation and chromatography on DEAE-cellulose. A 10-fold purification of the enzyme was obtained from these steps. Determinations of pH optima were made in 0.05M Tris-HCl buffers.
● DDC activity from whole organisms (●).
○ DDC activity from 7E10 cells (o).

we conclude that although the DDC found in cells exposed to 20-OH ecdysone is immunologically distinct from the enzyme present in the intact organism, it does resemble the latter in many respects.

2. Column Chromatography of Enzyme from the 7E10 Line on DEAE-Cellulose

In an attempt to elucidate the reason for the immunological differences between DDC molecules from cultured cells and adult organisms, chromatography on a DEAE-cellulose column was carried out. The data in Figure I-6 show that when extracts from the two sources are mixed and chromatographed on DEAE-cellulose, two peaks of activity were resolved. Loading the extracts separately confirmed that peaks I and II corresponded to the adult enzyme and the 7E10 enzyme, respectively.

3. Molecular Weight Determinations

A molecular weight determination of the cell line enzyme was performed by gel filtration on Sephadex G-200, as described in Materials and Methods, after calibrating a G-200 column with standard proteins. The cell line DDC sample had been partially purified through the G-200 stage of purification (see Protein Purifications, below). The apparent molecular weight of cell line DDC by this method was 155,000 and that of larval DDC was 112,000.

The estimated molecular weight of the cell line enzyme by comparison with sedimentation of BSA standard protein in sucrose gradients was 98,760 (average of 4 determinations). Its molecular weight by comparison with catalase was 88,900 (average of 3 determinations). In contrast, the molecular weight of adult fly DDC in these sucrose gradient determinations was 87,232 compared with catalase (average of 2 determinations) and 97,400 compared with BSA (average of 3 determinations). Previous sucrose gradient determinations for larval

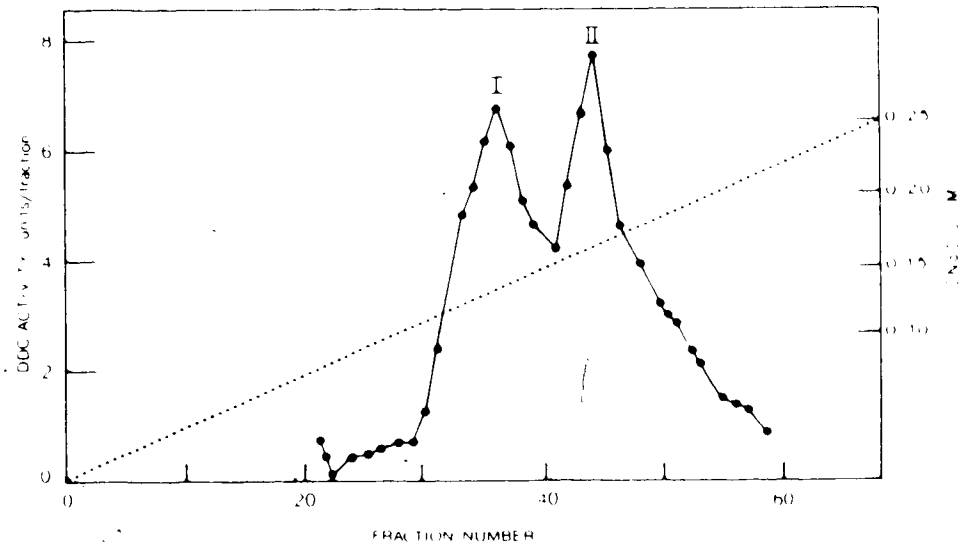


Figure I-6 Chromatographic Separation of DDC Molecules in a Mixture of Homogenates from Adults and 7E10 Cells.

One millilitre of a crude homogenate of young adults (20 mg./ml.) was mixed with 8 ml. of cell extract (1.3×10^8 cells/ml) and the material was loaded onto a DE-32 column. The chromatography was carried out as described in Materials and Methods. Each point represents a single enzyme assay.

DDC (10) gave an average molecular weight of 112,600 +/-6,200. For a summary of molecular weight estimates, see Table I-6.

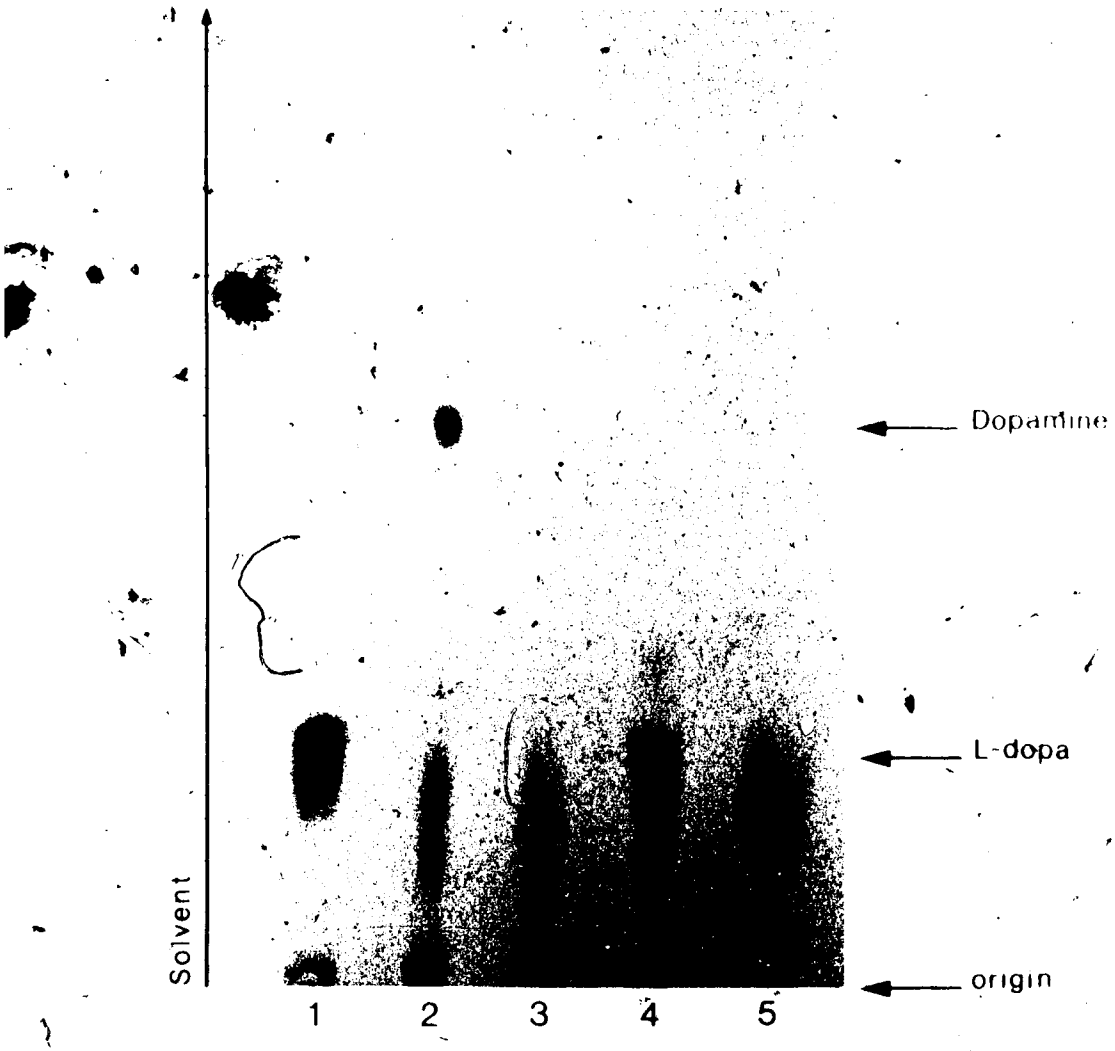
4. Thin Layer Chromatography of Reaction Products

During the course of enzyme purifications, it was noted that cell extracts which were active in the DDC microdiffusion assay were relatively inactive in the ^{3}H-dopa ring-labelled assay. A typical set of assay results is presented in Table I-4. These results suggest that cell line DDC was decarboxylating dopa (assayable as trapped $^{14}CO_2$), but that dopamine was not apparently present as the endproduct. To determine whether an inhibitor of the ^{3}H-dopa assay reaction or extraction step was present in cell line extracts, extract mixing experiments were performed. When 1 volume of cell line partially-purified extract (2200 cpm/50μl in the ^{14}C-dopa assay) was mixed with 1 volume of larval extract (16,500 cpm/50μl in the ^{14}C-dopa assay), there was only an 8% reduction in the expected combined activity when assayed by the ^{3}H-dopa method. This indicates that the presence of cell line extract does not inhibit the ability of Ddc from fly extract to produce extractable dopamine in the ^{3}H-dopa assay.

To determine whether the cell line enzyme was converting dopa to dopamine or to another end-product, thin layer chromatography was applied to reaction mixtures as described in Materials and Methods. These reaction mixtures were incubated for 1 hr, but were not subjected to the chloroform:DEHPA extraction step. Figure I-7 shows that the radioactive dopa spot, which has a migration Rf of 0.19 - 0.22 (22), has decreased in both the third instar larval and cell line extract reactions, - spots 2 and 3 respectively. The dopa spots can be clearly seen in the control, heat-denatured larval blank and a modified smear

TABLE I-4 - Comparison of Cell Line and Fly DDCs in the Two DDC Assays

Sample	CPM in $^{14}CO_2$-dopa Microdiffusion Assay (CO_2 liberated)	CPM in ^{3}H-dopa Ring-labelled Assay (Dopamine trapped)
7E10 cell line extract (ammonium sulfate cut)	2,508/50μl	213/3μl
7E10 purified extract	2,400/50μl	249/3μl
Adult fly extract	16,500/50μl	19,603/3μl


Dopamine
L-dopa
origin
Solvent
1
2
3
4
5

appears in the cell line heat-denatured blank. Dopamine, having an Rf of 0.44 - 0.49 (22), clearly is a product of the larval reaction and clearly is absent in the cell line reaction. The 2 extracts were adjusted to have similar DDC activities by the ^{14}C-dopa assay (ie. 15,500 cpm for cell line extract and 19,000 cpm for larval extract). A longer exposure of the autoradiograph showed no detectable dopamine in the cell line extract reaction.

One hypothesis to explain the apparent absence of reaction product in the cell line extract reaction was that the dopamine endproduct formed an insoluble complex with proteins in the extract. To test this, fly and cell line extracts were mixed and then treated with proteinase K and SDS to release the dopamine. Results are shown in Figure I-8. As in Figure I-7, dopamine appears in the larval extract reaction (spot 4), but not the cell line reaction (spot 5). Mixing extracts resulted in a loss of dopamine from the reaction (spot 2) which could not be released by proteinase K and SDS treatment (spot 3).

One feature evident in both Figure I-7 and I-8 is the non-uniform recovery of radioactive material from each spot. As an equal aliquot of reaction mix was spotted in each case, and each reaction mixture contained an equal amount of ^{3}H-dopa as a substrate, we are unable to account for the disparity between samples.

Northern Analysis

RNA extractions and Northern analyses were made on 7E10 cells before and after 20-hydroxyecdysone treatments (see Materials and Methods). Figure I-9 shows the presence of a transcript species homologous to the BamHI internal Ddc probe (probe 2 of Figure II-1 of

Figure I-8 Thin Layer Chromatography of Extract Mixes and Proteinase K-SDS Treatments.

Reactions were carried out as in Materials and Methods.

Spot 1 - Control = reaction mixture + 6 μl 0.1M Tris-HCl pH 7.5.

Spot 2 - Extract Mix = reaction as in Spot 4, with 6 μl cell line extract added after 1 hr incubation and incubated a further 1 hr.

Spot 3 - Proteinase K/SDS = Same as Spot 2, with 0.5 mg Proteinase K/ 0.5% SDS added and incubated a further 30 minutes.

Spot 4 - Larval DDC = reaction mix + 6 μl larval crude extract (19,000 cpm/50 μl in the $^{14}CO_2$-dopa DDC assay).

Spot 5 - Cell Line DDC = reaction mix + 6 μl of G-200 purified extract (4,000 cpm/50 μl in the $^{14}CO_2$-dopa DDC assay).

Spot 6 - Proteinase K/SDS = Same as Spot 5, with 0.5 mg Proteinase K/ 0.5% SDS added and incubated a further 30 minutes.

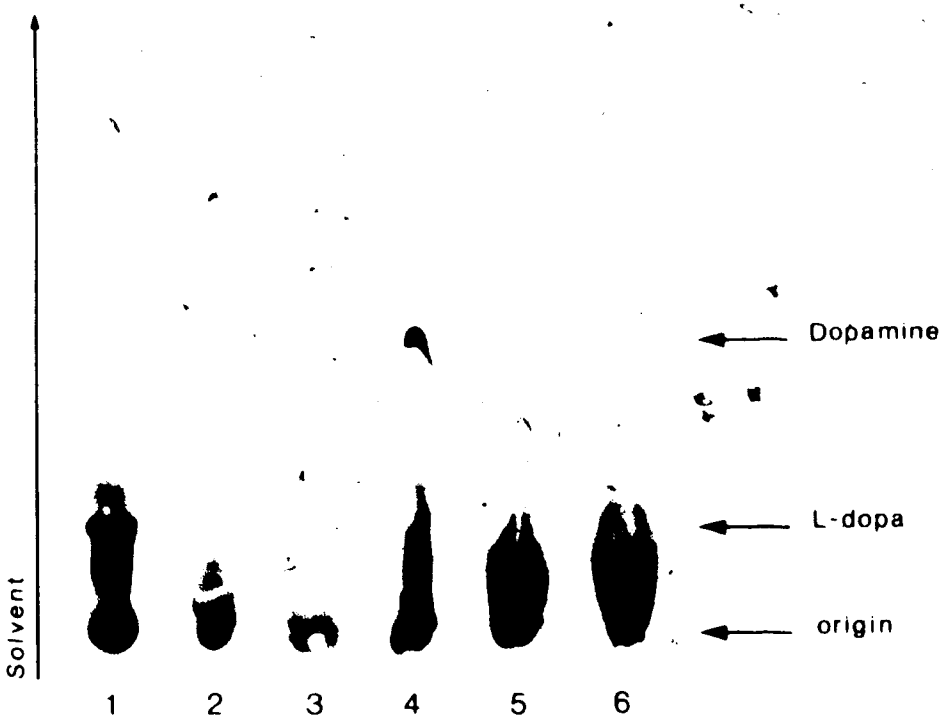

Solvent
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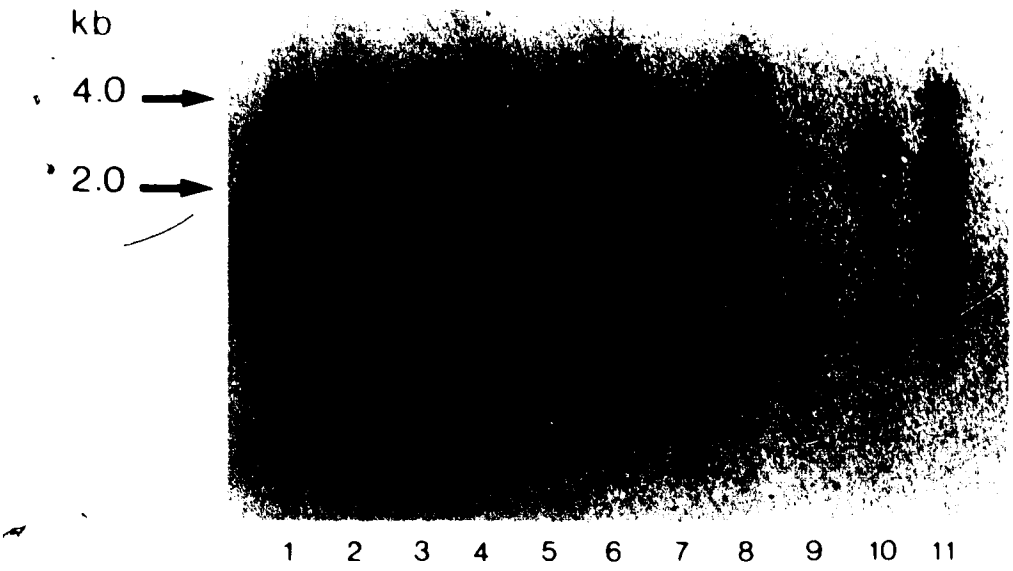
Figure I-9 Northern Analysis of Uninduced and Hormone Induced 7E10 Cells.

RNA extractions and Northern analyses were performed as described in Materials and Methods.

Slot 1 - Third instar larval poly(A^+) RNA - 0.4 μg.
Slot 2 - 16 hr embryo poly(A^+) RNA - 2.4 μg.
Slot 3 - Uninduced 7E10 cell line A^- RNA - 20 μg.
Slot 4 - Uninduced 7E10 cell line poly(A^+) RNA - 18 μg.
Slot 5 - 2 day induced 7E10 cell line A^- RNA - 18 μg.
Slot 6 - 2 day induced 7E10 cell line poly(A^+) RNA - 18 μg.
Slot 7 - 4 day induced 7E10 cell line A^- RNA - 16 μg.
Slot 8 - 4 day induced 7E10 cell line poly(A^+) RNA - 20 μg.
Slot 9 - 5 day induced 7E10 cell line A^- RNA - 16 μg.
Slot 10 - 5 day induced 7E10 cell line poly(A^+) RNA - 17 μg.
Slot 11 - Third instar larval poly(A^+) RNA - 0.4 μg.


kb
4.0
2.0
1 2 3 4 5 6 7 8 9 10 11

this thesis) in both uninduced cells producing no DDC activity and in those incubated for 2, 4 or 5 days in the presence of hormone. The size of the cell line RNA band, as calculated from a standard curve of ribosomal RNAs, is 2.4 kb. This is in contrast to in vivo Ddc mRNA species, which are of 2.0 kb in epidermal tissue and 2.3 kb in neural tissue (30). Precursor species were not apparent in this Northern analysis.

Enzyme Purifications and Characterizations of Purified Enzyme

In order to establish unequivocally the identify of the DDC enzymes from flies and cell lines, it was felt necessary to subject the 2 proteins to peptide mapping or amino acid sequencing. Therefore, enzyme purifications were attempted as described in Materials and Methods. A typical purification profile, through to the G-200 stage, is given in Table I-5.

Following the G-200 chromatography step, several methods were attempted with consistently poor yields or purification ratios. For instance, elution of enzyme from slices of native tube gels resulted in extremely high backgrounds in the DDC assay and loss of all enzyme activity. Carboxymethyl sepharose columns gave high recovery of activity but only a 1.02-fold purification. Hydroxyapatite chromatography resulted in either loss of all activity or inability to elute enzyme from the matrix.

Preparative electrophoresis was the only method that yielded, on one occasion, apparently pure, active cell line enzyme. Other attempts gave protein mixtures with severely-reduced activity. The purification obtained in this preparative electrophoresis run was 11-fold over the G-200 step (Table I-5). Figure I-10 shows a native acrylamide gel of fractions from this preparative electrophoresis run. Based on the extent of purification and the presence of one band on both native and SDS gels (Figure I-10 and I-11), the cell line enzyme was assumed pure and was used for the following tests. It was not possible, with the small amount of enzyme available, to rule out the presence of a co-migrating contaminant in this preparation. Also, replication of the experiments was not possible, as pure enzyme was not obtained later.

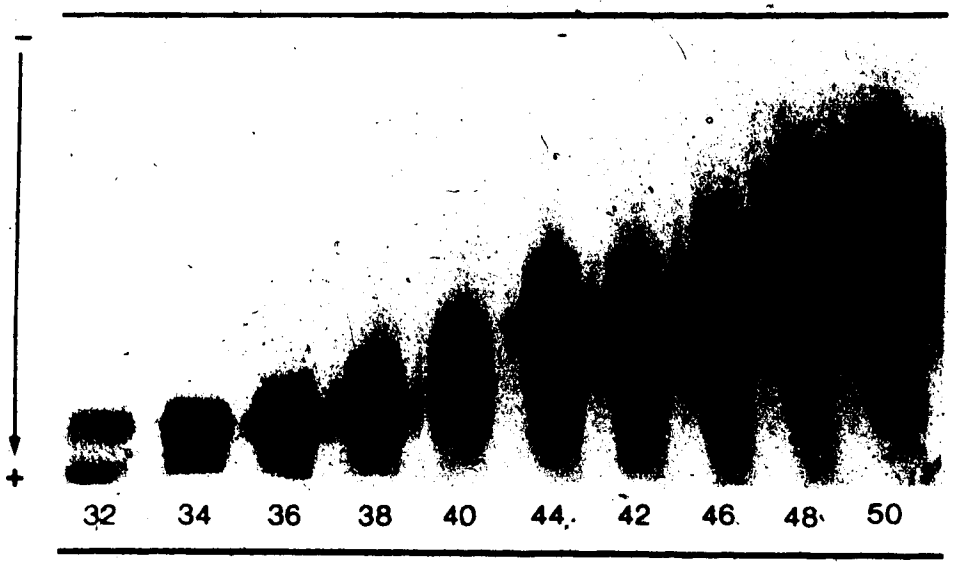
TABLE I-5 - Purification of Cell Line DDC

Purification Step	Total Protein (mg)	Enzyme Activity (units)	Specific Activity (units/mg)	Purification (fold)	Yield (%)
Crude extract	800	416.5	0.5	0	100
Ammonium sulfate precipitation	51.3	172.7	3.4	6.5	41
DEAE column chromatography	6.5	90.5	14.1	26.9	22
G-200 column chromatography	4.1	69.6	17.1	32.9	17
Preparative electrophoresis #2	0.04*	1.87	51.9	371.0	1.6

* 28% of the G-200 column material was loaded onto preparative electrophoresis run #2.

Figure I-10 Analysis of Preparative Electrophoresis Fractions.

A 7.5% native riboflavin acrylamide gel was run as described in Materials and Methods. The gel was stained with the Bio-Rad silver stain. Samples were 65 μl each of the indicated fractions from the preparative electrophoresis run #2. Fractions #36-#50 contained detectable DDC activity above background, the peak occurring in fraction #42. Activities above background for 200 μl samples of these fractions were: Fraction #32 - 0 cpm; #34 - 0 cpm; #36 - 280 cpm; #38 - 305 cpm; #40 - 581 cpm; #42 - 716 cpm; #44 - 634 cpm; #46 - 344 cpm; #48 - 213 cpm; #50 - 100 cpm.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1. Subunit Molecular Weight Estimate

The purified cell line enzyme migrated as a single molecular weight band of 53,000 when compared to standard proteins run on the same gel (see Figure I-11). Purified third instar larval DDC, purified by Bill Clark (10), migrated at 54,000, in close agreement with subunit molecular weight estimate of 53,950 made by Clark et al (10).

2. Native Protein Molecular Weight Estimate by Variable Porosity Native Gels.

Experiments using variable porosity non-denaturing acrylamide gel electrophoresis indicated a molecular weight of approximately 119,000 for the purified cell line enzyme (average of 2 determinations). Previous estimates for purified larval DDC by this method gave a molecular weight of 102,200 +/- 9,000 (10). See Table I-6 for summary of molecular weight estimates.

3. Glycoprotein Tests

One hypothesis to explain the loss of antigenicity of the cell line enzyme was that post-translational modifications such as glycosylation had occurred to bona fide DDC in the cell lines, hence altering or masking antigenic determinants. Therefore, tests for glycoproteins were performed on purified or partially purified cell line enzyme.

a) Anomalous migration on SDS acrylamide gels.

According to Bretscher (6), glycoproteins run more slowly than normal proteins in low percentage SDS gels, as SDS binds carbohydrate poorly. Increasing the acrylamide concentration would be expected to speed the glycoprotein with respect to others, as size becomes more important in the fractionation. However, when the samples shown in Figure I-11 were run on a

Figure I-11 SDS Polyacrylamide Gel of Purified Cell Line DDC and Standard Proteins.

Approximately 0.1 μg (20 μl) each of the active fractions from the preparative electrophoresis run analysed in Figure I-10 was loaded onto a 10% SDS denaturing gel and run as described in Materials and Methods. The gel was stained with the Bio-Rad silver stain. A linear plot of standard proteins allowed a subunit molecular weight estimate of larval DDC at 54,000 daltons and cell line DDC at 53,000 daltons. Slot 1 - Ovalbumin (m.wt. 43,000)

Slot 2 - Bovine serum albumin (m.wt. 68.000)
Slot 3 - Catalase (m.wt. 57.500)
Slot 4 - Larval DDC
Slot 5 - Cell line DDC - Fraction #44
Slot 6 - Cell line DDC - Fraction #42
Slot 7 - Cell line DDC - Fraction #46

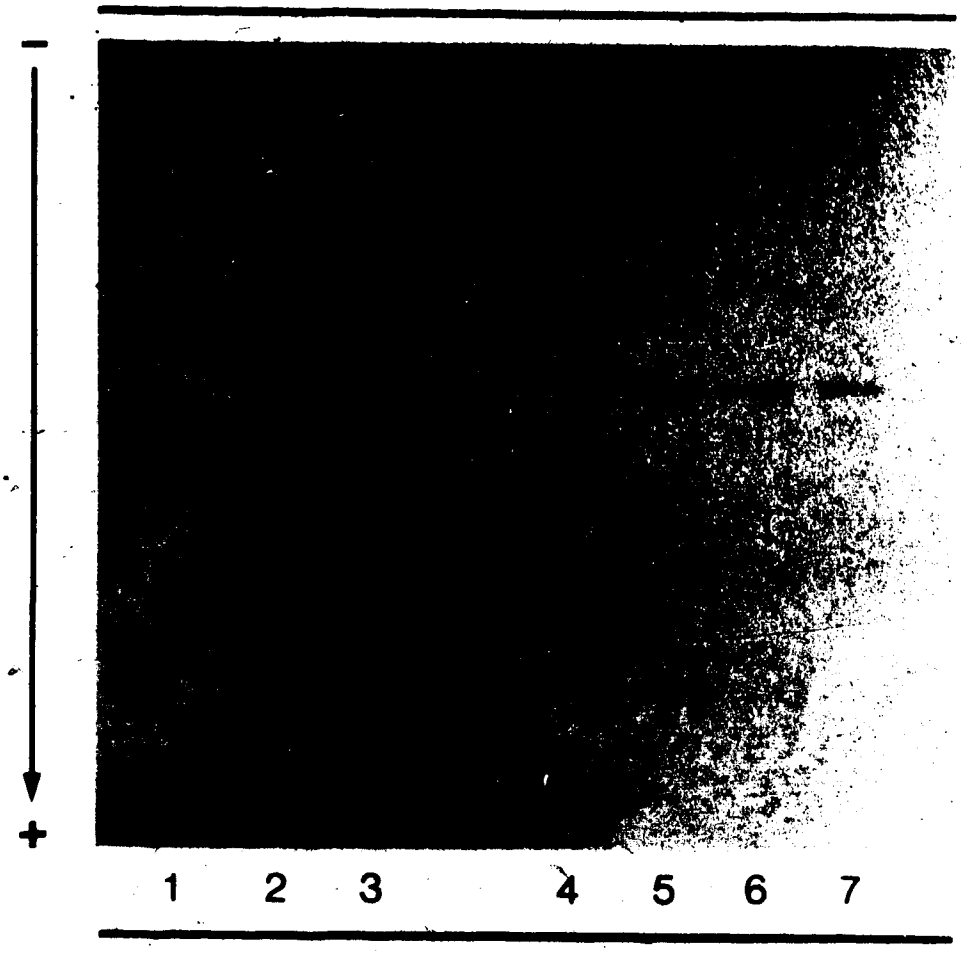
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TABLE I-6 - Summary of Molecular Weight Estimates

Method	Larval DDC	Cell Line DDC	Larval DDC (Previous estimate) (Ref. 10)
G-200 Sephadex	112,000	155,000	-
Sucrose gradients			
- vs. catalase	87,232	88,900	112.600+/-6,200
- vs. BSA	97,470	98,760	
Native variable porosity gels	-	119,000	102,200+/-9,000
SDS gel (subunit)	54,000	53,000	53,950

15% SDS acrylamide gel, the mobility of purified cell line DDC was the same in relationship to standard proteins as it was on the 10% gel. By this test, therefore, the cell line enzyme did not act as a glycoprotein.

b) ConA-Sepharose.

Partially purified cell line enzyme had no affinity for ConA-sepharose when chromatographed as in Materials and Methods, precluding the possibility that sugars containing C-3, C-4 or C-5 hydroxyl groups were present on the enzyme.

c) Phenyl boronate agarose chromatography.

Partially purified cell line DDC also displayed no affinity for phenyl boronate agarose (PBA-30 Matrix, Amicon Corp.) when used according to manufacturer's specifications. PBA has affinity for molecules containing 1,2 cis-diol groups and acts as a lectin. It is used for purification of glycoproteins, nucleic acids and carbohydrates.

Taken together, these tests indicate that glycosylation is unlikely to account for a loss of antigenicity of the cell line enzyme.

4. Peptide Map

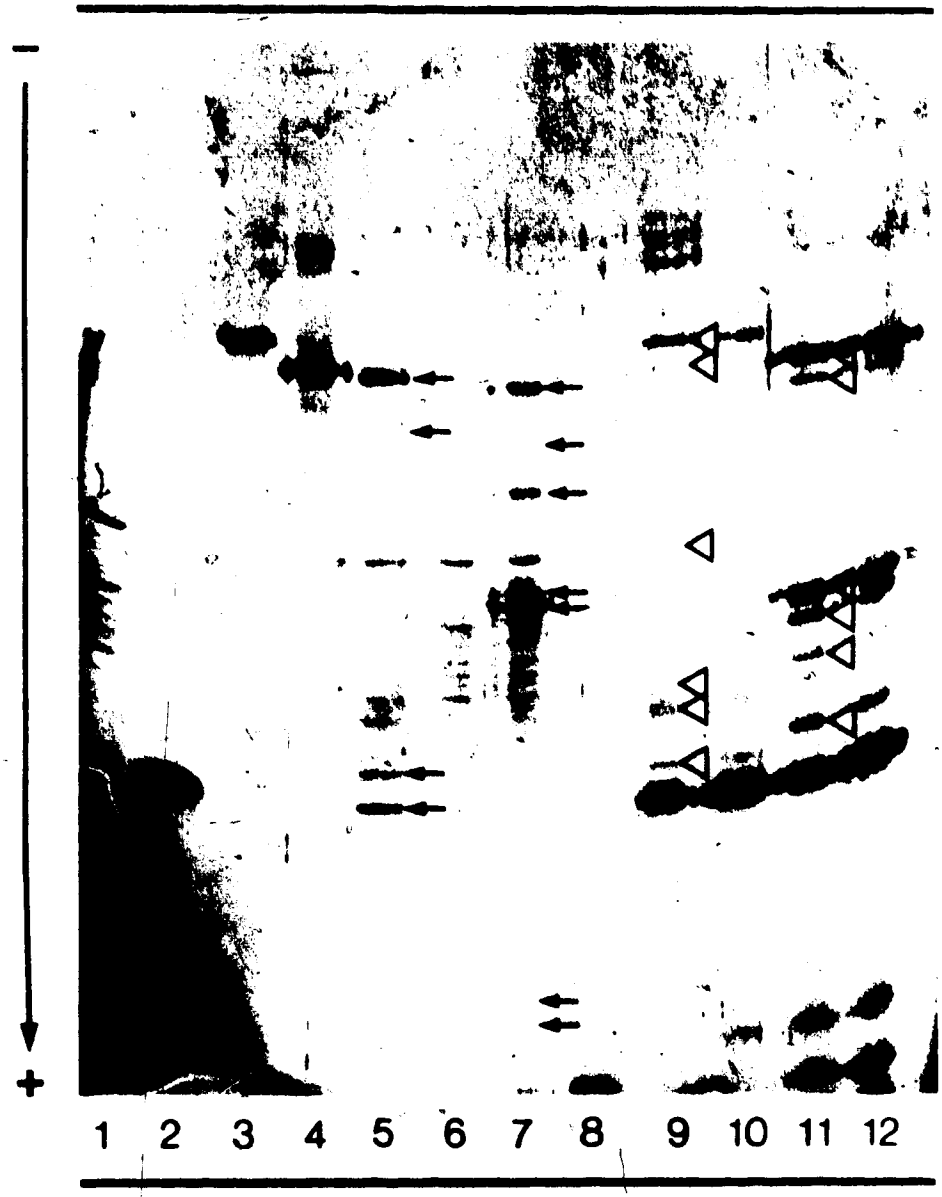
One-dimensional peptide maps using V8 protease and trypsin were made of purified larval and cell line enzymes as described in Materials and Methods. Results are shown in Figure I-12. The pattern of bands seen in control lanes 1 and 2 of Figure I-12 are contributed by V8 protease and trypsin (and their self-digestion products) respectively. The V8 protease peptide pattern is present in lanes 5,6,7 and 8, with the pattern of fly or cell line DDC peptides superimposed upon it. Similarly, trypsin and its peptides are present in lanes 9,10,11 and 12,

Figure I-12 One-dimensional Peptide Map of Purified Larval and Cell Line DDCs.

For peptide map protocol, see Materials and Methods.

Slot 1 - Control = reaction mix + 100ng V8 protease. Incubated 2 hr.
Slot 2 - Control = reaction mix + 100ng trypsin. Incubated 2 hr.
Slot 3 - Control = 20ng larval DDC only.
Slot 4 - Control = 20ng cell line DDC only.
Slot 5 - Larval DDC (20ng) digested with V8 protease (50ng) 2 hrs. Arrows show DDC-specific peptides.*
Slot 6 - Larval DDC (20ng) digested with V8 protease (100ng) 24 hrs.
Slot 7 - Cell line DDC (20ng) digested with V8 protease (50ng) 2 hrs. Arrows show cell line DDC-specific peptides.*
Slot 8 - Cell line DDC (20ng) digested with V8 protease (100ng) 24 hr.
Slot 9 - Larval DDC (20ng) digested with trypsin (50ng) 2 hrs. Triangles point to DDC-specific peptides.*
Slot 10 - Larval DDC (20ng) digested with trypsin (100ng) 24 hrs.
Slot 11 - Cell line DDC (20ng) digested with trypsin (50ng) 2 hr. Triangles point to cell line-specific peptides.*
Slot 12 - Cell line DDC (20ng) digested with trypsin (100ng) 24 hrs.

* All other bands are contributed by V8 or trypsin and their self-digestion peptides - see slots 1 and 2.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1 2 3 4 5 6 7 8 9 10 11 12

with fly and cell line DDC-specific peptides superimposed. The arrows in slot 5 point to DDC-specific peptides resulting from a 2 hr digestion of larval DDC with V8 protease. The arrows in slot 7 indicate cell line DDC peptides resulting from a 2 hr digestion of the cell line enzyme preparation. Likewise, the triangles in slot 9 point to DDC-specific peptides resulting from a 2 hr digestion of larval DDC with trypsin. The triangles in slot 11 show cell line DDC peptides resulting from a 2 hr trypsin digestion of the purified cell line enzyme preparation. It is clear that the digestion pattern of larval DDC is distinctive from that of the cell line enzyme when the purified enzymes are digested with either V8 protease or trypsin. Assuming that the single band obtained in the preparative electrophoresis run represented purified cell line enzyme, these results clearly indicate that purifed DDC from larvae is distinctly different from the molecule purified from tissue cultures.

As mentioned previously, replication of this peptide map was not possible with a different purifed enzyme preparation, as this was the only pure preparation obtained during these studies. The presence of trace amounts of bona fide DDC in this one purified preparation cannot be ruled out. Confirmation of the purity of this preparation and replication of these experiments are necessary before the unequivocal assignment of the cell line enzyme as a different molecular species from larval DDC.

DISCUSSION

The original finding that DDC produced by Drosophila cells differed immunologically from the enzyme found in the organisms was interesting and encouraged us to explore the reasons for this difference. Although not completely conclusive, the data presented in this chapter strongly suggest the non-identity of the dopa decarboxylating enzymes from 7E10 and whole organisms. The following conclusions can be drawn from these data:

1. Similarities between Fly and Cell Line DDCs

Many similarities between DDC enzymes from flies and cell lines were apparent in the early characterization of the cell line enzyme. The enzyme in cells was clearly able to decarboxylate the substrate in the $^{14}CO_2$-dopa microdiffusion assay, although dopamine was not demonstrable as an endproduct in the ^{3}H-dopa ring-labelled assay. Both enzymes required pyridoxal 5'-phosphate as a cofactor and were specific for L-dopa and 5-hydroxytryptophan. Both were sensitive, to similar degrees to the competitive inhibitors N-acetyl dopamine and α-methyl dopa and both were strongly inhibited by zinc ions and N-ethyl maleimide. The pH optima were very similar,

2. Differences

Despite these similarities, the two enzymes appeared distinct in most respects. DEAE-cellulose chromatography separated the two species, indicating a charge difference - the cell line enzyme being more negatively charged and eluting at a higher salt concentration.

Molecular weight estimates varied depending on the method (see Table I-6) but in each case the native molecular weight of the cell line enzyme was higher than that of larval DDC. In contrast, the subunit molecular weight of purified protein was lower than that of larval DDC. The apparently high molecular weight of the cell line enzyme on G-200 could be due to its net charge, as Sepharose is known to absorb basic proteins at low ionic strength (33b).

The antigenicity of the two enzymes was clearly different. These differences were not due to inhibitors in the cell extract, to lack of pyridoxal 5'-phosphate cofactor in cell extracts, or to the cell line enzyme's resemblance to an antigenically-distinct neural form of DDC. It was hypothesized that the immunological differences between the normal and novel forms of DDC could be due to some post-translational modifications which occur in the cells and alter antigenic determinants. Although phosphorylation and acetylation tests were not performed, glycosylation tests on purified and partially-purified cell line enzyme were negative. Also, different conformational states could have been responsible for altered antigencity. Such conformations have been associated with the same primary amino acid sequence in three isozymic forms of glutamate dehydrogenase (41). However, the quite distinct peptide map patterns for the enzymes from 7E10 and whole organisms argue against a conformational change to the same amino acid sequence.

Perhaps the most perplexing of the differences between the two enzymes was the lack of demonstrable dopamine as an endproduct of cell line reactions in the ^{3}H-dopa assay. A possible explanation for this was that cell line extracts contained bona fide DDC, but that another contaminating enzyme was rapidly metabolizing dopamine to a product that

was neither trapped in the chloroform:DEHPA phase nor soluble in the solvent during thin layer chromatography. (The chloroform:DEHPA phase is able to trap amine products such as dopamine, tyramine, histamine and 5-OH tryptamine (44).) However, the results of the two extract mixing experiments make this explanation unlikely. In the first experiment, the dopamine spot, produced by fly DDC, disappears from a TLC when the fly extract reaction is mixed with cell line extract (spot 2, Figure I-8). However, in a similar experiment where the 2 extracts are mixed and simultaneously assayed the release of near-normal levels of ^{3}H-dopamine is observed. This indicates that a contaminating enzyme using dopamine as a substrate was not present in the cell line extract.

An explanation for this disappearance of dopamine in the ^{3}H-dopa assay, when reaction products were run out on TLCs prior to the chloroform:DEHPA extraction step, is that proteins in the cell line extracts were complexing with dopamine to alter its solubility. The TLC extract mixing experiments, as shown in spot 2 of Figure I-8 did show a loss of the dopamine produced by the fly extract. However, treatment with proteinase K and SDS did not release a clearly-resolvable product which might have been complexed with cell extract proteins. It is possible, though, that the chloroform:DEHPA extraction step did liberate dopamine from such a complex, which would explain the near-normal levels of ^{3}H-dopamine that appeared in the second extract mixing experiment. If the chloroform:DEHPA extraction was able to liberate dopamine from a dopamine-protein complex, then dopamine should have been observed in the ^{3}H-dopa assay of cell line extracts, if any were present. From these data, therefore, it is not clear what endproduct is created after the decarboxylation of the dopa substrate or

what role the cell line enzyme plays in this metabolism.

The peptide maps, together with the immunological, charge and molecular weight differences, plus the absence of dopamine as a reaction endproduct in the ^{3}H-dopa assay, suggest the production, in 7E10 cells, of a different decarboxylating enzyme which is present in minor quantities in whole organisms.

It has recently been suggested that an enzyme other than DDC, with enzymatic activity like DDC, occurs in vivo (4,44) and may account for the low level (1-2%) of antigenically non-precipitable activity that Bishop and Wright (4) detect in whole Drosophila. This enzyme, dopa quinone imine decarboxylase (DQID), has not been characterized in Drosophila, but has been partially purified from Manduca sexta (1a). The Manduca enzyme converts dopa quinone imine to 5,6-dihydroxyindole but not dopamine quinone imine to 5,6-dihydroxyindole nor dopa to dopamine. The hypothesized role of this enzyme is shown in Figure 1 of the Introduction to this thesis. If the cell line enzyme is DQID, it is necessary to hypothesize that sufficient ^{14}C- or ^{3}H-dopa quinone (and its spontaneous cyclized product dopa quinone imine, the substrate of DQID) be present in reaction mixtures. This presence could be achieved in two ways. Firstly, sufficient dopa could spontaneously oxidize to the quinone form in the aqueous dopa stock solution of the DDC assay. And secondly, the presence of diphenol oxidase in cell extracts could also oxidize dopa to dopa quinone. Although it is possible that some diphenol oxidase could be present in cell line extracts and could co-purify with the cell line enzyme, phenylthiourea, an inhibitor of diphenol oxidase, was included in crude extracts and DDC reaction

mixes to inhibit diphenol oxidase activity. The decarboxylation observed with cell line extracts in the ^{14}C-dopa assay would then be due to the decarboxylation of ^{14}C-dopa quinone imine. Also, the product of the ^{3}H-dopa assay would be ^{3}H-5,6-dihydroxyindole, which may be insoluble in both the chloroform:DEHPA phase of the ^{3}H-dopa assay as well as in the butanol:acetic:water solvent of the TLC experiments. The disappearance of dopamine in the TLC extract mixing experiment (spot 2, Figure I-8) would require a separate explanation. As mentioned previously, it is possible that proteins in the cell line extract were complexing with the dopamine produced in the fly extract reaction. These complexes were not resolved by proteinase K/SDS treatment, but may have been extractable in the chloroform:DEHPA phase.

In order to test whether the cell line enzyme is acting as the enzyme DQID, the following tests are suggested. The production of spontaneously-generated, enzyme-generated or silver oxide-generated dopa quinone imine from dopa can be followed colorimetrically at 470nm (1a). Similarly, 5,6-dihydroxyindole can be followed at 540 nm, and by HPLC analysis. If the cell line extracts contain DQID and are added to mixtures containing the above generated dopa quinone imine, it should be possible to detect a decrease in absorbance at 470 nm and a concurrent increase at 540 nm. The production of 5,6-hydroxyindole could be verified by HPLC. Also, if cell line extracts contain DQID, dopa quinone imine should be able to compete with ^{14}C-dopa quinone imine in DDC assays with cell line extracts.

3. Inducibility of Ddc mRNA in 7E10 Cells

In whole organisms, each peak of DDC enzyme activity during development is accompanied by a corresponding increase in steady-state levels of Ddc mRNA (9,18,24). As the Northern analysis in Figure I-9 shows, the 7E10 cell line, in contrast, contains high levels of Ddc mRNA both before and after hormone treatment (Figure I-9) and at times when no DDC enzyme activity is apparent. Therefore, the cells are not responding in a regulatory fashion similar to that in Drosophila epidermal cells. It is possible that the Ddc gene in the cell lines is transcribed constitutively, similar to the constitutive expression of Ddc which occurs in neural tissue (34). This would be consistent with the presumptive neural origin of these cells, which is supported by enzymative and morphological evidence (1,3,12,13). Furthermore, the 2.4 kb transcript species in 7E10 cells (Figure I-9) is larger than either the 2.0 kb epidermal or 2.3 kb neural mature transcripts from whole flies (30). It is possible that the 2.4 kb transcript is a variant of the 2.3 kb neural transcript which is transcribed constitutively but remains untranslated in the cell lines.

Swiderski and O'Connor have recently completed a study of a sister line of Kc cells, the 7E10(4)EC line (40). This cell line originated from the same clone as the 7E10 line used in this study, but was maintained separately from 7E10 for approximately 10 years prior to their study. Their results on DDC enzyme activity, antigenicity and mRNA induction in hormone-treated cultures, are very different from those presented here.

They find a 26-fold increase in DDC activity and a 6-fold increase in Ddc mRNA in the presence of hormone. No Ddc mRNA is detectable until 72 hrs after addition of 20-hydroxyecdysone. Also, in contrast to our results, their immunoprecipitation experiments, using the same batch of epidermal DDC antiserum used in our experiments, showed a 70-75% precipitation of both fly and cell line DDC activities. In addition, in vitro translation products (55 kd and 20 kd) from polysomal poly(A^+) RNA from 7E10(4)EC cells were recognized by our epidermal DDC antiserum. The authors were also able to confirm, by HPLC analysis, the decarboxylation of [ring-^{14}C]dopa by 7E10(4)EC cells to ^{14}C-labelled dopamine.

It appears, therefore, that bona fide DDC is produced by the sister 7E10(4)EC cell line and that this line is also able to regulate Ddc expression at least partly at the level of transcription or transcript stability.

A further investigation of the reasons for the discrepancies between two isolates of the same clone is now beyond the scope of our research, and other approaches are being taken to the study of Ddc regulation in Drosophila.

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CHAPTER 2

Analysis of the Transcription Unit Adjacent to the 3'-End of the Dopa Decarboxylase Gene in *Drosophila melanogaster*

A version of this chapter has been published:

Spencer,C.A., R.D.Gietz and R.B.Hodgetts, 1986. Analysis of the Transcription Unit Adjacent to the 3'-End of the Dopa Decarboxylase Gene in *Drosophila melanogaster*. Developmental Biology 114: 260-264.

INTRODUCTION

The molecular mechanisms which lead to the expression of the dopa decarboxylase (Ddc) gene in the epidermis of Drosophila melanogaster are of interest since they are stage-specific. By exogenous feeding of the molting hormone, 20-hydroxyecdysone, to mature larvae, we have determined that Ddc transcript accumulation is a rapid consequence of the administration of the hormone (10). Considerable mRNA accumulation occurs even in the absence of protein synthesis (3), suggesting that the hormone has a direct effect on transcription of the Ddc gene. By contrast, at other stages in the life cycle, notably during embryogenesis (1,7) and in the imaginal discs following eversion (3), Ddc transcript accumulation may be triggered by a falling ecdysteroid titer.

A second feature of interest is the unusually high density of genes surrounding Ddc on chromosome 2L (9). Eighteen genes, as defined by mutation and complementation analysis (15) have been mapped within this 8 - 12 band region. Of these 18 lethal complementation groups, 8, including Ddc, are contained within 25 kb of DNA, of which 70% is transcribed (15). Although many of the genes in the Ddc cluster have been ordered relative to each other, their precise locations await cloning and sequencing of cDNAs.

It was recently drawn to our attention (Jay Hirsh, personal communication), that a transcription unit in addition to Ddc resided on the 4.6 kb genomic clone that was used in our previous study of embryonic Ddc transcripts (7). In this paper, we show that the hybridization response formerly seen in Northern analyses of early embryonic RNA was due

primarily to a transcript encoded by a gene adjacent to the 3'-Ddc terminus. Since this transcript is similar in size to Ddc mRNA, the earlier work (7) led to an overestimate of the amount of Ddc transcript actually present in early embryos. This present data therefore reopens the question of whether biologically significant amounts of Ddc mRNA are transmitted maternally as originally suggested by Gietz and Hodgetts (7). Also, these data indicate that the 3'-adjacent transcript is coded by a gene within the Ddc cluster which was previously undefined by complementation analysis and therefore represents an additional member of this dense cluster.

MATERIALS AND METHODS

Northern Blotting

RNA extractions and oligo-dT chromatography were performed as described in Gietz and Hodgetts (7). RNA samples were denatured by heating at 65°C for 5 min in the electrophoresis buffer (20 mM EPPS, pH 8.0, 10 mM sodium acetate, 0.1 mM EDTA) containing 50% deionized formamide and 6% formaldehyde. Following the addition of tracking dyes, samples were subjected to electrophoresis on a 1.35% agarose gel containing 3.7% formaldehyde. The above buffer containing 3.7% formaldehyde was used as the running buffer and was recirculated during the run.

A mixture of ribosomal RNA species from Drosophila and rabbit was used to generate a standard curve from which transcript sizes were calculated. The markers were visualized with UV light after staining for 1 hr in 200 mM ammonium acetate containing ethidium bromide (0.5μg/ml) and destaining for 1 hr in 200 mM ammonium acetate. Conditions for transfer and hybridization to nick-translated double-stranded probes were as described in Gietz and Hodgetts (7), using a probe concentration of 2-7 × 10^6 dpm/ml. Radioactivity was removed from blots as described by Thomas (14).

Hybridizations to single-stranded RNA probes (2×10^6 dpm/ml) were carried out for 12-20 hrs at 65°C in a solution of 50% formamide, 40 mM sodium phosphate, pH 6.5, 4× SSC, 1× Denhardt's, 1 mM EDTA, 0.1 mg/ml salmon sperm DNA, 0.5 mg/ml yeast tRNA, and 0.01 mg/ml polyadenylic acid. The blots were washed 3× at 70°C in 50 mM NaCl, 20 mM sodium phosphate, pH 6.5, 1 mM EDTA. Radioactivity was stripped from these blots as above except the temperature was raised to 95°C.

Southern Blotting

Techniques were as described in Estelle and Hodgetts (4) except the acid-depurination step prior to transferring to nitrocellulose was omitted. The hybridization conditions for the single-strand RNA probes were as described for Northern analysis, except that the hybridization temperature was 55°C and the washing temperature 65°C.

Preparation of Probes

Figure II-1 illustrates the probes constructed for this study. Double-stranded DNA probes (probes 1-3) were derived from the λ3 genomic clone isolated by Jay Hirsh and described in Gilbert and Hirsh (8). The cloned DNA was separated from the vector DNA by appropriate enzyme restriction and electrophoresis on low-gelling temperature agarose. Fragments were purifed by phenol extraction and chromatography using a NACS PREPAC (Gibco/BRL, Inc.) and nick-translated.

Single-strand RNA probes (probes 4-9,11,12) were prepared as run-off transcripts from either the T7 or SP6 promoter of the linearized vector pDDC14. This vector was constructed by inserting the 1.5 kb BamHI-PstI fragment (Figure II-1) into the pGEM-2 vector (Promega Biotech). Probe 10 was derived from a template in which the BamHI-HpaI DNA (and the SmaI site in the pGEM-2 polylinker region) had been removed from pDDC14.

As Figure II-1 shows, transcripts initiated at the SP6 promoter are not complementary to Ddc mRNA, while those initiated at the T7 promoter will hybridize to Ddc mRNA. In vitro transcriptions were carried out following Melton et al (12) in the presence of 25 μM UTP and 75-100 μCi α-[^{32}P]UTP (3000 Ci/mmole). The SP6 RNA polymerase (Promega Biotec)

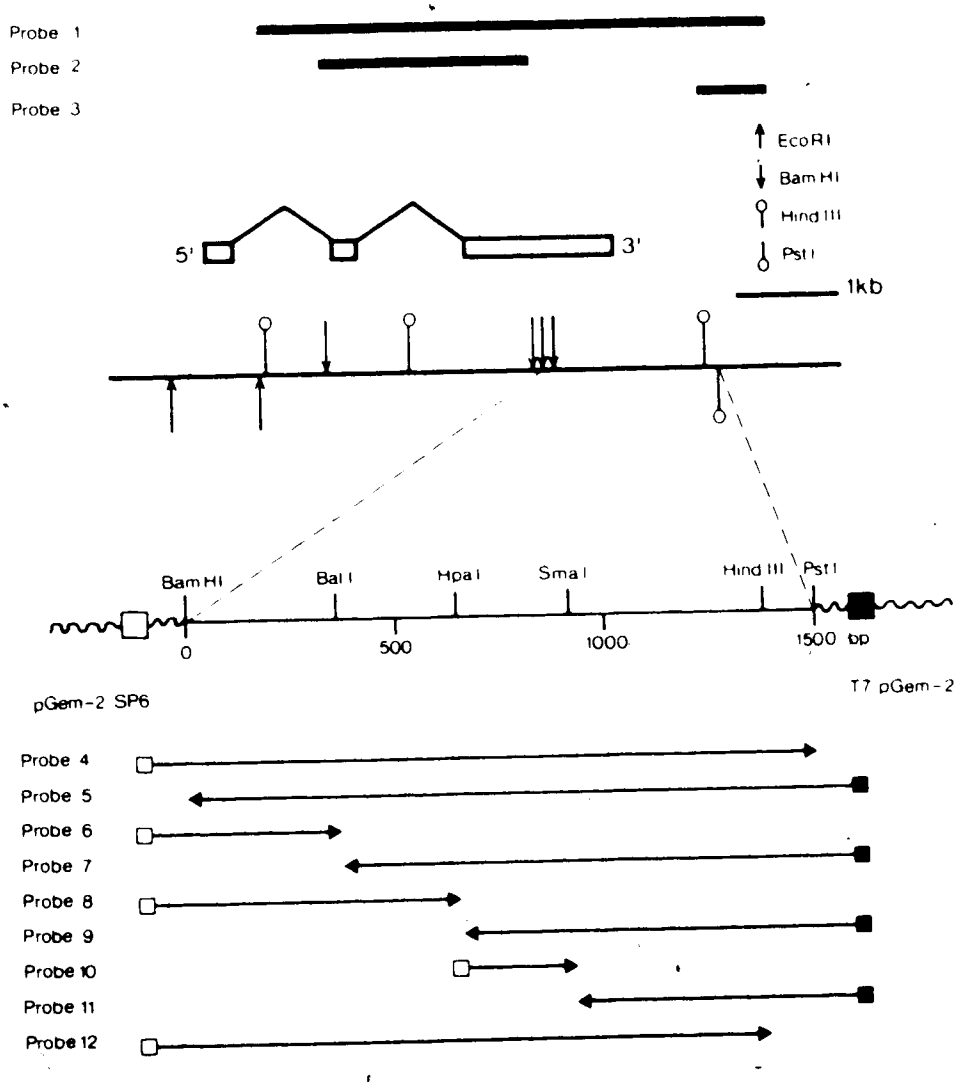


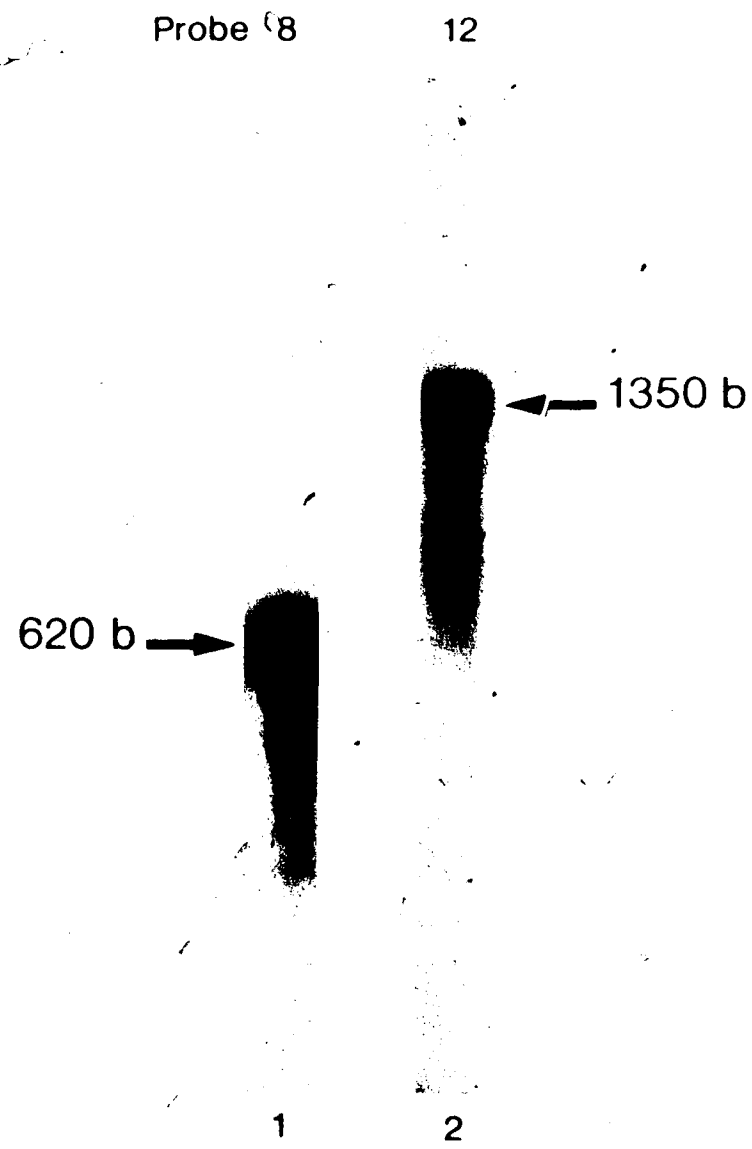
Figure II-1 Restriction Map of the Ddc Region and Probes Used in this Study.

The positioning of the Ddc gene on the restriction map and the direction of transcription were taken from Beall and Hirsh (1). Probes 1-3 were double stranded DNA fragments that were labelled by nick-translation. Probes 4-12 were single stranded RNA molecules labelled during in vitro transcription as described in Materials and Methods. Exons are indicated by open boxes in the figure.

and T7 RNA polymerase (U.S. Biochemical Corp.) were used at a concentration of 10 units/μg template DNA. Restricted template DNA was always visualized following electrophoresis on agarose to insure it had been completely cut at the restriction site chosen to terminate the probe. As a second check on the in vitro transcription product, all labelled probes were subjected to electrophoresis on a denaturing agarose gel, transferred to nitrocellulose, and visualized by autoradiography. Probes were used only if a majority of the molecules were full length and there was no evidence of transcripts extending beyond the restriction site chosen to linearize the template. A minority of prematurely terminated transcripts was always present but this had no influence on the interpretation of our hybridization results. Figure II-2 displays several typical probes and demonstrates the essential features of such probes; namely, a majority of molecules are full length and there is no evidence of transcripts extending beyond the restriction site chosen to linearize the template.

Figure II-2 Size Distribution of RNA Probes Produced by In Vitro Transcription.

About 5×10^4 dpm each of probes 8 and 12 (Figure II-1) were run on denaturing agarose gels as described for Northern analysis in Materials and Methods and blotted to nitrocellulose. The sizes indicated on the autoradiograph in the figure were calculated from a standard curve derived from the migration of Drosophila cytoplasmic and mitochondrial rRNA species included on the same gel. The expected sizes of full length transcripts based on the restriction map in Figure II-1 are 680 bases (probe 8) and 1380 bases (probe 12).


Probe 8
12
1350 b
620 b
1
2

RESULTS

Identification of the 3'-Adjacent Transcript in Early Embryonic RNA

The same poly(A^+) and polysomal RNA samples that were isolated from staged embryos by Gietz and Hodgetts (7) were subjected to electrophoresis on a denaturing agarose gel and transferred to nitrocellulose. The blots were then hybridized to double or single-stranded probes and the autoradiograms are displayed in Figure II-3. Figures II-3a and c contrast the hybridization responses of two identical blots to (a) a double-stranded DNA probe lying entirely within the Ddc gene (probe 2, Figure II-1), and (c) a double-stranded DNA probe lying just beyond the 3'-terminus of Ddc (probe 3, Figure II-1). Both probes lie within the 4.6 kb. genomic fragment (probe 1, Figure II-1) used in Gietz and Hodgetts (7).

Quite clearly, probes 2 and 3 give different results. In Figure II-3a, mature Ddc mRNA (2.0 kb) and two precursor species (3.0 kb and 4.0 kb) known to contain sequences from the 5'-proximal intron (7) are detected in 1 hr, 2 hr and 8 hr embryonic poly(A^+) RNA and in the mature larval sample. In the 14 hr embryonic sample, only the mature Ddc transcript was identified since this poly(A^+) RNA was extracted from polysomes. While the mature Ddc mRNA species is found in the early embryonic samples, it is far less abundant than the transcript of similar size which hybridizes to probe 3 in Figure II-3c. We have not been able to identify any precursors of this 3'-adjacent transcript in the early embryonic samples. Figure II-3c also shows that the 3'-adjacent transcript is present at very low levels in the late embryonic polysomal RNA and mature larval RNA, in sharp contrast to mature Ddc transcript.

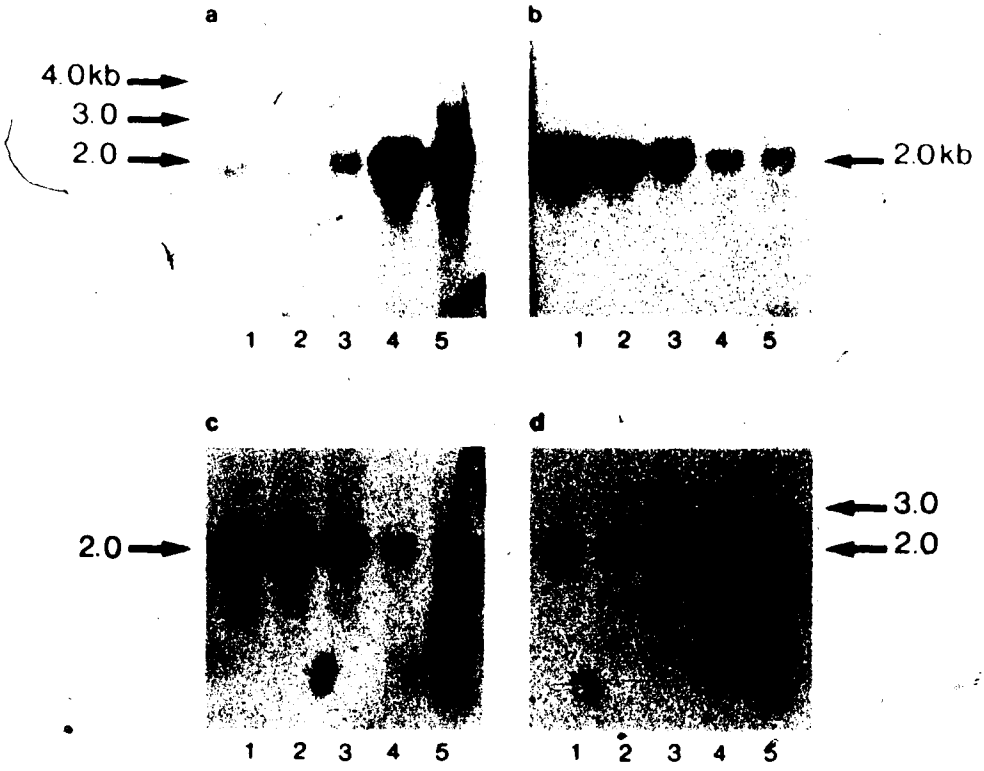
Figure II-3 Northern Analysis of Embryonic and Larval RNA.

Duplicate samples of poly(A^+) RNA (4 μg) were subjected to agarose gel electrophoresis in (a) and (c). Following transfer to nitrocellulose, the blot shown in (a) was hybridized to probe 2 while that shown in (c) was hybridized to probe 3 at a probe concentration of 2-7 × 10^6 dpm/ml of hybridization solution. After the autoradiograms shown in the figure had been obtained, radioactivity was removed from each blot; (a) was rehybridized to probe 4 and (c) was rehybridized to probe 5, producing (b) and (d), respectively. Both RNA probes were used at a concentration of 2 × 10^6 dpm/ml of hybridization solution. The RNA samples were the same ones analyzed by Gietz and Hodgetts (7).

Lane 1 = 1 hr embryonic poly(A^+) cellular RNA.
Lane 2 = 2 hr embryonic poly(A^+) cellular RNA.
Lane 3 = 8 hr embryonic poly(A^+) cellular RNA.
Lane 4 = 14 hr embryonic poly(A^+) polysomal RNA.
Lane 5 = mature larval poly(A^+) cellular RNA.

Autoradiograph a) = hybridized to nick-translated DNA probe 2, internal to the Ddc gene and specific for Ddc mRNA.
Autoradiograph b) = autoradiograph a), rehybridized to single-stranded RNA probe 4, specific for the 3'-adjacent transcript.
Autoradiograph c) = hybridized to nick-translated DNA probe 3, specific for the 3'-adjacent transcript.
Autoradiograph d) = autoradiograph c), rehybridized to single-stranded RNA probe 5, specific for Ddc mRNA.

Probe diagrams are shown in Figure II-1.


a
b
4.0kb
3.0
2.0
2.0kb
1 2 3 4 5
1 2 3 4 5
c
d
2.0
3.0
2.0
1 2 3 4 5
1 2 3 4 5

An unequivocal demonstration that two different transcripts are revealed by the hybridization results in Figures II-3a and c is provided in Figures II-3b and d. Radioactive probes 2 and 3 were removed from the blots shown in Figures II-3a and c as described in Materials and Methods and the blots were rehybridized to the single-stranded RNA probes 4 and 5 (Figure II-1). In Figure II-3b the blot of Figure II-3a was hybridized to probe 4; this RNA probe is transcribed from the same strand as Ddc and is therefore not complementary to Ddc transcripts. The results are virtually identical to those shown in Figures II-3c using the double-stranded 3'-flanking probe 3. Conversely, in Figure II-3d the blot of Figure II-3c was hybridized to probe 5; this RNA probe is complementary to Ddc transcripts and produces a hybridization pattern similar to that of Figure II-3a. A longer exposure of similar blots revealed the 4.0 kb precursor which is not visible in Figure II-3d. The results of these hybridizations identify a transcription unit, oriented in the opposite direction to the Ddc gene and lying within the PstI - HindIII interval common to probes 3 and 4.

Localization of the 3'-Termini of the Ddc and the 3'-Adjacent Transcription Units.

The obvious question raised by the results presented above concerns the relative positions of the two 3'-termini. Given their proximity and opposite orientations, we explored the possibility that these two transcription units actually overlapped. The analysis was carried out using the strand specific RNA probes shown in Figure II-1. RNA probes produced from the T7 promoter will hybridize to Ddc mRNA; those produced from the SP6 promoter will hybridize to the 3'-adjacent transcript.

Hybridizations to both mature larval and young adult poly(A^+) RNA samples were carried out by Northern analysis and a typical result is shown in Figure II-4. In Figures II-4a and c, larval and adult RNA samples were first hybridized to nick-translated probe 1 (Figure II-1) which contains regions homologous to both Ddc and the 3'-adjacent transcript. The results observed are therefore the composite hybridization patterns of both the Ddc and the 3'-adjacent probes. Radioactivity was removed from the blot in Figure II-4a and the RNA was rehybridized to the single stranded Ddc-specific, probe 5. As Figure II-4b shows, Ddc transcripts were present in species 1-3 and 5 in the larval samples and in species 1 and 4 in the adult RNA. Although species 5 was not visualized in the adult RNA using either probe 1 or 5, a repeat of this experiment using longer exposures revealed its presence in both cases. In summary, the transcript pattern revealed by the double stranded probe 1 is identical to that revealed by the single stranded RNA probe 5.

The results obtained by reprobing the blot shown in Figure II-4c with probe 4 are very different. As Figure II-4d shows, the 3'-adjacent transcript is virtually undetectable in mature larvae (as shown by Figure II-3) but is present in adults. Longer exposures of this and other Northern blots used for mapping the 3' termini show trace amounts of the 3'-adjacent transcript in larval RNA.

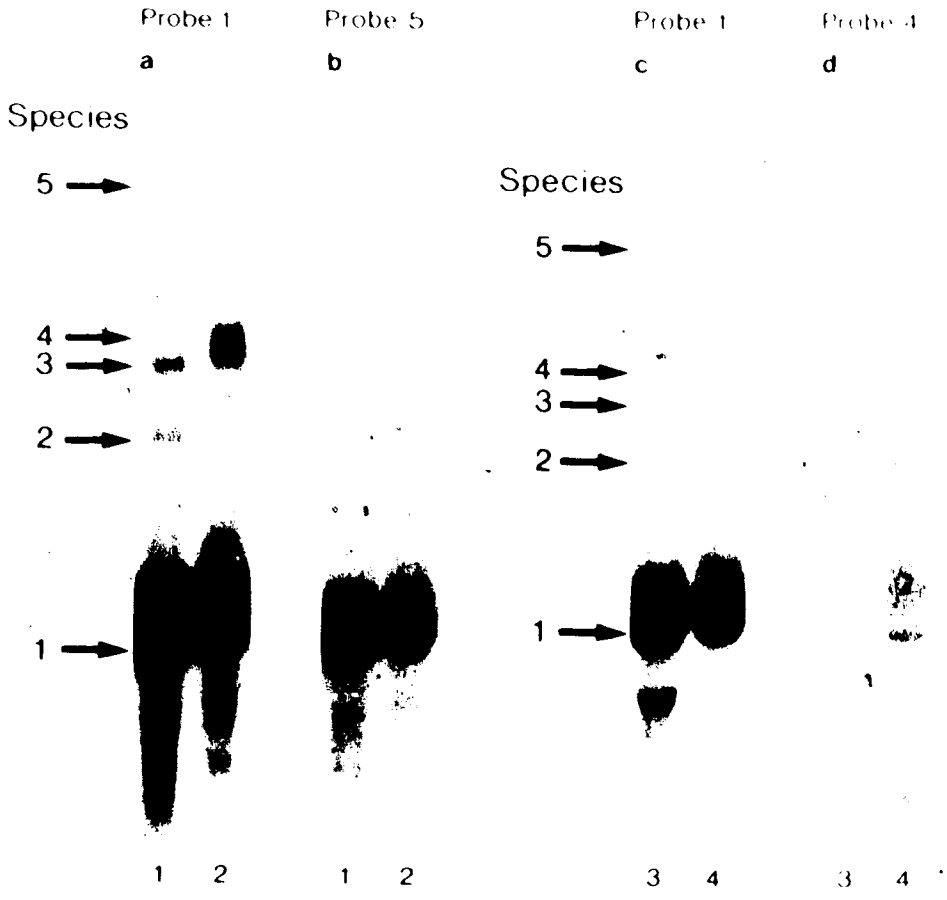
The entire set of RNA probes from Figure II-1 was used on Northern blots similar to those shown in Figure II-4, and the results are shown in Figure II-5. In these experiments, when a negative hybridization response was obtained, the blots were rehybridized to a known positive probe in order to ensure that RNA had not been stripped from the blots.

Figure II-4 Developmental Specificity of the Hybridization Responses Used to Map the 3'-Termini.

Poly(A^+) RNA was obtained from mature larvae and newly-eclosed adults and 20 μg samples were subjected in duplicate to Northern analysis in (a) and (c). These autoradiograms show the hybridization responses to probe 1, a nick-translated DNA probe homologous to both the Ddc and 3'-adjacent transcripts, at a probe concentration of 2-7 × 10^6 dpm/ml. Radioactivity was then removed from both blots; blot (a) was rehybridized to probe 5, a single-stranded RNA probe specific for Ddc transcripts. Blot (c) was rehybridized to probe 4, a single-stranded RNA probe specific for the 3'-adjacent transcript. Both probes were used at a concentration of 2 × 10^6 dpm/ml of hybridization solution, and hybridization and washes carried out under stringent conditions.

Lanes 1,3 = larval RNA.
Lanes 2,4 = adult RNA.

Species 2 and 5 in autoradiograph b) are more evident after longer exposures (data not shown).


Probe 1
Probe 5
Probe 1
Probe 4
a
b
c
d
Species
5
4
3
2
1
Species
5
4
3
2
1
1 2
1 2
3 4
3 4

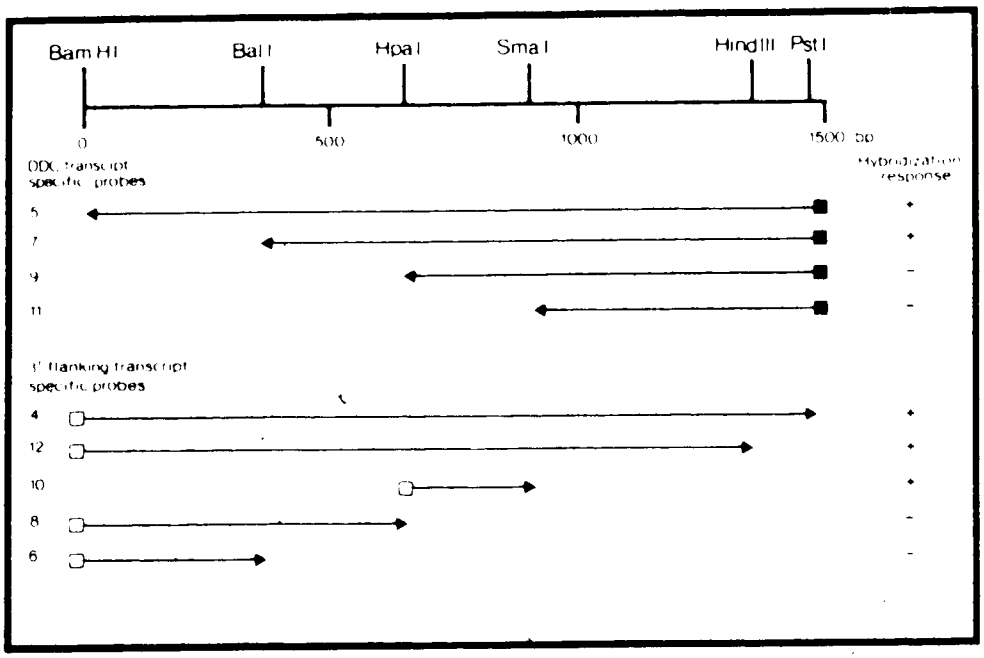


Figure II-5 Mapping the 3'-Termini of the Ddc and Adjacent Transcription Units.

Mapping data were based on hybridization responses of the above RNA probes to Northern blots similar to the example shown in Figure II-4. Poly(A^+) RNA (15-20μg each) from both larvae and adults was present on each Northern. Hybridization conditions were as described in Materials and Methods. Probe concentrations were 2 x 10^6 dpm/ml in all experiments. A positive hybridization response to the Ddc-specific probes (5,7) indicates that transcripts were visualized in both mature larval and newly-eclosed adult poly(A^+) RNA samples. A positive response to the RNA probes specific for the 3'-adjacent transcript (4, 10, 12) indicates that a transcript was visualized in the adult but not the larval poly(A^+) RNA.

Examples of the types of hybridization responses to both Ddc-specific and 3'-adjacent transcript-specific probes are shown in Figure II-4. The data show that the 3'-terminus of the Ddc transcript is located in the BalI-HpaI interval, whereas the 3'-end of the adjacent transcript is located in the HpaI-SmaI interval. Thus, at the level of resolution provided by the hybridization response, the mature transcripts from these adjacent transcription units do not overlap.

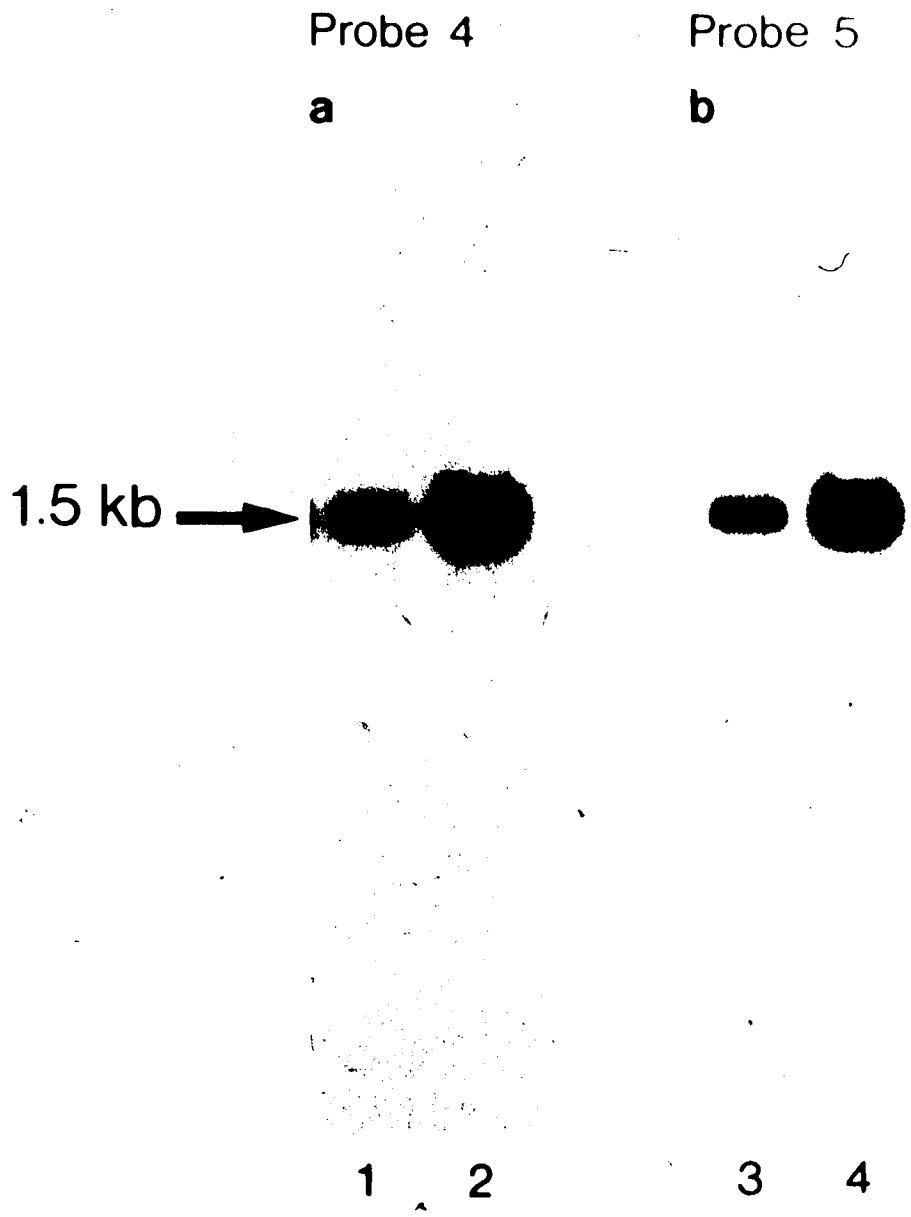
The 3'-Adjacent Transcript is Transcribed from Genomic Sequences Adjacent to Ddc

The data presented to this point do not exclude the possibility that the 3'-adjacent RNA species is actually transcribed from genomic sequences that bear some homology to those lying 3' to the Ddc gene, but are actually located elsewhere. However, the genomic Southern analysis, presented in Figure II-6 rules this out. Drosophila DNA was digested with BamHI and PstI, and following electrophoresis and transfer to nitrocellulose, hybridized to either probe 4 or 5. A single band of 1.5 kb was visualized with both probes. As both probes hybridized to fragments the same size as the expected BamHI-PstI fragment shown in Figure II-1, this confirms that the 3'-adjacent transcript originates from a DNA located in this region, immediately downstream of the Ddc gene.

Figure II-6 Southern Analysis of Genomic Sequences Homologous to the BamHI-PstI Fragment.

Samples of Drosophila DNA obtained by Estelle and Hodgetts (4) were restricted with BamHI and PstI and subjected to Southern analysis using (a) probe 4, or (b) probe 5. The band shown migrated the same distance as the 1.5 kb. BamHI - PstI band present in pDDC14.

Lanes 1,3 = 1 μg DNA.
Lanes 2,4 = 5 μg DNA.


Probe 4
Probe 5
a
b
1.5 kb
1
2
3
4

DISCUSSION

The region of the genome surrounding the Ddc gene is characterized by a very high density of genes (9,15). The 18 complementation groups so far identified in the 160 kb Ddc region are functionally related, being involved in catecholamine metabolism, the formation, sclerotization and pigmentation of cuticle and in male and female fertility (15). Also, recent discoveries of sequence homologies between transcribed regions within the cluster indicate that the genes may be evolutionarily related (5).

Deletion mapping shows that the 2 lethal complementation groups closest to the 3' end of Ddc are l(2)amd and l(2)37Bd (15). A cDNA of the amd gene has recently been cloned, sequenced and mapped by Marsh et al. (11). They find that the amd transcript is coded from the opposite strand to that of the Ddc transcript and terminates approximately 2.5 kb from the 3'-terminus of the Ddc gene, precluding the identity of the 3'-adjacent transcript with the amd gene product. Also, the developmental expression of the 3'-adjacent transcript is inconsistent with that of the amd transcript and with the lethal phases of amd mutants (13). The 2.0 kb amd transcript is barely detectable in early embryos, reaches a maximum at 12-16 hrs of embryogenesis and is expressed at very low levels in adults (11).

The possibility that the 3'-adjacent transcript is coded by the Bd gene is also unlikely, as l(2)37Bd mutants act as larval lethals (15) and we detect very little 3'-adjacent transcript in third instar larvae. It appears, therefore, that the 3'-adjacent transcription unit

represents a gene located between Ddc and amd, in which no mutant lesions have so far been identified.

Placing the gene for the 2.0 kb 3'-adjacent transcription unit between Ddc and amd requires that the 5' transcription start site of the 3'-transcript lies within 500 bp of the amd poly(A) cleavage site. Thus, the 3 mature transcripts in this region (Ddc, 3'-adjacent gene and amd) account for a total of 8 kb of coding sequence within 8.5 kb of genomic DNA.

One consequence of such abnormally high transcript densities near a particular gene is the possible inclusion of a second transcription unit on genomic fragments used in molecular studies. The presence of both Ddc and the 3'-adjacent transcription units on the 4.6 kb probe (probe 1 of Figure II-1) used in previous studies of early embryonic RNA (7) led to an overestimate in the level of Ddc transcript present in early embryos. Using probes which are specific to Ddc, we show here that Ddc transcript levels are actually very low in early embryos. In a previous study, Beall and Hirsh (1) were unable to detect Ddc transcripts at this stage. At present we are unable to account for this discrepancy, as Beall and Hirsh used different RNA extraction methods (urea-phenol-chloroform), Northern analysis techniques (methyl mercury gels) and hybridization conditions. The functional significance of low levels of Ddc transcript in early embryos is unclear, although we detect low levels of DDC activity as early as 3 hr's after egg deposition.

The hybridization results shown in Figure II-5 indicate that the 3' end of the mature 3'-adjacent transcript lies within the HpaI-SmaI interval. However, an examination of the DNA sequence in this region

(D.Eveleth and L.Marsh, personal communication) reveals that no sequence resembling the canonical polyadenylation sequence (2) is present in this region. The most likely polyadenylation sequence, 5'-AATAAA-3', actually lies in the BalI-HpaI interval, contiguous to the HpaI restriction site itself. This would place the polyadenylation site of the 3'-adjacent gene 10 - 30 bases into this interval. While this is inconsistent with our hybridization data, our failure to detect a response to probe 8 could be explained by insuffient homology between the probe and the 3'-transcript at the hybridization stringency used. Since we have shown that the 3'-terminus of the Ddc gene lies in this same BalI-HpaI interval (Figure II-5), it is possible that the mature Ddc transcript overlaps that from the adjacent gene slightly. However, proof of this will require direct sequencing of the respective transcripts or the corresponding cDNA molecules.

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CHAPTER 3

Overlapping Transcription Units in the Dopa Decarboxylase Region of *Drosophila*

A version of this chapter has been published:

Spencer,C.A., R.D.Gietz and R.B.Hodgetts, 1986. Overlapping transcription units in the dopa decarboxylase region of *Drosophila*. Nature 322: 279-281.

INTRODUCTION

The many examples of overlap in the genes of various viruses and bacteria illustrate that the parsimonious utilization of the coding capacity of DNA is relatively common amongst procaryotes (for review, see reference 12). The recent discoveries of a pupal cuticle gene within an intron of the completely unrelated Gart locus in Drosophila (8) and of 2 overlapping mRNAs in mouse cell lines (17) shows that overlapping transcription units also exist in higher organisms. However, the prevalence of such phenomena is unknown. We report here a situation of overlap between the 3' termini of a pair of convergent transcription units in another region of the Drosophila genome. This 88 base-pair (bp) genomic region encodes the 3' terminus of the messenger RNA for the enzyme dopa decarboxylase (Ddc) and, in opposite orientation, the 3' terminus of the adjacent gene whose function is unknown. An analysis of the temporal and spatial distribution of the two transcripts within the organism shows that high levels of both transcripts are never concordant. However, within the testes, where the 3' transcript is maximally expressed, low levels of Ddc transcript were detected. This result raises the possibility that a hybrid molecule involving the two transcripts forms in vivo or that transcription interference occurs, with concomitant regulatory implications.

Previous work has shown that the dopa decarboxylase region of Drosophila, as defined by the deficiency DF(2L)TW130 (19), contains a cluster of 18 genes, 14 of which appear to function in cuticle development and catecholamine metabolism (18). Four genes, including Ddc, have been mapped within a 12 kilobase (kb) region and primary

transcripts from these genes account for ≈10.5 kb of the genomic DNA (5). This tight clustering of genes has complicated our previous analysis of Ddc transcripts (7), because the DNA probes we used in that study were found to encompass both Ddc and its 3'-adjacent transcription unit (reference 15 and Chapter 2 of this thesis). Strand-specific RNA probes revealed that the 3'-adjacent transcript originated on the opposite strand from that of the Ddc transcript and a hybridization of Northern blots to a series of nested single-stranded probes showed that the 3' ends of the Ddc and the adjacent gene transcripts apparently lie on opposite sides of the HpaI site shown in Figure III-1. However, subsequent analysis of the DNA sequence in this region showed that putative polyadenylation signals for the two genes are present distal to the HpaI site in each case (see Figure III-1). This result suggested the possibility of a slight amount of overlap between the two transcripts in the vicinity of the HpaI site. We have obtained complementary DNA clones for both Ddc and the 3'-adjacent gene, and a comparison of their DNA sequences with the genomic DNA sequence confirms that an 88 bp overlap between the two 3' termini of the mature mRNAs exists.

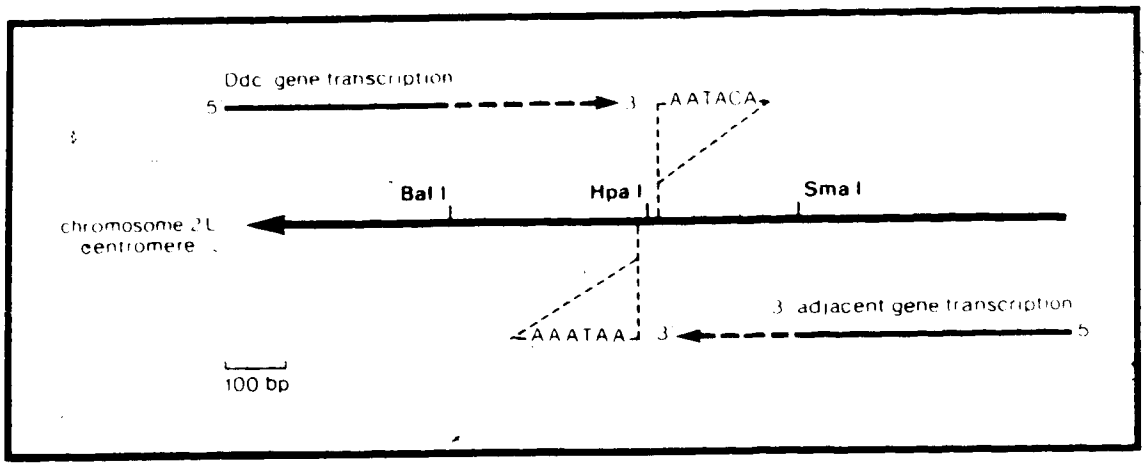


FIGURE III-1 Mapping the 3' Termini of Ddc and the Adjacent Gene.

The figure summarizes the results of our earlier work in which strand-specific RNA probes were used in a Northern analysis of poly(A^+) RNA from the mature larval and young adult stages of Drosophila (reference 15 and Chapter 2 of this thesis). Single-stranded Ddc-specific probes indicated that mature transcripts of this gene terminated in the BalI-HpaI interval, while probes specific for the flanking gene indicated that its transcripts terminated in the HpaI-SmaI interval. Possible polyadenylation signals, chosen on the basis of their similarity to the eucaryotic consensus sequence ^{5}'AATAAA^3' (see reference 2), were obtained from genomic DNA sequence analysis. Their precise locations with respect to the HpaI site are shown in Figure III-2.

MATERIALS AND METHODS

Isolation of cDNAs

Poly(A^+) RNA was prepared from whole 0-2 hr (newly-eclosed) adult Drosophila (Canton-S) as described below, and a cDNA library in λgt10 was prepared following methods in Eveleth et al (6).

Ddc-specific clones were identified using a nick-translated HpaI genomic fragment which extends 3.6 kb upstream from the HpaI site shown in Figure III-1. Although this fragment includes the 3'-terminal 26 bp of the 3'-adjacent gene, the extent of homology between the probe and the 3' gene was insufficient to generate a stable hybrid under the stringent hybridization conditions used. Clones specific for the 3'-adjacent transcription unit were identified using the strand-specific probe 10 (Figure II-1 of this thesis) which is homologous to sequences in the HpaI-SmaI interval shown in Figure III-1. Two cDNA clones each of Ddc and the 3'-adjacent gene were selected and shown to contain inserts of between 1.1 and 2.0 kb.

DNA Sequencing

Inserts of the above clones were re-cloned into M13mp19 and were sequenced on single stranded templates following the methods of Sanger (13) or on double stranded templates following by Chen and Seeburg (3).

Drosophila Dissections and Quantitation of Total RNAs

Newly-eclosed adult male and female Drosophila (48 of each) were dissected into head, thoracic and abdominal sections in D-20 tissue culture medium (4). Sections were immediately frozen in liquid nitrogen

and stored at -70°C prior to RNA extractions. Testes and ovaries (48 of each) were also dissected in D-20, stored briefly on ice, then frozen as above. Total RNA was extracted as described in Materials and Methods of Chapter 1 of this thesis.

A fraction of each RNA sample, equivalent to 12 flies, was denatured by dissolving the sample in 50% formamide/6% formaldehyde, heating for 1 hr at 50°C, then chilling on ice. Samples were dotted onto GeneScreenPlus (Dupont) using a Tyler Research Corporation Dot Blot Apparatus. Dot blots were hybridized to either single-stranded probe 5 or 12 (Figure II-1 and Materials and Methods of Chapter 2).

Extraction of Poly(A^+) RNAs and Northern Analysis of Adult Drosophila

Newly-eclosed (0-2 hr old) male and female Drosophila were collected by emptying culture bottles every 2 hrs, selecting newly-eclosed, unpigmented organisms and freezing samples at -70°C prior to RNA extractions. Aged samples were obtained of newly-eclosed females or males, aged for 4 days at 25°C on standard medium (11), then frozen at -70°C. RNA extractions, oligo-dT chromatography, Northern blotting and hybridization to either probe 4 or probe 5 were carried out as in Materials and Methods of Chapter 2.

RESULTS

Sequencing of cDNA clones ACP, ACQ and ACR (Figure III-2) showed that they represented intact 3' termini, as revealed by tracts of oligo(dT) (or oligo(dA), depending on their orientation within the cloning vector) not found in the genomic sequence. The polyadenylation site of the 3'-adjacent gene was assigned unambiguously from two cDNA clones of different length (ACQ and ACR); it is indicated by an asterisk beneath the genomic sequence in Figure III-2. A canonical polyadenylation signal sequence, $^{5'}$AATAAA$^{3'}$, lies 16 bases upstream of the terminal C residue (boxed in Figure III-2). The polyadenylation site for the Ddc gene (indicated by an asterisk above the genomic sequence in Figure III-2) could not be assigned unambiguously from the cDNA sequence because of the uncertainty caused by a run of adenine residues in the genomic sequence. However, we believe the site chosen at the T residue in Figure III-2 is the most likely one, based on its similarity to the eucaryotic polyadenylation addition sequence $^{5'}$..Py↓A..$^{3'}$ (reference 2); a hexameric sequence ($^{5'}$ATTACA$^{3'}$) which could control poly(A$^+$) addition at this residue lies 16 bases upstream of it. Although this sequence appears to be an inefficient signal for transcription end formation (16), the other possible hexamers, $^{5'}$AACAA$^{3'}$ and $^{5'}$AATTAA$^{3'}$ lie 38 and 43 bases upstream of the oligo d(A) tract, farther than any reported in the most recent review (2).

The extent of overlap that we have found between the 3' termini of the poly(A$^+$) mRNAs may reflect an even greater overlap between the unprocessed, non-adenylated transcripts, assuming that transcription termination occurs in Drosophila 1 kb or farther downstream of the

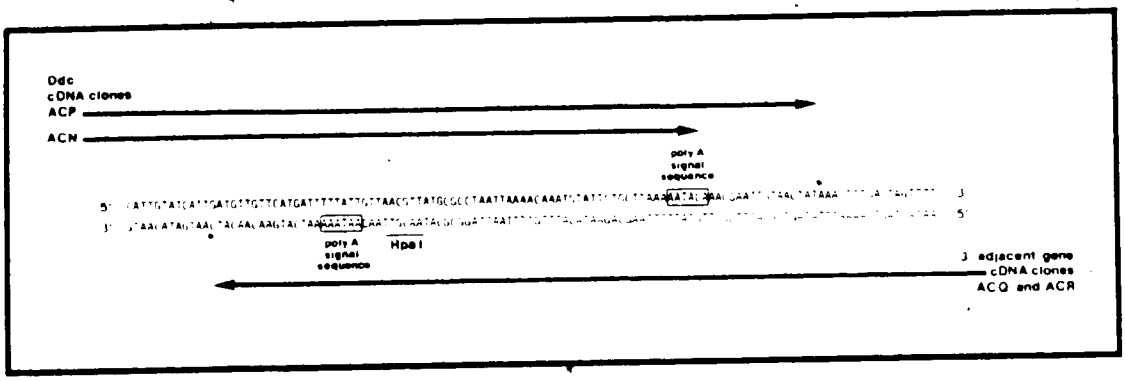


FIGURE III-2 Genomic DNA Sequence in the Region of Overlap Between the Ddc Transcription Unit and the 3'-Adjacent Gene.

The sequence of both strands of the genomic DNA was determined by the dideoxynucleotide method of Sanger (13) and constitutes part of a 5 kb sequence including the entire Ddc gene (6). Poly(A) signal sequences are boxed. Sequences of the cDNA molecules near their 3' termini were obtained from single- or double-stranded (3) templates of the cDNA constructs cloned into M13mp19. The cDNA libraries were prepared from poly(A^+) adult RNA as described in Materials and Methods.

poly(A^+) signal sequence, as is known to occur in other systems (2). The overlap also raises the intriguing possibility that a sense/anti-sense hybrid might exist in vivo, with regulatory implications for the expression of one or both mRNAs. We therefore undertook analyses of the temporal and spatial distribution of Ddc transcripts and of the 3'-adjacent transcripts during the life cycle of Drosophila. Our earlier work indicates that the developmental expression of the two genes is quite different. Maximum levels of dopa decarboxylase occur in 18 hr embryos, at each larval molt, at pupariation and at adult eclosion (9). The 3'-adjacent transcript, however, is abundant only in adult stages and in 1-4 hr embryos (reference 15 and Chapter 2 of this thesis). These data make it unlikely that the region of overlap between the two transcripts has biological significance during embryonic or larval life. However, both transcripts are present in adult flies (Figure III-3). Between 0 and 4 days, the level of mature 2.0 kb Ddc transcript (1,7) decreases, although substantial amounts of the 3.0 kb Ddc species are still present (Figure III-3a). High levels of the 3'-adjacent transcript are present in both newly-eclosed and 4 day old adults and an obvious sexual dimorphism in both the amount and size distribution of this transcript is apparent (Figure III-3b).

To further define the distribution of the two transcripts within adults, we prepared RNAs from several dissected fractions of newly-eclosed adults and quantitated the two transcripts by dot hybridization (Figure III-4). Control experiments (not shown) indicated no detectable hybridization to either of the strand-specific probes in purified ribosomal or poly(A^-) cytoplasmic RNA from young adults. The pattern of Ddc expression in whole heads, thoraces and abdomens of both

FIGURE III-3 Northern Analysis of Transcript Levels in Male and Female Adults.

Duplicate samples of poly(A^+) RNA (5 μg) were subjected to Northern analysis as described in Materials and Methods. The autoradiogram in a) shows the hybridization response to the Ddc-specific RNA probe 5 prepared by in vitro transcription of a 1.5 kb genomic fragment including ~600 bp from the 3' end of Ddc (15 and Chapter 2). b) shows the response to probe 4 (reference 15), transcribed from the opposite strand of the same genomic fragment and therefore complementary to transcripts of the 3'-adjacent gene. Both probes were used at a concentration of 2×10^6 dpm/ml of hybridization solution. Hybridization conditions and exposure times were identical for both blots.

Lanes 1 = newly-eclosed male
Lanes 2 = newly-eclosed female
Lanes 3 = 4 day old adult male
Lanes 4 = 4 day old adult female.

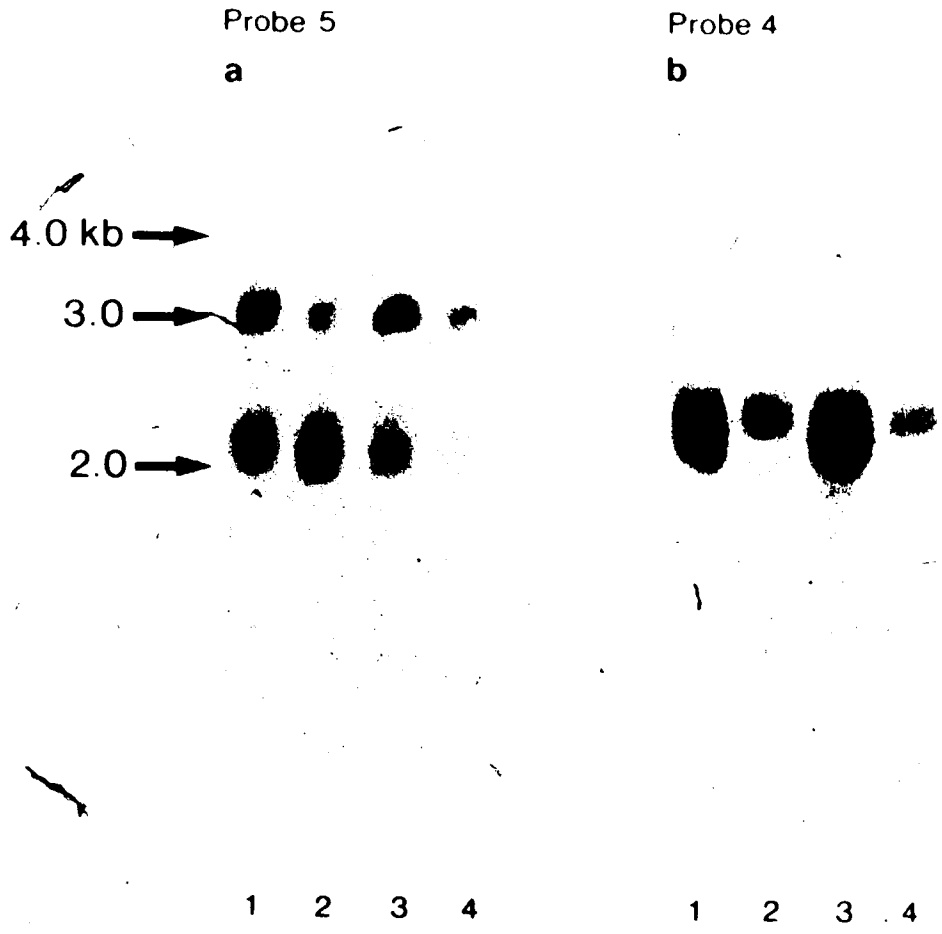

Probe 5
a
Probe 4
b
4.0 kb
3.0
2.0
1 2 3 4
1 2 3 4

FIGURE III-4 Tissue Distribution of Transcripts of Ddc and the 3'-adjacent Gene.

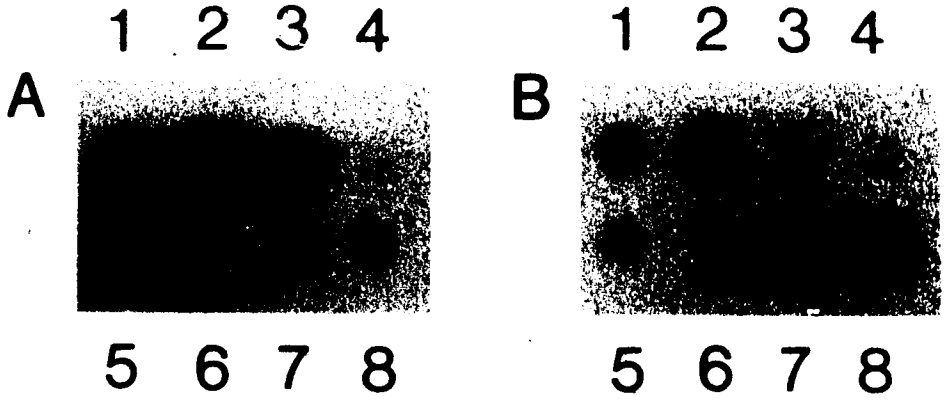
Dot blots of total cellular RNA were hybridized with:

a) RNA probe 5, which is homologous to only Ddc transcripts.
b) RNA probe 12, which is homologous to only RNA molecules transcribed from the 3'-adjacent gene (15).

Twelve animal equivalents were spotted in each case. RNAs in dots 1-4 were obtained from newly-eclosed female samples, those in dots 5-8 from newly-eclosed males.

Dots 1,5 = heads
Dots 2,6 = thoraces
Dots 3,7 = abdomens
Dots 4,8 = isolated gonads

As hybridization conditions were identical and the lengths of the two probes and their respective specific activities were similar, the intensities of the dots in a) and b) represent the relative levels of the two transcripts.


1 2 3 4
A
5 6 7 8
1 2 3 4
B
5 6 7 8

males and females (Figure III-4a) is consistent with a distribution of the enzyme in epidermal (14) and neural tissue (10). The higher concentration of Ddc transcripts in male abdomens (Figure III-4a, dot 7) than in female abdomens (a, dot 3) probably results from the contribution of testicular Ddc transcripts (a, dot 8) to the male abdominal sample. Longer exposures indicated a very low but detectable level of Ddc transcript in dissected ovaries (Figure III-4a, dot 4).

The pattern observed for the 3'-adjacent transcript appears almost complementary to that of the Ddc transcripts (Figure III-4b): low, but detectable levels of the former transcript occur in both male and female heads and thoraces, and in female abdomens and ovaries. Clearly, though, the 3'-adjacent gene transcripts accumulate primarily in the testes (Figure III-4b, dot 8). A Northern analysis of total RNA samples from the dissected tissue samples confirmed that the male-specific size distribution of the 3'-adjacent gene transcripts (Figure III-3b) is also seen in the testes. Similarly, the female-specific size distribution (Figure III-3b) is identical to that contributed by ovaries and 2 hr embryos (data not shown).

The high levels of 3'-adjacent transcript seen in early embryos (Figure II-3) contrast with the weak signal for this transcript seen in the ovary dot blot (Figure III-4, B, dot 4). This disparity is probably due to the fact that the ovary dot blot was obtained from newly-eclosed (0-2 hr) adult females. If the 3'-adjacent transcript is maternally deposited in embryos, we would not expect ovaries to produce the transcript until oocyte maturation, approximately 2 days after eclosion.

DISCUSSION

The data presented here, and our previous work (15 and Chapter 2 of this thesis), indicate that high levels of the two overlapping transcripts are found only within young adults. However, the maximal steady-state levels of the two transcripts occur in different tissues. Ddc transcripts are confined primarily to the epidermis of males and females whereas transcripts of the 3'-adjacent gene accumulate primarily in the testes. The presence of low levels of Ddc transcript in the testes makes this the most likely place for formation of a sense/anti-sense hybrid molecule containing both transcripts. However, confirmation of this possibility must await further detailed functional analysis of the 3'-adjacent transcription unit and in situ studies of the cell-specific distribution of both species of transcript.

Although many examples of antisense RNA regulation are known in procaryotes, the extent to which this mode of regulation is utilized naturally in eucaryotes remains to be determined. Instances of alternate transcripts being created from the same DNA strand of some eucaryotic genes have been described. These usually involve either multiple transcription initiation sites, polyadenylation sites or splicing patterns. The Introduction to this thesis provides a discussion of these instances. However, to date, only 3 cases of naturally-occurring complementary RNAs in eucaryotes have been reported. The first, reported in this chapter, involves an 88 bp overlap of the 3' untranslated ends of the Ddc and the 3'-adjacent transcription units. The second describes a similar situation and involves two adjacent genes of unknown function which are expressed in

mouse tissue culture cells (17). Analysis of cDNAs shows that the 2 mRNAs are transcribed convergently and the processed molecules overlap by 133 bases at their 3' untranslated ends. The transcripts occur simultaneously in several cell lines, but their presence in whole tissues has not been determined. The third case of a naturally-occurring antisense transcript is that of a Drosophila pupal cuticle gene which is located entirely within the first intron the Gart locus (8). The 2 mRNAs are transcribed from opposite strands and their developmental profiles differ from each other. The Gart locus is a "housekeeping" gene, expressed constitutively at low levels in all tissues, whereas the pupal cuticle gene is expressed predominantly in abdominal epidermis over a 3 hr period during the prepupal stage. Whether the 2 transcripts actually are present simultaneously in epidermal cells during this period remains to be established.

It is possible to hypothesize several regulatory consequences in those cases where sense and antisense RNAs can be demonstrated to be present simultaneously in the same cells. Firstly, transcription interference could occur due to the steric effects of converging polymerase molecules. This could result in reduced transcription rates of one or both transcription units, or in a kind of switch mechanism resulting in complementary expression patterns. Such a complementary pattern appears to occur in the tissue and temporal expression of the Ddc and 3'-adjacent transcription units. Secondly, 3' cleavage, polyadenylation or splicing could be hindered or qualitatively altered as a result of masking of processing signals by antisense RNA sequences. Thirdly, stability and transport from the nucleus could be affected by sense/antisense hybrids. And lastly, once in the

cytoplasm, antisense RNA could inhibit translation, as has been demonstrated in procaryotes. However, more data on the functional relationships between sense and antisense transcripts is necessary in order to establish whether a regulatory role for complementary RNAs exists in eucaryotes.

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CHAPTER 4

An Investigation of the Function of a Sequence Element Upstream from the Dopa Decarboxylase Gene in *Drosophila melanogaster*

INTRODUCTION

Control of transcription initiation in eucaryotes is effected through the presence of two types of cis-acting DNA elements - promoters and enhancers. A promoter is defined as a sequence located near the site of transcription initiation which is responsible for directing the site of transcription initiation in a position and orientation dependent manner. Enhancers, as first studied in DNA viruses, are defined as elements which stimulate the rate of transcription initiation from a linked promoter. They characteristically act in an orientation and position independent manner and in a variety of cell types. Since the discovery of viral enhancer elements in 1981 (1,13,43), they have been intensively studied in several eucaryotic systems and some interesting features of these elements are emerging.

Initially the viral enhancer was considered promiscuous with respect to promoter and cell type. However, many viral and cellular enhancers are now known to contribute to strong tissue specific and temporal expression patterns. For example, the rat insulin I gene contains an enhancer element whose activity is restricted to pancreatic B cells (15). Also, the Drosophila yolk protein 1 gene contains a 125 base pair segment of DNA which specifies sex, stage and tissue specific expression (22). A further example is afforded by the mouse α-fetoprotein gene, which contains three enhancers, each of which directs different levels of expression in different tissues (yolk sac, fetal liver and gastrointestinal tract) (28).

Many enhancers are known to operate in response to an inducer. For instance, the enhancers of the mouse mammary tumor virus and the Moloney

murine sarcoma virus respond to glucocorticoids (42,65); the metallothionein gene enhancer responds to glucocorticoids and heavy metals (37,62); and the human β-interferon gene enhancer is induced by viruses or poly(I)-poly(C) (27).

Another interesting feature of enhancers is their conditional binding to trans-acting regulatory proteins, reminiscent of the binding of repressors and activators to procaryotic operators. The binding of these factors is often accompanied by DNase 1 hypersensitivity within an enhancer, suggesting that alterations in chromatin configuration are associated with transcriptional activation (47,68). Some regulatory proteins that recognize enhancer sequences are ubiquitous, such as the Sp1 factor (3,5,37), while others are tissue or stage specific (11,37,61). Regulatory proteins may facilitate either positive or negative control through their interaction with enhancers (46,69). Also, numbers of distinct binding factors may act in combination, to mediate transcriptional enhancement (12).

The mechanisms by which short sequences of DNA may activate a promoter, sometimes over great distances, are still a mystery. However, Ptashne (49) suggests that several proteins may bind to DNA, and it is their interaction with each other and the concomitant looping out of intervening DNA that simulates an adjacent promoter, presumably through the influence of these proteins on RNA polymerase, present at the promoter.

A rapidly emerging concept of the enhancer is not of an isolated element providing an ON/OFF switch, but of an interactive module, influencing both its own promoter and other enhancer elements. It now appears that enhancers stimulate certain promoters more efficiently than

others (23). Also, many examples now exist of multiple elements occurring within and between enhancers. These can cooperatively contribute towards both a modulation in the rate of transcription initiation and an alteration in cell specificity. For example, the SV40 enhancer contains 3 units, each of which can cooperate with others or with duplicates of itself to further enhance transcription (31). The consensus sequence of the metal regulatory element of the mouse metallothionein I gene is repeated 5-fold. At least 2 copies of these elements are necessary to confer metal inducibility to the thymidine kinase promoter in tissue culture cells (62). The synergism which can occur between promoter and enhancer elements has been graphically illustrated in recent recombinant studies with both viral and cellular genes. For instance, when the B enhancer of polyomavirus is replaced by one repeat of the MMLV virus enhancer, the recombinant virus gains expression in mouse pancreas, a tissue in which neither parent virus is expressed (50). Another such example is provided by the Drosophila transformation studies from Steve Beckendorf's laboratory at UC, Berkeley. Upstream sequences of the Sgs-4 glue protein gene, when fused to the larval promoter of the Drosophila Adh gene and introduced into flies via germ line transformation, confer a classical orientation and distance independent enhancement to Adh expression. However, interactions of Adh and Sgs-4 regulatory sequences create anomalous expression of Adh in second instar salivary glands, where neither gene is normally active (58).

The phenomenon of negative regulation through enhancers provides a further complication to the simple classical view of the viral enhancer. Goodbourn et al. (26) have shown that the enhancer of the

human β-interferon gene contains a 19 bp region responsible for maintaining low levels of expression before induction. Removal of these sequences results in a large increase in the basal level of β-interferon mRNA. The remaining 22 bp of the enhancer act as a strong constitutive transcription element. Therefore, it appears that the β-interferon enhancer is repressed by a negative regulatory factor which is somehow inactivated upon induction. Negative enhancer effects have also been identified in the adenovirus EIIa region (35).

It now seems that tissue specific and temporal regulation of transcription probably involves a number of cooperating and perhaps overlapping regulatory sequences which themselves interact with combinations of trans-acting regulatory factors. This presence of interactive and multiple regulatory elements affecting a single gene can interfere with the interpretation of a simple mutation or deletion analysis of promoter function. For instance, the gene under study may utilize multiple regulatory elements, each of which augments the others, but can act alone. In this case, deletion constructs may identify only the last remaining element.

The case of the Ddc gene in Drosophila illustrates some complications arising from deletion analyses. The initial transformation experiments from J.Hirsh's laboratory involved deleted genes retaining 800, 383, 208 or 24 bp of upstream flanking Ddc sequence (32). These experiments indicated that only sequences downstream of -208 were necessary for normal levels and patterns of neural and epidermal expression at hatch, pupariation and eclosion. Paradoxically, though, Ddc deletion genes entirely lacking sequences between -208 and -38, but retaining 2.3 kb of Ddc sequences upstream of -208, still expressed

10-50% of wild-type levels at hatch and eclosion (57). Even severely deleted Ddc genes, retaining only 24 bp of sequence upstream from the transcription start site, and deleting even the Ddc tata box, appeared to show some components of normally regulated expression (32). Therefore, it is possible to conjecture that Ddc contains multiple equivalent regulatory elements, members of which may reside within the gene or upstream of -208, and which can partially compensate for elements operating within the -208/-24 region. Even elements in adjacent vector sequences may make up for missing components of Ddc's multiple element family.

The situation with regard to the central nervous system (CNS) regulation of Ddc is equally complicated. The initial deletion analysis from Hirsh's laboratory (57) revealed a CNS-specific element between -83 and -59, deletion of which abolished Ddc expression in the CNS. However, more recent studies from the same laboratory (Jay Hirsh, unpublished), which involve Ddc antibody staining of individual neurons in transformed larvae, reveal that normal staining patterns are dependent upon retention of 2 further sequence regions - the first from -800 to the transcription start site and the second, approximately 2.2 kb upstream of Ddc. This second region is actually within the exon of an adjacent transcription unit.

Our feeling is that problems of interpretation could arise in a functional analysis of a gene with an expression pattern as complicated as that of Ddc, if deletion studies alone are used. We have therefore undertaken an alternative approach to this problem. We have chosen to analyse a naturally-occurring strain of Drosophila melanogaster (Ddc^{+4}) which exhibits temporally specific variation in Ddc levels. Ddc^{+4} is a

50% underproducer of epidermal Ddc activity and mRNA at pupariation, a 40% and 50% overproducer at the hatch and 2-3 molt respectively and an 18% overproducer at adult eclosion. CNS levels appear unaffected in Ddc^{+4} (16,17,18). Characterization of Ddc^{+4} by Estelle and Hodgetts (17,18) established that the variant phenotype at adult eclosion and pupariation mapped genetically to within 0.15 map units of the Ddc structural gene. The variant contained 7 small (less than 100 bp) deletion or insertion restriction length polymorphisms within intronic and 5' non-coding DNA.

In the study described in this chapter, a region containing 2 of these polymorphisms was subcloned, sequenced and compared to wild-type in an attempt to identify altered sequences which may be responsible for one or more of the stage-specific variations in expression. An enhancer-like element was then selected for functional study in isolation of its flanking material. An oligonucleotide was synthesized corresponding to this short (37 bp) element, it was cloned upstream from a reporter gene, Adh, and introduced via P element germ line transformation into an Adh^{-} host strain. Preliminary characterization of Adh levels and histochemical staining reveal that a modest Adh expression level effect is associated with the presence of the Ddc element, but that the element itself does not contribute a tissue specificity distinct from that of Adh.

MATERIALS AND METHODS

Construction of M13M Subclones

1. Source of DNA

M13M (a gift from Dr.Larry Marsh, UC, Irvine) was used as a sequencing vector for all sequencing experiments. M13M was constructed by replacing the polylinker of M13mp10 with a synthetic oligonucleotide polylinker (EcoRI EcoRV XbaI XhoI SacI NruI HindIII). The reason for choosing M13M as a sequencing vector was the presence of the EcoRV site, making possible the subcloning of L1RV3 and L2RV5, described below.

Plasmid pDDC40-2 (18) was used as a source of Ddc^{+4} DNA which was subcloned into M13M for sequencing. This plasmid was constructed by inserting the 5.6 kb BamHI fragment (spanning 4.6 kb of 5' sequences and 1.0 kb of Ddc transcribed sequences) from λ15 into pBR322 (18).

Plasmid preparations were obtained using standard procedures (38). Replicative form was prepared by inoculating 500 ml of LB Broth with 10 ml of overnight JM103 plus 0.5 ml of M13M phage stock and shaking the culture at 37°C for 8-12 hrs. Cells were harvested and lysed as for plasmid preparation, using the SDS lysis procedure (38).

2. Construction of L1 and L2 Subclones

The 0.8 kb EcoRI fragment (Figure IV-1) from pDDC40-2 was gel purified on 0.7% low melting point agarose (Sigma) and passed through an Elutip-d column (Schleicher & Schuell) according to manufacturer's instructions. DNA was recovered by ethanol precipitation with the addition of 0.3M sodium acetate, pH7.0.

EcoRI-digested M13M (0.03μg) was ligated with purified 0.8 kb fragment (0.03μg) at 15°C for 10 hrs in ligation buffer (50mM Tris-HCl pH7.8, 10mM magnesium chloride, 2mM dithiothreitol, 1mM adenosine triphosphate, 2mM spermine and 50μg/ml BSA) with the addition of 0.1 unit of T4 DNA ligase. Transfection was performed in JM103, as described by Messing (41). Single stranded M13 DNA was prepared from white plaques by picking a single white plaque to 25 ml of LB broth (1% Bacto-tryptone, 1% NaCl, 0.5% Bacto-yeast extract, pH7.5) containing 100 μl of fresh overnight JM103 cells grown in minimal medium (-proline). The culture was shaken at 37°C for 8 hr, the cells pelleted at 15K for 30 min and the supernatant removed to a new tube. One ml of supernatant was pasteurized at 65°C for 20 min and retained as a phage stock. The remaining supernatant was mixed with 6.5 ml of 10% polyethylene glycol, 2.5M NaCl and refrigerated overnight. The pellet of a 10K, 20 min spin was resuspended in 600 μl of phenol extraction buffer (0.3M NaCl, 1.0mM EDTA and 0.1M Tris-HCl pH7.9) and incubated at 37°C for 60 min after the addition of 10 μl of 10% SDS and 10μl of 3 mg/ml Proteinase K. Digestion was followed by 2 phenol and 2 chloroform:isoamyl alcohol (24:1) extractions and the single stranded DNA recovered by ethanol precipitation.

M13 clones containing inserts were selected for sequencing based on their slower migration relative to single stranded DNA of parent M13M bearing no inserts, on 0.6% agarose gels. Sequencing (A tracks) of a selection of single stranded DNA preparations allowed selection of 2 M13M clones (L1 and L2) representing opposite orientations of the Ddc^{+4} 0.8 kb fragment relative to the sequencing primer site (Figure IV-1).

3. Construction of L1RV3 and L2RV5 Subclones

These subclones were generated from L1 and L2 respectively by removing the EcoRV fragment which extends from the EcoRV site in the M13M polylinker to that found in the insert.

L1 and L2 RF's were digested with EcoRV and an aliquot of each digest re-ligated at 0.3 μg/30μl ligation buffer. Transfection and preparation of single stranded DNAs were performed as described for L1 and L2. Single stranded DNAs with the appropriately-sized deletions were selected for sequencing.

4. Construction of L1RV3-NcoRV Subclone

This subclone was obtained by cutting L1RV3 RF with NcoI and EcoRV, blunt-ending with T4 DNA polymerase and ligating. Conversion to blunt ends was performed following Maniatis et al (38) except that the reaction was stopped at 70°C for 5 min, the DNA recovered by ethanol precipitation and redissolved in ligation buffer to a concentration of 10μg/ml. Blunt end ligation was performed by adding 1 unit of T4 DNA ligase and incubating at 12°C for 20 hr.

5. Exonuclease III Deletions

The subclones described above allowed sequencing of both strands from -381 to -208 and from -50 to +376 (Figure IV-1). However, due to a lack of useful restriction sites in the 3' half of the 0.8 kb insert, sequence for one strand between -50 and -208 remained to be determined. Therefore, deletion clones of L2 were obtained using the Exonuclease III technique of Henikoff (30) with variations. RF DNA (18μg), digested with AccI and SacI, was precipitated and resuspended in ExoIII reaction

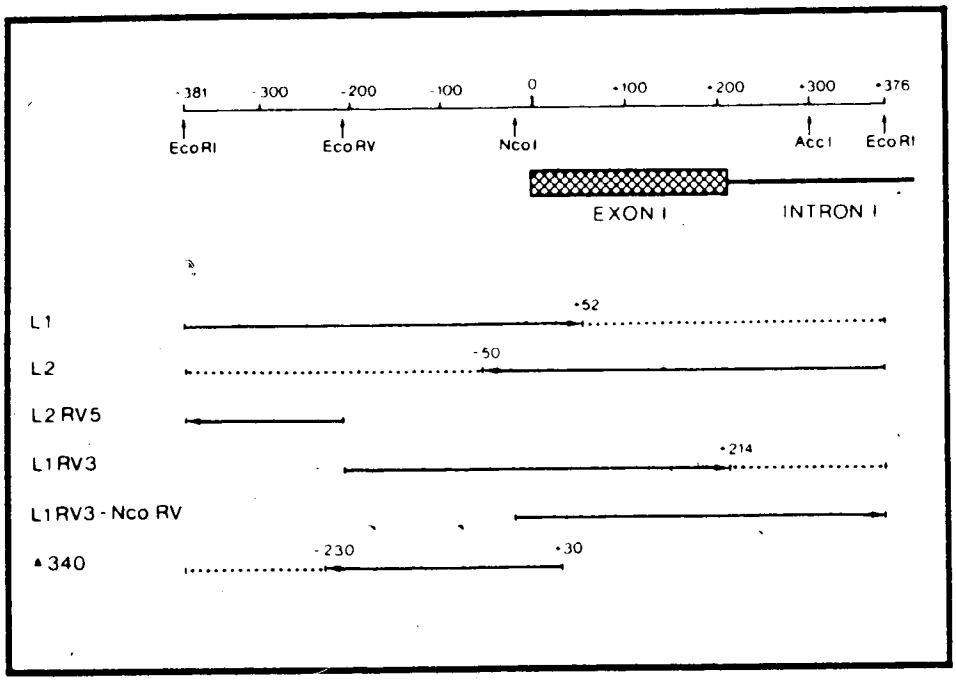


Figure IV-1 Summary of Clones and Sequencing Strategy.

The top scale is in base pairs corresponding to the sequence in Figure IV-7. The Ddc transcription start site, exon 1 and 5' end of intron 1 are indicated below the scale (19). The solid arrows below indicate the extent of genomic sequence obtained from each M13 clone. The full length of each clone is completed with a dotted line. Subclones are listed at the left of the diagram and were generated as described in Materials and Methods. The Δ340 clone was generated from Exonuclease III digestion of L2.

buffer (50mM Tris-HCl pH 8.0, 5mM magnesium chloride and 10mM β-mercaptoethanol) to a concentration of 0.16μg/μl. Exonuclease III (BRL, 67 units/μl) was added to the reaction at 30°C at a concentration of 6 units/μl reaction. At 30 seconds, 1 min, 1.5 min, 2 min and 4 min after addition of Exonuclease III, 15 μl aliquots were removed to 45 μl stop buffer (0.2N NaCl, 5mM EDTA, pH8.0), heated to 70°C for 10 min and ethanol precipitated. Pellets were resuspended in 50μl S1 reaction buffer (0.25M NaCl, 30mM potassium acetate pH4.6, 1mM zinc sulfate and 5% glycerol). S1 nuclease (BRL, 1250 units/μl) was diluted 1:5000 in S1 reaction buffer and 1 unit S1 added to each of the Exonuclease III digested aliquots. After digestion for 10 min at room temperature, the reaction was terminated by adding 6 μl of S1 stop buffer (1M Tris-HCl pH 8.0, 0.25M EDTA and 250μg/ml tRNA) and ethanol precipitated. Ends were blunted with T4 DNA polymerase and blunt end ligated for 20 hr at room temperature.

DNA Sequencing

DNA sequence from single stranded M13M clones was determined by the dideoxy chain termination method of Sanger, Nicklen and Coulsen (53). Urea acrylamide gels were run at 40 watts for 3 hrs (short gels) or 6 hrs (long gels) and exposed to X-ray film at -20°C overnight.

Double-stranded sequencing of pARP-11 derived plasmids was obtained following the method of Chen and Seeburg (7). A 17-base oligonucleotide (homologous to a region of the pARP-11 vector, 20 bp 3' to the SalI site within the P element - see Figure IV-2) was synthesized by the Regional DNA Synthesis Lab, University of Calgary, and was used as the sequencing primer for double stranded sequencing reactions.

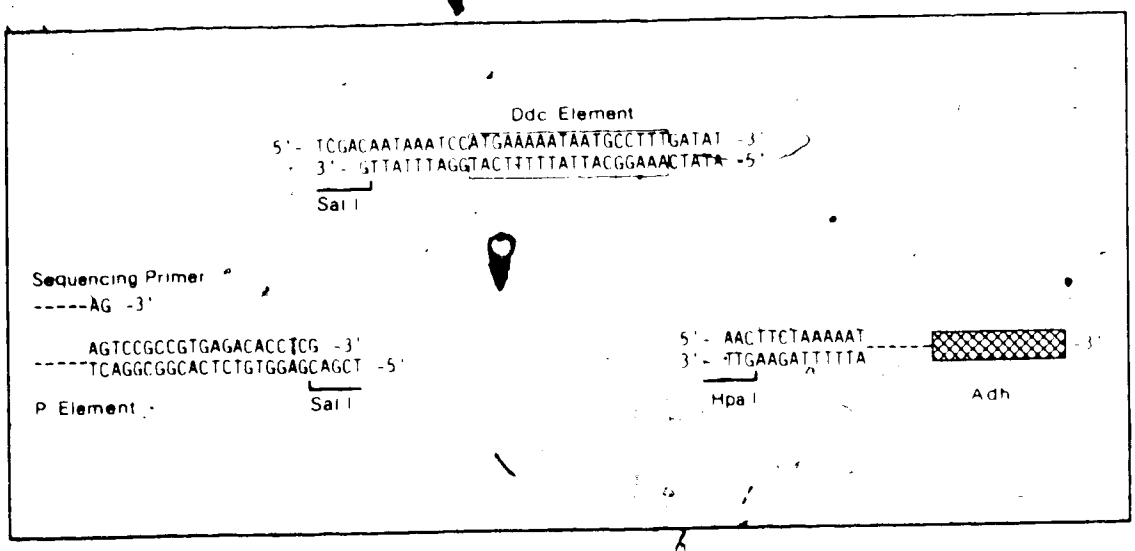


Figure IV-2 Sequence of Ddc Synthetic Oligonucleotide and Site of Insertion in pARP-11.

Northern Analysis of Ddc^{+4} and CS Transcripts

1. *Drosophila* Stocks

The Canton-S laboratory strain of *Drosophila* was used as a wild-type control in these studies. The Ddc^{+4} variant strain was constructed by Estelle and Hodgetts (17), from strain WGM-40, by crossing the variant Ddc gene into a second chromosome marked with *pr* and generating a stock homozygous for this second chromosome. Stocks were maintained at room temperature (23.5°C) on standard food (45).

2. Collection of Organisms

Late third instar larvae ("wandering thirds") were collected by removing larvae from the sides of trays of standard food on which eggs had been deposited. Larvae were then aged for 2 hr on slightly moist paper towels, then frozen pending RNA extractions. Newly-eclosed adults were collected by clearing bottles and selecting unpigmented adults at 2 hr intervals.

3. RNA Extractions and Northern Analysis

Extractions, oligo-dT chromatography and Northern analyses were performed as described in Chapter 2 of this thesis. The probe used for these Northerns was the nick-translated BamHI DNA fragment of 2 kb, specific for Ddc transcripts and located internal to the Ddc gene (Probe 2 of Figure II-1).

P Element Transformation

1. Fly Strains

The recipient strain for injection was the ACR strain (Adh-fn^{6} cn ry^{506}) constructed by James Posakony and obtained from Steve Beckendorf's laboratory at UC, Berkeley. The Adh-fn^{6} allele contains a 6 bp deletion and a 5 bp substitution which inactivates the splice donor sequence of IVS1. This Adh null produces low levels of partially-processed Adh mRNA but no CRM^{+} (21,58). The ry^{506} allelle is null for xanthine dehydrogenase (XDH) activity (21). Both recipient and transformed stocks were maintained at room temperature, 60-80% humidity in the dark. The tARP-9 strain, transformed with the ΔAdh plasmid, was obtained from James Posakony of UC, San Diego.

2. DNA Constructs

The pARP-11 plasmid, from which both control and test plasmids were derived, was kindly provided by James Posakony. The pARP-11 plasmid contains an 8.1 kb fragment containing the ry^{+} gene, a 4.6 kb fragment containing the Adh-F gene, the P-element ends and pUC-9 sequences (Figure IV-3).

The ΔAdh (control) plasmid, also shown in Figure IV-3, was constructed by digestion of pARP-11 with SalI and HpaI, blunt-ending with T4 DNA polymerase and ligating at a DNA concentration of 5 μg/ml. This deletion removes 274 bp of Adh upstream sequence including the adult promoter and larval enhancer, and retains 380 bp of sequence upstream of the larval promoter plus the entire Adh structural gene and 700 bp of 3' sequence (36,48,54).

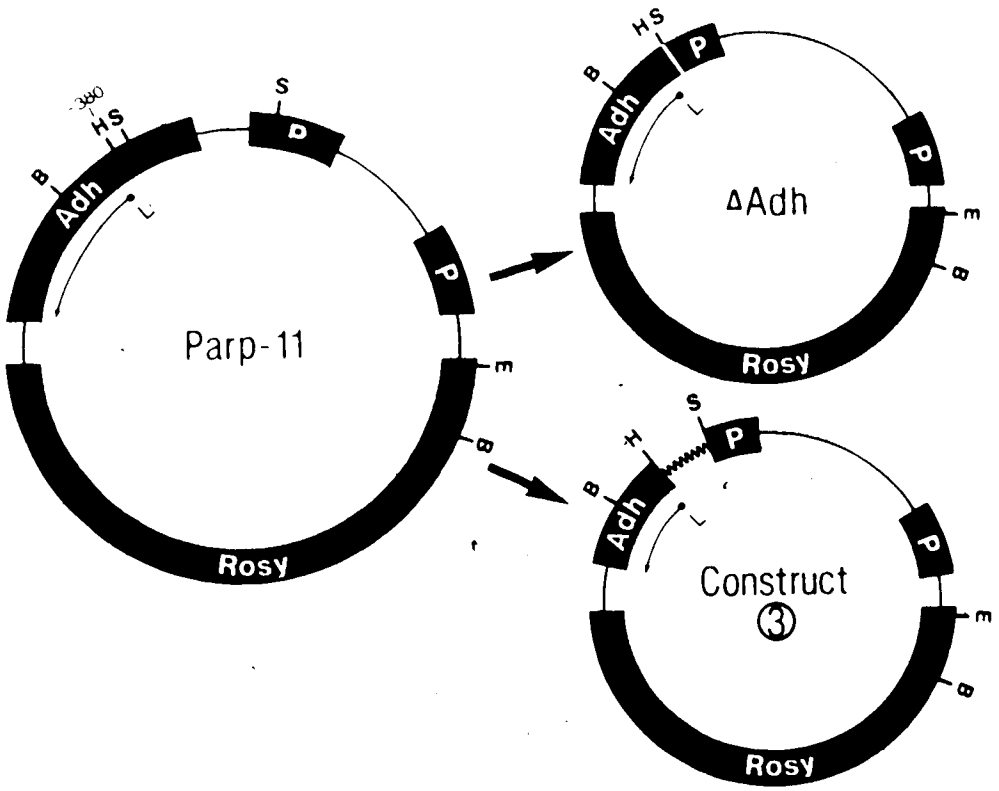


Figure IV-3 Plasmid Constructions Used for Transformation.

The pARP-11 plasmid was the parent plasmid from which Ddc-Adh hybrid plasmids were constructed. The larval promoter (L) and larval transcript (arrow) of Adh are shown. The plasmid constructs contain the rosy (Xdh) gene as well as the P element ends (P). The control plasmid (ΔAdh) serves as a control for the Ddc-Adh hybrid construct and has the HpaI and SalI sites of pARP-11 fused to eliminate Adh sequences upstream of -380, including the adult promoter and larval enhancer. The test transformant (Construct-3) has the Ddc synthetic oligonucleotide (Ddc sequences from between -238 and -206) inserted at the HpaI-SalI site in the same orientation as the Adh gene. Ddc sequences are shown as a wavy line.

H = HpaI
S = SalI
E = EcoRI
B = BamHI

Construct-3 (the Ddc-Adh chimeric test construct) was created by inserting a double-stranded synthetic 37 bp oligonucleotide into the HpaI-SalI site of pARP-11. Two complementary single stranded oligonucleotides were synthesized at the Regional DNA Synthesis Lab, University of Calgary, and together contain the sequence of the Canton-S Ddc gene between positions -238 and -206. An additional 4 bases were added onto the 5' end of one strand in order to engineer a SalI site, facilitating cloning into the HpaI-SalI site of pARP-11. The sequence of the 2 complementary oligonucleotides and their site of insertion in pARP-11 are shown in Figure IV-2. The synthetic oligonucleotide was cloned into pARP-11 as follows:

a) Phosphorylating the Single Stranded Oligomers:

Each oligonucleotide was individually phosphorylated by heating 0.5μg of oligomer in 6μl ddH_2O at 65°C for 6 min in order to denature any secondary structure. It was then cooled to 37°C and maintained at 37°C throughout the reaction, which is a variation of Maniatis et al (38). The oligomer was then mixed with 1μl of 10x linker kinase buffer (0.7M Tris-HCl pH7.6, 0.1M magnesium chloride, 50mM dithiothreitol, 5mM spermidine and 1mM EDTA), 2μl of 10mM ATP and 5 units of T4 Polynucleotide Kinase (PL). After incubation at 37°C for 15 min, a further equal volume of buffer, ATP and kinase was added and the reaction proceeded another 30 min at 37°C. The phosphorylated oligomers were then stored at -70°C prior to use.

b) Ligations and Transformations:

The 2 phosphorylated oligomers were mixed (0.125μg each), heated to 70°C for 10 min to denature any secondary structure, then allowed to cool slowly to 40°C to allow annealing of single strands, and maintained

at 40°C to prevent SalI ends from annealing. The pARP-11 plasmid was digested to completion with HpaI and SalI, heated to 72°C for 10 min to inactivate enzymes and spin dialysed on 0.05μ pore size VM Filters (Millipore Corp.) against 1 liter double distilled H_2O for 30 min to remove salts. The digest was then reconstituted with 10x linker kinase buffer, heated to 72°C for 5 min, then maintained at 40°C. The annealed oligomers were added to digested pARP-11 at a ratio of 1:100 ends (0.84μg pARP-11: 0.25μg oligomer), and the mixture cooled to room temperature. ATP was added to a final concentration of 1mM, spermine to 1mM and BSA to 50μg/ml. T4 DNA ligase (0.1 unit) was added and ligation proceeded for 24 hr at 15°C. ATP was then brought to a final concentration of 2mM and a further 3 units of ligase added. Blunt end ligation then proceeded at 22°C for 20 hr. Transformations were made in E.coli HB101 competent cells (38).

Rapid plasmid preparations (70) of both ΔAdh and Construct-3 colonies were made and plasmids of the expected size were sequenced by the double stranded sequencing method to verify either deletion of the HpaI-SalI fragment or insertion of the synthetic oligomer in the correct orientation. The SalI-HpaI fragment of Construct-3 was subcloned into M13mp10 and single stranded sequencing verified that the cloned synthetic oligonucleotide had not been altered during cloning.

3, Germ Line Transformations

Transformation was carried out essentially as described in Rubin and Spradling (51) and Karess (34). ACR embryos from 0-2 hours old were injected with either ΔAdh or Construct-3 DNA, plus the defective

P element helper plasmid, pπ25.7wc (35). DNA concentrations were 0.22μg/ml:0.1μg/ml for plasmid:pπ25.7wc respectively. G^0 adults were mated individually to the ACR parent strain and ry^+ G^1 flies selected. The ry^+ transformants were mated to either sibling ry^+ transformants or to the parent ACR stock to establish homozygous lines.

Histochemical Assays

Tissue staining for Adh was performed as described in Shermoen et al (58). After 15 min staining, the reaction was stopped with 70% ethanol:5% acetic acid and stained tissues were preserved on a microscope slide in Euparal (Carolina Biologicals).

Enzyme Assays

1. Staging of Organisms

10.5 hr embryos - Eggs were collected for 2 hr onto hard grape agar (10% concentrated Welch's Grape Juice, 2% sucrose, 4% agar, pH7.5) with a drop of yeast paste. A sample was checked microscopically to verify less than 10% post-blastoderm embryos. Plates were incubated for 10.5 hr at 25°C, then the embryos collected and frozen at -70°C until assay.

Hatch - Eggs were collected as above, then newly-hatched organisms collected every 2 hrs after the initial collection at 24 hr incubation at 25°C. Larvae were counted and frozen as above.

Mid-first Instar - Newly-hatched organisms were collected as above, placed on grape plates with yeast paste and incubated 12 hr at 25°C. They were then harvested as described above.

1-2 Molt - A sample of organisms from the mid-first instar plates were incubated a further 12 hr, then selected for molting, based on the

on the appearance of anterior spiracles.

Mid-second Instar - These larvae were placed on grape plates with yeast paste at the 1-2 molt and incubated 12 hr at 25°C, then harvested as described previously.

Late Second Instar - These organisms were selected as mid-second instar larvae from the grape plates described above, or from vials of standard food, based on the morphology of the anterior spiracles, which appear broad and fuzzy approximately 2 hr prior to the 2-3 molt.

2-3 Molt - For this timed collection, 150 newly-hatched organisms were placed on a slice of standard food fortified with a drop of yeast paste in a petri dish, and incubated at 23.5°C for 3 days until they reached late second instar. At this point, 30 individuals were removed to grape plates with yeast paste and maintained an average of 12 hr until the molt. Plates were cleared every 2 hr and newly molted organisms were removed, washed free of food and frozen prior to assay. Newly molted 3rd instar larvae were selected based on the appearance of bold finger-like projections on the anterior spiracles.

Third Instar Samples - Newly molted third instar larvae were selected by removing late second instar larvae from tubes of food to grape plates with yeast and then clearing the plates of 3rd instar larvae every 2 hrs. Newly molted organisms were placed on standard food in tubes at a density of 10/tube and incubated for 22-72 hrs at 23.5°C. They were collected, washed and frozen prior to assay.

White Prepupae - White prepupae were selected from the sides of standard food vials and either frozen for assay or incubated at 23.5°C for various times for assays throughout the pupal period.

Newly-Eclosed Adults - Adults, 0-2 hr old, were collected by

clearing tubes and collecting unpigmented flies every 2 hrs.

ADH enzyme activity measurements were performed as described by Shermoen et al (58). Samples were homogenized in 300-500μl of homogenization buffer (0.1M Tris-HCl pH8.5, 1mM EDTA, 7mM β-mercaptoethanol). Debris was pelletted, 100μl of supernatant was added to 900μl of assay buffer (homogenization buffer, plus 0.1M 2-butanol and 1.4mM NAD^+) and NAD^+ reduction followed at 340nM. One unit of ADH activity was defined as the change of 0.001 absorbance units per minute. Protein measurements were made by the Bradford micrbprotein assay (4,59).

Southern Analysis

DNA was extracted from small numbers of adult flies by the sarcosyl/EDTA/proteinase K method (R.Hodgetts, personal communication). Digestion of DNA, gel electrophoresis and blotting to GeneScreenPlus were as described in Chapter 2 of this thesis. Probes were either the 1 kb BamHI-HpaI fragment of Adh, used to determine the type of insert in each transformed line, or the 1.7 kb EcoRI-BamHI fragment of Xdh, used to determine the number of inserts in each line. Probes were labelled by the oligo-labelling technique (20). Blotting to GeneScreenPlus, hybridization and wash conditions were as described in GeneScreenPlus manufacturer's specifications (Dupont).

RESULTS

Northern Analyses

In an attempt to detect any aberrant Ddc transcripts in the Ddc^{+4} variant strain, a high resolution Northern analysis was made of nuclear and cytoplasmic poly(A^+) RNA from both Canton-S (CS) and variant late third instar larvae and newly-eclosed adults. The Northerns depicted in Figures IV-4, IV-5 and IV-6 were able to reveal size differences of between 20 and 50 nucleotides.

Figure IV-4 shows a comparison of poly(A^+) RNA species from Canton-S and variant organisms at late third instar and newly-eclosed adult stages. Several interesting features are apparent in this comparison. The major transcript sizes in third instar RNA (the 4.0 kb, 3.0 kb and 2.8 kb precursor species, as well as the 2.0 kb mature transcript), which were observed by Gietz and Hodgetts (24) are apparent in both variant and wild-type samples. No obvious size differences exist between Canton-S and Ddc^{+4} transcripts. The apparent size difference in the 2.0 kb transcripts in lanes 1 and 2 is an artifact of this gel and did not appear in subsequent analyses (Figure IV-5). Similarly, adult RNAs of 4.0 kb and 2.0 kb show no major differences in size between strains. However, the 3.0 kb precursor species from Ddc^{+4} shows an increase in size of approximately 50 bases compared to the CS species. This difference is also apparent in Figure IV-6. The slower migration of the variant 3.0 kb species, which contains intron 1 material (24), is probably due to polymorphism 6 of Estelle and Hodgetts (18) which is an approximately 20 nucleotide increase in the size of the EcoRI-BamHI fragment within the first intron. The 4.0 kb species (which

Figure IV-4 Northern Analysis of Canton-S and Ddc^{+4} mRNAs.

Approximately 20μg of poly(A^+) RNA from Canton-S and Ddc^{+4} organisms was run on a 1.35% agarose formaldehyde gel. The RNA was blotted onto nitrocellulose and the blot probed with nick-translated 2.0kb BamHI fragment (probe 2 of Figure II-1) specific for Ddc transcripts. The size species marked are the major Ddc transcripts present in larval A^+ RNA (24). Drosophila and rabbit ribosomal RNAs were run as molecular weight standards and visualized with ethidium bromide. Autoradiograph b) is an underexposure of autoradiograph a).

Lane 1 = Canton-S larval poly(A^+) RNA.
Lane 2 = Ddc^{+4} larval poly(A^+) RNA.
Lane 3 = Canton-S newly-eclosed adult poly(A^+) RNA.
Lane 4 = Ddc^{+4} newly-eclosed adult poly(A^+) RNA.
Lane 5 = repeat of Lane 1.


a
b
kb
4.0
3.0
2.8
2.0
1 2 3 4 5
1 2 3 4 5

contains both major Ddc introns) appears identical in both variant and wild-type. This may be due to the fact that the ≈20 nucleotide increase in the size of intron 1 (polymorphism 6) in the variant is balanced by a less than 50 decrease in the size of intron 2 (polymorphism 7).

The most striking aspect of Figure IV-4 is the apparent difference in kinetics of splicing between larval and adult Ddc transcripts, which is suggested by the different steady-state levels of the 4.0, 3.0 and 2.8 kb precursor RNAs at these stages. Also, the size heterogeneity of the "3.0 kb" adult species (3.0 to 3.2 kb) is not seen in larval RNA.

A more detailed comparison of variant and Canton-S larval poly(A^+) RNAs was attempted by loading alternate slots on a Northern gel with Ddc^{+4} and Canton-S samples from late third instar larvae. Figure IV-5 shows that, at the resolution of this system, differences between sizes of RNA species between the 2 strains are undetectable. The 20-50 base increase in the size of the variant 3.0 kb species, seen in adult RNA, was not observed on this gel.

Figure IV-6 shows a similar "alternate slot" comparison of adult RNAs of variant and wild-type. No major size differences in the 4.0kb and 2.0kb transcripts can be detected. The approximately 50 base increase in size of the variant 3.0 kb species is confirmed on this gel.

Sequence Analysis

Figure IV-1 summarizes the clones and strategy used to obtain DNA sequences of both strands of the Ddc^{+4} gene between -381 and +376 relative to the transcription start site. The wild-type sequence, as determined by Eveleth et al (19) is shown in Figure IV-7, with the variant sequence indicated beneath. The following features are noted:

Figure IV-5 Northern Analysis of Canton-S and Ddc^{+4} Larval mRNAs.

Approximately 20μg of poly(A^+) RNA from late third instar larvae of Canton-S and Ddc^{+4} organisms was treated as in Figure IV-4. Autoradiograph b) is an underexposure of autoradiograph a).

Lanes 1,3,5 = Ddc^{+4} larval poly(A^+) RNA.
Lanes 2,4 = Canton-S larval poly(A^+) RNA.
Lane 6 = Mix of 10μg each of Canton-S and Ddc^{+4} RNAs.

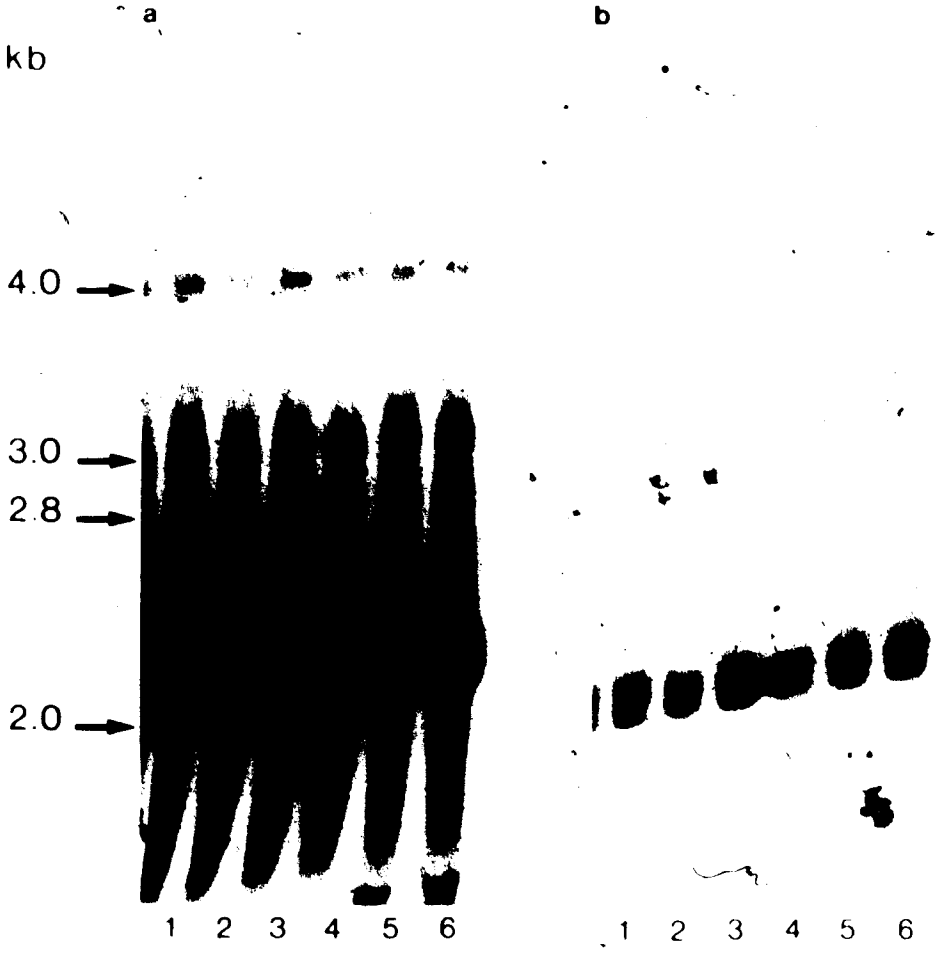
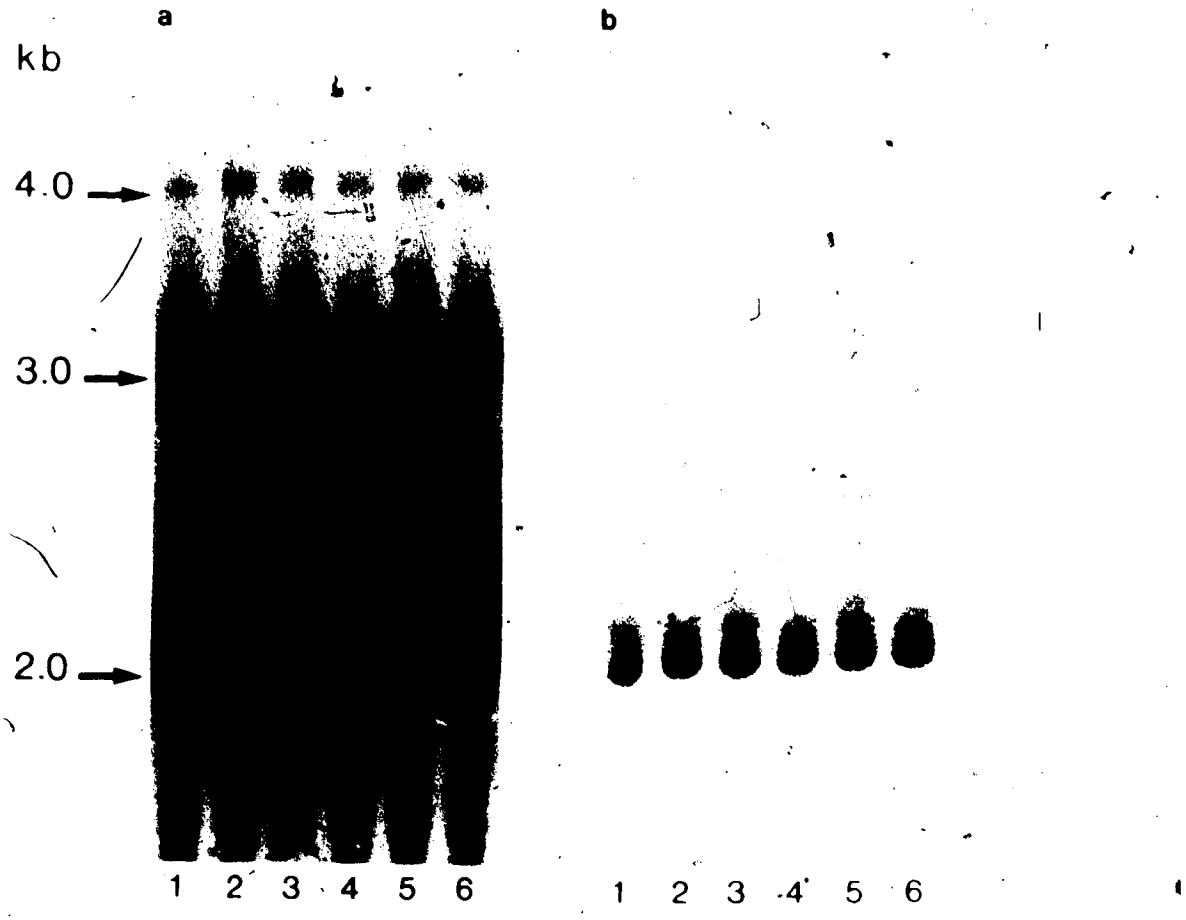

a
b
kb
4.0
3.0
2.8
2.0
1 2 3 4 5 6
1 2 3 4 5 6

Figure IV-6 Northern Analysis of Canton-S and Ddc^{+4} Adult mRNAs.

Approximately 20μg each of poly(A^+) RNA of Canton-S and Ddc^{+4} newly-eclosed adults was treated as described in Figure IV-4. Autoradiogaph b) is an underexposure of autoradiograph a).

Lanes 1,3,5 = Canton-S adult poly(A^+) RNA.
Lane 2,4,6 = Ddc^{+4} adult poly(A^+) RNA.


a
b
kb
4.0
3.0
2.0
1 2 3 4 5 6
1 2 3 4 5 6

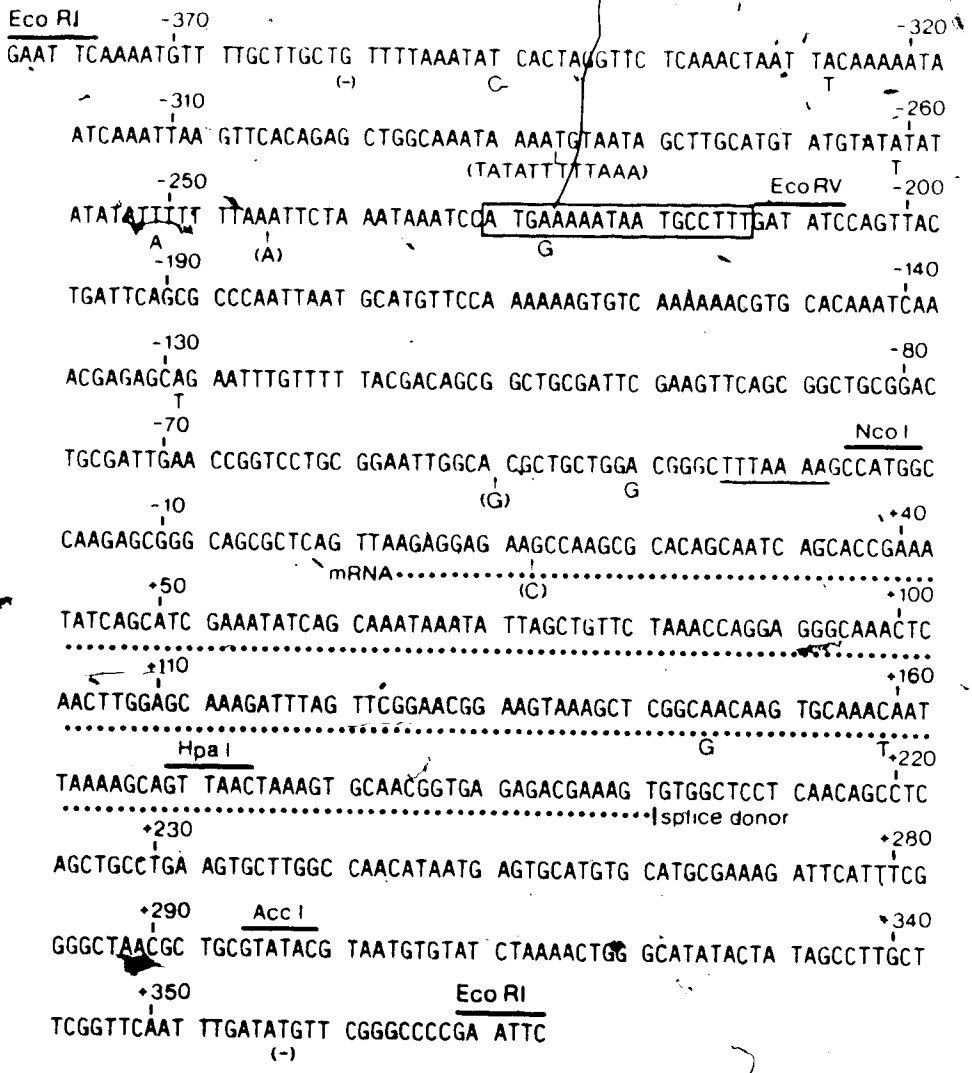


Figure IV-7 Sequence of Canton-S and Ddc^{+4} Genomic DNAs.

The nucleotide sequence of the non-coding strand of the Canton-S Ddc gene, as obtained from Eveleth et al. (19), is shown on the top line. The coordinates are relative to the transcription start site (19). In the lower lines, bases which fail to match in the Ddc^{+4} sequence are printed. Inserts are indicated with an (+), deletions with a (-). Relevant restriction sites are indicated, as well as the TATA box, exon 1 (dotted line) and splice donor site of intron 1. The sequence element chosen for functional analysis is outlined in a box.

1. Polymorphism 4 of Ddc^{+4} was previously identified as a less than 20 nucleotide increase in the size of the EcoRI-EcoRV fragment between -381 and -208 (18). At the nucleotide level, this polymorphism consists of a 12 bp duplication, at -284, of sequences located between -254 and -243. A 1 bp insert at -243, a 1 bp deletion at -358 and 5 single bp substitutions also occur within this EcoRI-EcoRV fragment.

2. Polymorphism 5 was described as an increase of less than 20 nucleotides in the size of the EcoRV-EcoRI fragment (18). The actual sequence reveals 2 single bp inserts (-48 and +15), one single bp deletion (+359) and 4 single bp substitutions in this region. It is not known how these minor sequence differences could have produced a detectable restriction fragment length polymorphism in the previous study.

3. Of the total 26 nucleotide differences between Canton-S and Ddc^{+4} genomic DNAs in this region, 22 (85%) occur 5' to the transcription start site. Three (12%) occur within the first non-coding exon and only 1 (4%) occurs within the first 172 bp of the first intron. Such conservation of non-coding sequence between strains may indicate a possible important role for Ddc exon1/intron1 sequences.

4. As the Ddc gene responds to the steroid hormone, 20-OH-ecdysone at pupariation, a search was made for sequences homologous to those upstream of other hormonally-regulated genes. A perfect MMTV glucocorticoid receptor binding hexamer (56), TGTTCT, is found at +80. Imperfect homologies occur at -173 (TGTTCC), at -371 (TGTTTT) and at -342 (GGTTCT). Regions homologous to the progesterone receptor binding site of the ovalbumin gene are not present.

5. Ddc sequences between -228 and -211 bear a strong homology to sequences between -352 and -336 of the ecdysterone-regulated Sgs-4 gene of Drosophila (39). Figure IV-8 shows a comparison of these and a related sequence element found in the 74EF gene of Drosophila. A single bp change within the Sgs-4 element at -344, in a naturally-occurring variant, is associated with a 50% underproduction of Sgs-4 mRNA (39). A 7 bp inverted repeat, which is an isomer of the Sgs-4 and Ddc elements, is present upstream of another ecdysterone-regulated gene cloned from the 74EF puff region (44). All three of these sequence elements contain the canonical enhancer core sequence of SV40, (G)TTGGAAA(G) (67). Intriguingly, the Ddc^{+4} variant sequence contains a single bp change, at position -225, which creates a more perfect enhancer core sequence and also creates a closer homology to the Sgs-4 element. The element consensus sequence (ATGGAAA-( )-TTTCCAT) has also been identified upstream of the Drosophila ecdysone-regulated genes HSP23, HSP26, HSP27, HSP22, 74F and Genel (71,72). Mestril et al (71) have shown that this element is important for HSP23 induction by ecdysone.

Functional Analysis of the Ddc Sequence Element

In order to examine the possible functional significance of the "enhancer-like" Ddc sequence element discussed above, a synthetic oligonucleotide homologous to wild-type sequence between -238 and -206 was cloned upstream of a truncated Adh gene in an Adh/ry/P element vector (Construct-3) and introduced via germ line transformation into Adh^{-} ry^{-} flies. Another series of injections with a control plasmid lacking the synthetic oligonucleotide (ΔAdh) provided control fly

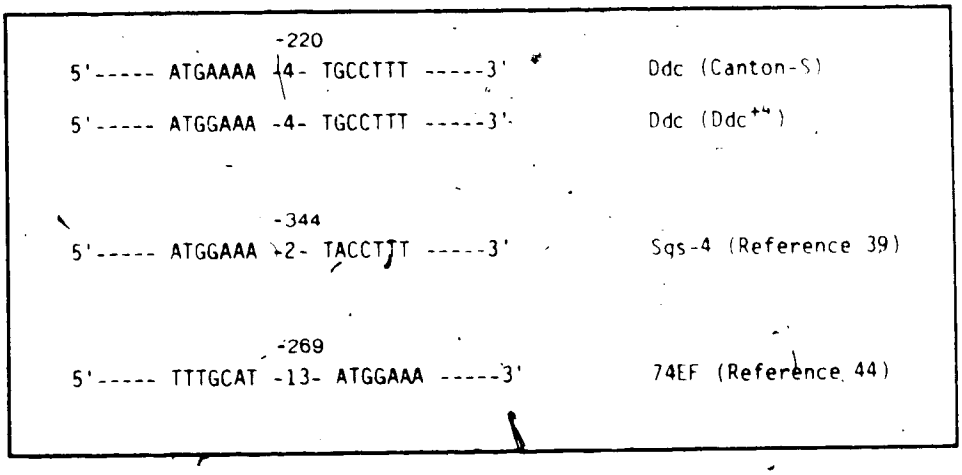


Figure IV-8 Comparison of Homologous Sequences Upstream of Three Ecdysterone-Regulated *Drosophila* Genes.

strains. Transformants were selected as ry^+ G^1 flies, and single pairs crossed to establish homozygous lines which were then tested for ADH activity.

Two control lines (tARP-9 and G) and three test lines (P-7, P-14 and P4-7 were selected for study. Each transformant carried a single insert as demonstrated by the Southern analysis shown in Figure IV-9. For this Southern, DNA was digested with BamHI and blots were hybridized to the oligo-labelled 1.7 kb EcoRI-BamHI fragment of the Xdh gene (Figure IV-3). The parent ACR line as well as all transformants show a 4.7 kb fragment generated by BamHI digestion of the endogenous Xdh gene. Transformants also show a single, variably-sized BamHI fragment extending from the Xdh gene into flanking genomic DNA. Each transformed line was also confirmed as containing the correct P element vector construct as shown in Figure IV-10. In this case, DNA was digested with BamHI and SalI and blots hybridized to the oligo-labelled 1 kb BamHI-HpaI fragment of Adh (Figure IV-3). All transformants show a 1.3 kb fragment representing the BamHI-SalI fragment of the endogenous Adh gene. Control transformants tARP-9 and G contain the ΔAdh construct and therefore lack the SalI site in Adh and have had the SalI site within the P element destroyed after blunt-end ligation at the HpaI site. Hence, the larger molecular weight bands seen in lanes 1 and 2 of Figure IV-10 represent restriction fragments extending from the BamHI site of Adh out into flanking genomic DNA. The test transformants contain a 1.0 kb band which extends from the BamHI site of Adh to the engineered SalI site within the Ddc synthetic oligonucleotide of Construct-3.

Figure IV-9 Southern Analysis of ACR and Transformed Lines.

DNA was digested with BamHI and gels run and blotted as described in Materials and Methods. Blots were hybridized to the oligo-labelled 1.7 kb EcoRI-BamHI fragment of the Xdh gene. All transformants and parent ACR line show a 4.7 kb fragment generated by BamHI digestion of the endogenous Xdh gene. Transformants tARP, G, P-7, P-14 and P4-7 show one additional band representing integrated Xdh genes at new locations.

Lane 1 = ACR
Lane 2 = tARP-9
Lane 3 = G
Lane 4 = P-7
Lane 5 = P-14
Lane 6 = P4-7

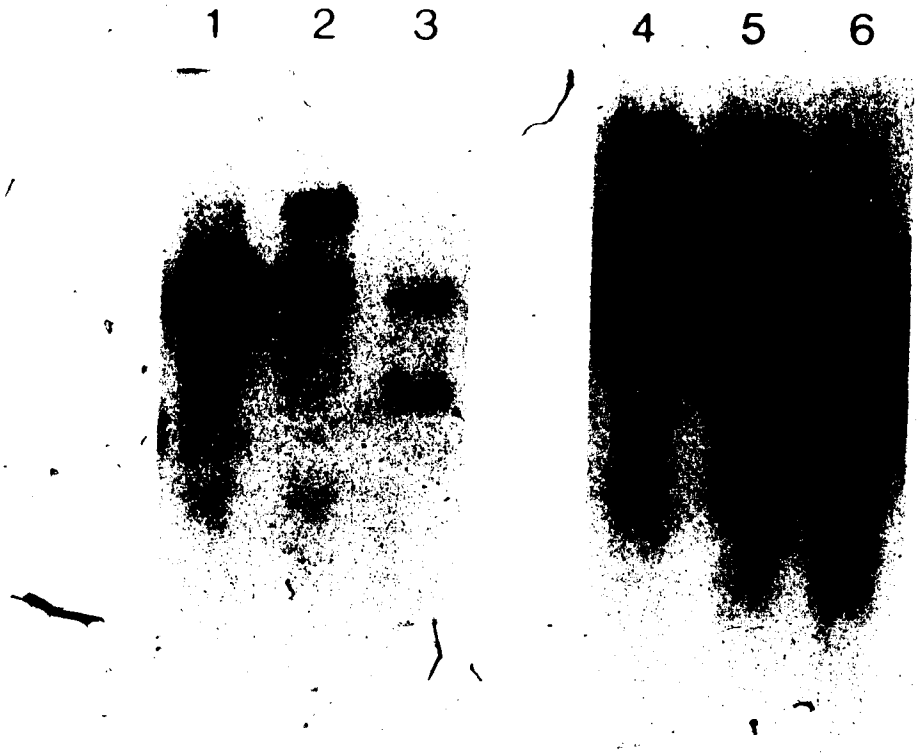

1 2 3
4 5 6

Figure IV-10 Southern Analysis Confirming Type of Insert.

DNA was digested with BamHI and SalI and gels run and blotted as described in Materials and Methods. Blots were hybridized to the oligo-labelled 1 kb BamHI-HpaI fragment of Adh. All transformants and the parent line ACR (not shown in this autoradiograph) show a 1.3 kb fragment generated from the endogenous Adh gene. This fragment extends from the BamHI site to the SalI site of Adh. Control transformants containing the ΔAdh plasmid (lanes 1 and 2) show one extra band extending from the Adh BamHI site out into flanking genomic DNA. Test transformants containing Construct-3 (Lanes 3,4,5) show a unique 1 kb band extending from the Adh BamHI site to the Sal site within the Ddc synthetic oligonucleotide inserted at the HpaI-SalI site of pARP-11. THe high molecular weight bands in lane 4 are partial digests of this DNA sample.

Lane 1 = tARP-9
Lane 2 = G
Lane 3 = P-7
Lane 4 = P-14
Lane 5 = P4-7


1
2
3
4
5

1. Tissue Specificity of the Ddc-Adh Hybrid Gene.

The tissues in which ADH activity is normally detected are the malpighian tubules, anterior and middle midgut and fat body of larvae, and the hindgut, malpighian tubules and genital structures of adults (21,25). In contrast, the tissue-specificity of Ddc is restricted to the epidermis, CNS and possibly the testes (60).

Whole organisms of Canton-S, ACR, tARP-9 and P-14 lines were dissected and stained according to Materials and Methods. The stages examined were mid-2nd instar (CS, tARP-9, G, P-14), the 2-3 molt (CS, tARP-9, G, P-14), mid and late 3rd instar (CS, ACR, tARP-9, G, P-14) and adults of both sexes (CS, ACR, tARP-9, P-14). The ACR parent line showed only light background staining at larval and adult stages. In contrast, all transformed strains, both control and test lines, displayed the characteristic wild-type ADH staining pattern during larval stages, although the intensity of staining was about half that observed in CS. Even the expected adult structures showed detectable ADH staining, presumably as a result of the residual expression in the adult that occurs from the larval promoter (21). No detectable difference was observed in the larval tissue specificity of transformants containing the synthetic Ddc sequence element. Neither CNS nor epidermis displayed ADH activity by histochemical staining. The only staining difference between control and test transformants was a faint staining of the distal half of adult testes in transformants containing the Ddc element.

2. Enhancement and Temporal Regulation of the Ddc-Adh Hybrid Gene.

To determine the contribution of the Ddc sequence element to either expression levels or temporal specificity, ADH enzyme activity levels were compared in Canton-S, ACR and transformed flies at frequent intervals in whole larvae, pupae and adults.

In whole wild-type larvae, ADH specific activity rises from hatch through to the 2-3 molt, then rapidly declines through to mid-3rd instar, when a second smaller peak occurs (58). Activity then declines in wandering larvae. Expression at these stages is primarily a result of transcription from the Adh larval promoter, and enzyme levels are reflected in steady-state mRNA levels. Adh mRNA levels are minimal during pupal stages, then, within 30 minutes of adult eclosion, a large and rapid accumulation of transcript from the adult promoter occurs. Savakis and Ashburner (54), Savakis, Ashburner and Willis (55), Shermoen et al (58) and Ursprung (64) provide summaries of normal developmental Adh expression.

Both control and test transformants show a similar larval developmental pattern in ADH activity to that of wild-type flies, although the mid-3rd instar peak is reduced, as is the activity level generally. ADH activity levels in the parent ACR line are virtually undetectable. ADH activity in both 2 day and 4 day old adults of all transformants is also undetectable by our assay system.

The hypothesis which inspired this functional study was that the enhancer-like Ddc sequence element may contribute to the peaks of Ddc expression which occur at the larval molts. Also, the single base pair change within this element in the Ddc^{+4} variant may be responsible for the 50% overproduction of Ddc mRNA and activity at these stages. For

Table IV-1 ADH Activity at the 2-3 Molt for Wild-type and Transformed Lines.

	ADH Activity (units x100 per μg protein)			
Strain	Expt. 1	Expt. 2	Expt. 3	Expt. 4
Canton-S	1.69	1.92	1.90	2.22
tARP-9	0.29	0.49	0.49,0.47*	0.31
G [illegible]	0.38	0.50	0.72	0.50
Mean, Controls	0.34	0.49	0.56	0.41
P-7	0.33	-	0.55,0.73*	0.42
P-14	0.62	0.69	1.05,0.88*	0.72
P4-7	0.51	0.64	0.82	0.50
Mean, Test transformants	0.49	0.67	0.81	0.55
Ratio of Test:Control transformants	1.41	1.37	1.45	1.34

Assays are single determinations on 100μl of crude extract.
*Assays on two separate samples collected during the same experiment.

this reason, and because ADH activity is high in the transformants at this time, measurements of ADH activity in larvae at the 2-3 molt stage were made in both control and test transformants. This developmental stage was also chosen because larvae can be accurately staged by collecting them as they molt, as described in Materials and Methods. Table IV-1 summarizes the values obtained for the 2-3 molt in 4 separate experiments in Canton-S, control transformants tARP-9 and G, and for test transformants P-7, P-14 and P4-7. Although considerable variation occurred in ADH levels between experiments, the ratio of activity in the test transformants to that of control transformants was consistently between 1.34 and 1.45.

To examine the ratio of ADH activity in test and control transformants beyond the 2-3 molt, samples were collected at 4 timed points following the 2-3 molt and ADH activity determined. Table IV-2 shows enzymatic activity and ratios of test to control lines, for organisms aged 22 hr, 30 hr, 44 hr and 58 hr following the 2-3 molt. Portions of each of these samples were incubated until pupariation in order to determine the length of third instar for these strains. All transformants pupariated between 68 and 74 hr following the 2-3 molt. It appears, therefore, that the enhanced activity in test transformants persists until approximately half way through third instar. By 44 hr after the molt, all strains exhibited similar low ADH activity levels which then persisted throughout pupal stages.

Table IV-2 ADH Activity in Larvae Aged from the 2-3 Molt

Hrs from 2-3 Molt	ADH Activity (units x100 per μg protein) tARP-9	G	Mean, Controls	P-7	P-14	P4-7	Mean, Test	Ratio Test: Controls
22 hr (#1)	0.23	0.39	0.31	0.46	0.61	-	0.54	1.73
22 hr (#2)	0.25	0.56	0.41	0.50	0.73	0.50	0.58	1.41
30 hr (#1)	0.27	0.38	0.33	0.45	0.58	-	0.51	1.56
30 hr (#2)	0.27	0.38	0.33	0.47	0.68	-	0.58	1.74
44 hr	0.31	0.25	0.28	0.27	0.34	0.16	0.26	0.93
58 hr	0.18	0.13	0.16	0.14	0.21	0.19	0.18	1.13

DISCUSSION

Northern Analyses

Previous characterization by Estelle and Hodgetts (17,18) of a naturally-occurring activity and mRNA variant of the Ddc gene revealed 7 small (less than 100 bp) insertion or deletion polymorphisms in 5' and intronic sequences of the Ddc^{+4} gene. The finding that small sequence changes were associated with a variant exhibiting complex temporal alterations in Ddc gene expression encouraged us to further characterize these sequences and perhaps correlate one or more of these with aspects of Ddc expression. The preliminary work on the Ddc^{+4} variant (16,17) showed that the steady-state levels of Ddc mRNA reflected activity differences seen at hatch, pupariation and adult eclosion. The ratio of Canton-S:Ddc^{+4} activity levels were 1:1.4 (hatch), 1:1.5 (2-3 molt), 1:0.5 (pupariation) and 1:1.2 (adult eclosion). Also, no major qualitative differences in mature transcript were noted between Ddc^{+4} and Canton-S. Whether the Ddc^{+4} phenotype was due to an altered level of transcription or of processing or stability of mRNA was not established.

The Northern analyses presented in this chapter show that there are no size differences of 20 nucleotides or greater between mature mRNA species of the variant and Canton-S. This analysis, however, does not rule out the use of alternate RNA start sites or subtle differences in the use of splice donor or accceptor sequences in wild-type and variant, which could affect the stability of transcripts. S1 nuclease mapping or primer extension analysis could be used to examine transcription start sites and intron-exon boundaries. It will be important to establish the role that transcriptional control plays in the phenotype

of this variant, as well as the role it plays in Ddc regulation throughout development in the wild-type organism.

Sequence and Functional Analyses

Our goal in studying the Ddc^{+4} variant was to identify elements, through a comparison of wild-type and variant sequences, which may play a role in the complex pattern of Ddc expression. Polymorphisms 4 and 5 of Estelle and Hodgetts (18) were chosen for sequence analysis, as they lie within the 5' flanking region where regulatory sequences have been found in other eucaryotic genes.

The two most striking sequence differences in the variant were the 12 bp AT-rich duplication at -284 and the A→G transition within the "enhancer-like" element at -225. AT-rich insertions in an oligo-dA:dT tract 222 bp upstream of a yeast Adh gene contributes a promoter-up phenotype to the mutant (52). The progesterone receptor is known to bind to an 18 bp AT-rich sequence between -135 and -247 of the ovalbumin gene (10). However, the exact role of these AT-rich sequences in the regulation of these genes is still not known.

Of greater interest to us than the AT-rich region was the Ddc element between -228 and -211 which contains the SV40 enhancer core sequence and which bears considerable homology to elements upstream of the ecdysterone-controlled Drosophila genes Sgs-4 and 74EF. Such strong homologies between the Ddc element and that of Sgs-4, compounded with the single base pair transition in the variant sequence within this element, encouraged us to speculate that this element may contribute to

regulation of the Ddc peaks at the larval molts and perhaps the hatch, and that the single base pair change in the variant sequence may be responsible for the 40-50% up-promotion of expression seen at these stages. If so, functional analysis of this element would help define one aspect of a complex control region operating on the Ddc gene at several developmental stages.

The larval molts were selected as a time for possible operation of this element for two reasons. Firstly, although overexpression occurs in the variant at 3 stages (hatch, 2-3 molt and eclosion), the level of overproduction at the hatch and molts is distinct from that at eclosion (40-50% overproduction at hatch and molt as opposed ~~to~~ 18% overproduction at eclosion). We therefore hypothesized that adult eclosion provides a regulatory situation distinct from that occurring at the hatch and molt, which may be regulated similarly to each other. Secondly, Hirsh's transformation experiments, described below, suggest a role for sequences upstream of -208 at the hatch (but not eclosion) and hence perhaps also at the molts.

Hirsh's initial deletion and transformation experiments (32) showed that sequences upstream of -208 were not necessary for proper regulation (level or timing) at pupariation and adult eclosion. However, these experiments did not examine the situation at the larval molts. Also, the results obtained for Ddc regulation at the hatch in these transformants were suggestive of a role for upstream sequences. Although a Ddc peak occurred at the hatch in Ddc transformants lacking sequences upstream of -208, levels between individual transformed lines were highly variable. Also, deletion constructs lacking all Ddc upstream material including the TATA box, still showed Ddc induction near the hatch, although timing

was altered (4 hrs later than normal) and there was no evidence of a decline in levels after the hatch "peak". In all of these constructs involving gross deletions of upstream Ddc material, there appeared to be interactions occurring between Ddc and vector sequences creating some novel patterns of expression. For these reasons, Scholnick et al (57) made follow-up transformation studies of the Ddc control region, deleting sequences downstream of -208, while retaining 2.3kb of material upstream of -208. All of the deletion constructs in these experiments retained the Ddc element at -229 to -211. Although both pupariation and eclosion peaks were affected by deletions extending downstream from -208, normal induction of Ddc at 16-18 hrs of embryogenesis was observed in all deletion strains assayed. Again, as in the initial experiments, the larval molts were not examined. These results are suggestive of a role for sequences upstream of -208 in the normal Ddc hatch peak and therefore, possibly at the larval molts.

Our observations of transformed strains containing the Ddc element upstream from Adh are suggestive of an enhancing role for this element at times surrounding the 2-3 molt. Despite the variability observed between experiments, the ratios of ADH activity at the 2-3 molt between transformed strains containing the Ddc element and control strains were consistently between 1.34 and 1.45. The approximately 40% enhancement conferred by the Ddc element upon the Adh promoter is not as dramatic as that contributed by other enhancer sequences (48,58), but is not inconsistent with the 2-fold level of Ddc induction which occurs in vivo at the 2-3 molt (Figure 2 of Introduction to this thesis).

Whether the Ddc element confers temporal specificity cannot be determined from these initial experiments. Adh expression from the

larval promoter itself peaks just prior to the 2-3 molt and would mask any developmental specificity contributed by the Ddc element at this stage. The enhancement effect of the element persists up to 30 hrs after the 2-3 molt, as shown in Table IV-2. Whether this is due to a persistence of stable Adh enzyme 30 hr past the molt, or whether it is the result of continuing transcription and enzyme synthesis is not known. That similar sequence elements to the Ddc element are found associated with 2 other Drosophila genes which are not expressed at the 2-3 molt, may argue against a temporal role for this element.

The histochemical staining results suggest that the Ddc element alone does not confer any tissue-specific differences to the Adh reporter gene. The light staining of the distal ends of the adult testes is interesting but cannot be assessed until more is known about Ddc expression in testes. That the Ddc element is probably not in itself responsible for Ddc tissue specificity is also suggested by the presence of a nearly identical element upstream of Sgs-4, a gene showing very different tissue expression (salivary glands of mid to late 3rd instar larvae). Also, Hirsh's deletion transformants lacking sequences upstream of -208 exhibit normal distribution of Ddc in epidermis and CNS at pupariation and eclosion (32).

The preliminary results on the function of the Ddc element reported here are intriguing, but will require a more elaborate analysis using a different assay system.

One of the limitations of using Adh with its larval control region intact as a reporter gene is its restricted window of assayable expression, ie. from mid-second instar to mid-third instar. There is also evidence that Adh levels are somewhat influenced by dietary alcohol

and carbohydrate levels (40), making uniformity of growth conditions between vials of food and between experiments difficult. This inducibility may partially account for the variability noted between experiments in Table IV-1. In addition, the use of a reporter gene which requires considerable upstream control sequence to operate imposes several other limitations and problems when fused to a simple sequence such as that tested here. Heberlein et al. (29) find that at least 3 distinct DNA binding proteins interact with 3 regions within 380 bp upstream of the Adh larval promoter. Two of these overlap regulatory sequences that mediate promoter activity in in vitro transcription assays. If, for example, any of these Adh transcription factors are lacking in a Ddc-specific tissue such as the epidermis, and if Ddc transcription factors necessary for epidermal expression (which may act through the Ddc element) are lacking in Adh-specific tissues, the effects of the Ddc element would be missed in the Ddc-Adh chimeric gene. A final possible problem with this system is the interactions that occur between promoter and enhancer regions (32,50,58) which may result in novel expression patterns, different from either parent gene.

In order to minimize these restrictions and allow a further assessment of this Ddc element, the following transformation experiments are suggested. An Adh fusion gene lacking its own promoter and 9 bp of sequence downstream of the mRNA start site is still able to produce functional ADH enzyme when linked to the heterologous hsp70 promoter and leader sequence (14). If the Ddc transcription start site plus 32 bp of upstream sequence including the TATA box could be linked to Adh just downstream of the Adh larval cap site at +9, the resulting gene would be under the control of the Ddc promoter, but no Ddc induction should occur

at any stage without further upstream Ddc sequences. Such a construct, when linked to appropriate control sequences, would allow tissue-specific staining of functional ADH enzyme, one of the major advantages of using Adh as a reporter gene. The feasibility of this system could be tested initially by linking the Ddc promoter and 208 bp of upstream Ddc sequence to the promoter-less Adh gene in the pARP-11 vector (the "-208/+1/Adh" construct) and transforming into ACR. Both staining patterns and Adh level changes during pupariation and eclosion could be examined. If production of Adh within the CNS and epidermis were detectable and inducible at the proper times, the chimeric gene system could be used for several tissue and temporal studies, including a further assessment of the Ddc element.

As a confirmation of the experiments reported in this chapter, the wild-type Ddc element could be added to the -208/+1/Adh construct and enhancement and tissue-specificity examined at the larval molts. The element of the Ddc^{+4} variant sequence could then be tested to detect any up-promotion above wild-type levels at the 2-3 molt. To further confirm the element's function, a construct containing 381 bp of upstream Ddc sequence fused to Adh at +9 (the "-381/+1/Adh" construct) could be made. The EcoRI site at -381 provides a convenient restriction site which would yield a fragment containing the Ddc element and sufficient normal upstream sequence to act as a buffer against the influence of vector sequences. This construct could be tested with and without the presence of the Ddc element between -230 and -208. Experiments to determine whether the element is acting as a classical enhancer could be made by reversing the element's orientation or altering its position relative to the Ddc promoter/Adh structural gene.

If these experiments confirm the conclusions presented here in our preliminary characterization of the Ddc element, we will have identified sequences within a complex regulatory region which contribute to Ddc expression levels at the hatch and larval molts.

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DISCUSSION

In this thesis, I have described several studies that were undertaken in our pursuit of the mechanisms that govern the complex developmental regulation of the dopa decarboxylase gene. The first study led to the discovery of a novel dopa decarboxylating enzyme in Drosophila tissue culture cells; the second to the discovery of an antisense transcription unit overlapping that of the Ddc gene. And the results described in the last chapter provide information about the cis-acting elements that control the pattern of Ddc gene expression. However, the molecular details of how the Ddc gene is expressed in a precise spatial and temporal manner remains largely unknown.

Our interest in the Ddc system continues to center around the multifactorial (including hormonal) regulation of epidermal Ddc. The task of sorting out the effects of multiple regulatory elements and factors could, in the future, be approached in several ways.

I. Transcriptional Control Studies

The role of the ecdysterone-receptor complex and tissue specific transcriptional factors in determining either the rate of transcription initiation or mRNA stability is central to our understanding of the Ddc system. The protein synthesis inhibition studies of Clark et al (4) suggest that at least part of the Ddc transcript accumulation that occurs at pupariation is due to a direct hormonal effect, perhaps at the level of transcription initiation. However, at this point, a more direct assessment of the relative contributions of transcription and mRNA turnover is required. This could be achieved by the use of run-on

transcription assays on isolated nuclei.

Run-on transcription assays monitor the elongation of RNA molecules which were initiated in vivo and is a measure of the number of RNA polymerase molecules present on a gene at the time of nuclear isolation and hence transcription rate (10).

Hybridization of labelled RNA, transcribed in vitro, to filter-bound clones covering regions of the Ddc gene would allow an estimate of transcription rates throughout the gene. Rates of transcription could be determined from nuclei isolated from Drosophila epidermal cells at several developmental stages, from ecd^1 epidermis which was treated or untreated with 20-OH-ecdysone, or from imaginal discs undergoing the kinds of hormonal treatment regimes described in Clark et al (4). Results of these in vitro transcription assays would provide estimates of the relative contributions of transcription initiation and mRNA stability to mRNA accumulation (1,18). An effort to establish protocols for isolating purified preparations of nuclei from epidermis or imaginal discs would facilitate these and other studies, such as the chromatin and footprinting experiments suggested below.

2. DNase I Hypersensitivity Studies

The detection of regions of altered chromatin configuration, as assayed by the presence of DNase I hypersensitive sites, could help define important sequences within Ddc's complicated control region. The nature of DNase I hypersensitive sites is speculative, but they are presumed to be sites of altered DNA structure, perhaps resulting from protein binding at or near these sites (2,14). DNase I hypersensitivity studies have been used to pinpoint regulatory sequences in several

systems, including the Sgs-4 gene of Drosophila (9) and the mouse mammary tumor virus (MMTV) (19). For example, in MMTV DNA, a DNase I hypersensitive site appears when transcription is stimulated by glucocorticoids and disappears upon hormonal withdrawal. The hypersensitive site coincides with sequences within the MMTV long terminal repeat that specifically bind glucocorticoid receptor in vitro and which act as a hormone-dependent enhancer in vivo.

The pattern of DNase I hypersensitive sites surrounding the Ddc gene could be determined in isolated nuclei of imaginal discs or ecd[1] epidermis. A comparison of the hypersensitivity patterns before and after the types of hormonal treatments described in Clark et al (4) may give us clues as to sequences involved in the hormonal responses of Ddc. These sequences would then be selected for closer examination by footprinting or functional assays.

3. Footprinting Studies

Like DNase I hypersensitivity studies, footprinting assays have been used to complement gene transfer studies in an attempt to identify important regulatory elements. Both in vivo and in vitro footprinting techniques for identifying specific DNA-protein interactions now exist (3,5,6,11,15).

The ability to correlate DNA-protein binding sites with transcriptional control sequences can be illustrated with two recent studies. Firstly, Ohlsson and Edlund (11) find that nuclear factors from an insulin-secreting cell line interact with three regions within the insulin gene enhancer. All three footprints coincide with DNase I hypersensitive domains and one encompasses 46 bp including a sequence

homologous to the SV40 enhancer core sequence. Second, Heberlein et al (6) detect a promoter-specific transcription factor that binds to and protects a region upstream from the distal transcription start site of the Drosophila Adh gene. In vitro transcription experiments with deletion constructs indicate that this same region is required for RNA synthesis. In addition, three sequence-specific DNA binding proteins interact with sequences upstream of the Adh proximal promoter. Two of these footprint regions coincide with sequences necessary for in vitro transcription of the Adh gene.

Results of footprinting experiments could be valuable in defining transcriptional control sequences for the Ddc gene. Nuclear extracts could be derived from epidermal cells or imaginal discs which were being treated as described above for the DNase I hypersensitivity studies. Binding of tissue- or developmentally-specific nuclear factors to cloned Ddc sequences could provide a valuable complement to functional assays of selected sequence elements. Detection of transcription factors would also be a first-step towards the purification of these factors and their testing under in vitro transcription conditions.

The availability of cloned or purified ecdysterone receptor would make possible binding studies (7,16) which would resolve sequences within or surrounding the Ddc region which may mediate the hormonal responses of the Ddc gene.

4. Functional Studies - In Vitro Transcription Assays

In vitro transcription assays, when used in conjunction with in vivo functional tests, can provide a valuable mechanism for defining transcriptional control sequences. They also offer several advantages to a solely in vivo approach. Drosophila cell-free systems that allow accurate RNA polymerase II transcription of cloned DNA templates have been described (6,12,13) and, to date, derive from tissue culture cells.

One advantage of in vitro transcription assays is that they allow a rapid assessment of the transcriptional ability of a large number of deletion or mutated constructs of the gene under study. Furthermore, fractionation of nuclear transcription extracts can lead to identification of the multiple factors that may be necessary for transcription. These can then be assessed for their binding sites using footprinting techniques. In vitro transcription systems also allow nuclear extract reconstitution experiments to evaluate the roles of suspected tissue-specific or temporal regulatory factors. These assays are also amenable to competition assays, which are used to confirm the factor-dependence of a defined DNA sequence. For instance, the addition, to in vitro transcription assays, of excess competitor DNA of a suspected regulatory sequence will titrate-out the necessary trans-acting factors and hence inhibit transcription. Finally, in vitro transcriptions allow templates of actin or other DNAs to be added to identical reactions, as a built-in control for the transcriptional efficiency of an extract.

An effort to develop an in vitro transcription system for Ddc would greatly facilitate the definition of control sequences and factors that mediate Ddc's hormonal and non-hormonal responses. These in vitro

transcription extracts could be derived from hormonally-naive or stimulated Drosophila tissue culture cells which are known to transcribe Ddc, such as the 7E10(4)EC line of Swiderski and O'Connor (17). It would also be of great interest to develop in vitro transcription systems from "in vivo" cell types such as imaginal discs and epidermal cells. These novel transcription extract systems would allow transcription of Ddc with both the tissue and developmental specificity inherent in the source of nuclei.

5. Functional Studies - Transformation Assays

Both the in vitro transcription assays and footprinting studies suggested above will help us locate the regions surrounding the Ddc gene that are relevant to transcriptional aspects of its control. However, in vivo functional assays of these sequences will be necessary to confirm their actions. The heterologous Ddc/Adh/P element vector system, suggested in Chapter 4, could be used to verify the in vitro findings.

I will suggest one final experimental protocol which could facilitate our attempts to define the tissue-specific control elements of the Ddc gene. This protocol involves the use of the in vivo transient expression assay developed by Martin et al (8). They report that Drosophila Adh genes, cloned into any vector, can be injected into Adh^- preblastoderm embryos, similar to injections for P element transformations. These genes, as assayed by histochemical staining for ADH, are expressed in somatic tissues of both larvae and adults that develop from these injected embryos. They find that Adh expression is tissue-specific to normal Adh-expressing cells and they were able to

define, through the use of deletion constructs, the 5' flanking sequences necessary for this tissue-specific expression.

This in vivo transient assay system, if applied to Ddc/Adh chimeric clones, could provide a rapid method for detecting elements necessary for Ddc tissue specificity.

In summary, it is apparent that sorting out the mechanisms of Ddc's multifactorial and multi-element regulation will be challenging. However, the Ddc system has provided and will continue to provide a readily manipulable experimental model with which we can pursue our knowledge of gene regulation.

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