Hox Gene Function and Cell Identity in Drosophila

by

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Thesis submitted to the University of London, for the degree of Doctor of Philosophy

October 2001
“A fool can ask more questions than a wise man can answer”

ANON
ACKNOWLEDGEMENTS

Firstly I would like to thank Alex Gould, my supervisor, for taking me on as his first PhD student and for his guidance and continual enthusiasm over the past four years. I am hugely indebted to Patricia Serpente and Bruno Bello, who were the only lab members for much of my PhD. Patricia provided support and humour from day one when I arrived in an essentially empty lab. I am very grateful to Patricia for efficiently establishing and running the lab. Patricia was an invaluable source of conversation and advice, whether the topic was the intricacies of molecular biology or how our respective football teams were performing. I thank Bruno for teaching me all that he knows about Drosophila and how to be “Lord of the Flies”. I do not think Bruno has ever answered one of my questions with a simple yes or no, however this lead to many stimulating discussions during which I learnt a great deal. I often found myself in Room 101 during my PhD; pushing flies, listening to CDs and teaching Bruno the finer points of the English language – Northern style. I am very grateful to Bruno for all his guidance and companionship. I owe a big thank you to Véronique Brodu who joined the lab approximately 6 months before my departure and unselfishly helped with experiments for the Development paper. I express my thanks to JP Vincent and Cyrille Alexandre for sharing their knowledge and reagents, and to other members of Mammalian Development for their advice. I would like to thank Frank Johnson and Lesley MacNiell for help with the cartoons presented in this thesis.

The late Rosa Beddington was a scientist for whom I had the utmost respect, she was a great inspiration and I feel privileged to have known her.

I would like to say a huge thank you to all of my PhD friends for the forest treks, snooker matches, the kicking I received in TaeKwondo and football, and for making life at Mill Hill as fun as it could be. Finally I am indebted to Claire, my parents and family for all of their support throughout my PhD.
PREFACE

The research reported in this thesis was carried out in the Division of Mammalian Development at the National Institute for Medical Research (Mill Hill, London) under the supervision of Dr. Alex Gould.

This thesis describes my own original work with the exception of Figure 4.1A, D-F and Figure 5.3B. These panels show the preliminary work on oenocytes carried out by Dr. Alex Gould and were included to provide the necessary background information for the studies described in this thesis.
ABSTRACT

The Hox/Homeotic genes pattern the anteroposterior axis of animal embryos. However, the mechanisms by which these conserved transcription factors generate morphological diversity remain largely unknown. Here I describe both a molecular and a cellular study of Hox gene function. In the molecular approach, a model Hox target enhancer, the late neural enhancer (LNE) of the mouse Hoxb4 gene, was dissected in Drosophila. Individual analysis of two essential HOX binding sites (HS1 and HS2) revealed that each site has a different Hox specificity and modulates responses to more than one Hox input. Activation of the LNE requires the group 4-6 Hox genes and is largely dependent on the Hox cofactor Extradenticle. Furthermore, three conserved regions, remote to HS1/2, were found to influence LNE activity along the anteroposterior and dorsoventral axes. In summary, the LNE contains at least five regulatory modules required for correct enhancer expression.

In a cellular approach, I have investigated how the Hox gene abdominal A micromanages segment identity in Drosophila by studying its role in specifying a single cell identity: the larval oenocyte. An initial study of this cell type revealed three stages of morphogenesis: 1) induction, 2) anterior movement out of the posterior compartment and 3) ventral migration. Induction occurs in response to EGFR signalling from primary chordotonal sensory organ precursors. Ectodermal cells are primed to become oenocytes by virtue of a genetic prepattern, one component of which is spalt. SPALT also suppresses EGFR-mediated induction of an alternative cell fate, the chordotonal organ. Given that both abdominal A and EGFR ligand can induce ectopic oenocytes, I propose that abdominal A might specify oenocytes non-cell autonomously, through regulating local EGFR ligand production and thus oenocyte induction.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acknowledgements</td>
<td>3</td>
</tr>
<tr>
<td>Preface</td>
<td>4</td>
</tr>
<tr>
<td>Abstract</td>
<td>5</td>
</tr>
<tr>
<td>List of Figures</td>
<td>10</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>13</td>
</tr>
<tr>
<td><strong>Chapter One: General Introduction</strong></td>
<td></td>
</tr>
<tr>
<td>1.1 <em>Hox</em> gene function and segment identity in <em>Drosophila</em></td>
<td>17</td>
</tr>
<tr>
<td>1.2 Establishing the <em>Drosophila</em> body plan</td>
<td>17</td>
</tr>
<tr>
<td>1.3 The <em>Hox/Homeotic</em> genes</td>
<td>21</td>
</tr>
<tr>
<td>1.4 Systematic screens for <em>Hox</em> target identification</td>
<td>25</td>
</tr>
<tr>
<td>1.5 Focussed candidate approaches to <em>Hox</em> target identification</td>
<td>27</td>
</tr>
<tr>
<td>1.6 <em>Hox</em> target genes – a summary</td>
<td>34</td>
</tr>
<tr>
<td>1.7 <em>Hox</em> genes and the micromanagement of segment identity</td>
<td>35</td>
</tr>
<tr>
<td>1.8 Co-operative HOX-cofactor interactions in HOX target gene regulation</td>
<td>37</td>
</tr>
<tr>
<td>1.9 Two models for achieving HOX functional specificity: binding site selection versus activity regulation</td>
<td>40</td>
</tr>
<tr>
<td>1.10 A composite binding site selection-activity regulation model</td>
<td>46</td>
</tr>
<tr>
<td>1.11 Co-operative interactions with EXD do not account for all <em>Hox</em>-regulated morphological events</td>
<td>48</td>
</tr>
<tr>
<td>1.12 Synopsis</td>
<td>49</td>
</tr>
<tr>
<td><strong>Chapter Two: Materials and Methods</strong></td>
<td></td>
</tr>
<tr>
<td>2.1 <em>Drosophila</em> stocks and genetic manipulations</td>
<td>52</td>
</tr>
<tr>
<td>2.2 <em>Drosophila</em> transformation</td>
<td>54</td>
</tr>
<tr>
<td>2.3 Generation of LNE <em>cis</em> mutations and transformation constructs</td>
<td>57</td>
</tr>
<tr>
<td>2.4 <em>S10</em> cloning strategy</td>
<td>59</td>
</tr>
<tr>
<td>2.5 Embryo immunolabelling and X-gal reactions</td>
<td>61</td>
</tr>
</tbody>
</table>
Chapter Three: Molecular Mechanisms of Hox Gene Function

3.1 Introduction 64
3.2 Results
   3.2.1 LNE expression in the maxillary segment is associated with the common salivary duct 69
   3.2.2 A role for Deformed during salivary duct development? 75
   3.2.3 HS1 and HS2 are targets for regulation by multiple Hox genes 76
   3.2.4 Remote sequences and the restriction of the HOX response 82
   3.2.5 EXD is required for the in vivo activation of the LNE 86
3.3 Discussion
   3.3.1 HS1 and HS2 have different Hox specificities in Drosophila 94
   3.3.2 Conserved sequences remote to HS1 and HS2 modulate Hox activation of the LNE 101
   3.3.3 A major role for cofactors in Hox activation of the LNE 102
   3.3.4 Perspectives 103

Chapter Four: The oenocyte: a model for studying cellular mechanisms of Hox gene function

4.1 Introduction 106
4.2 Results
   4.2.1 A screen for new oenocyte genes 109
   4.2.2 The embryonic development of oenocytes 109
   4.2.3 Ventral migration of oenocytes; a role for seven up 117
   4.2.4 Anterior movement of oenocyte precursors 128
   4.2.5 A requirement for engrailed but not wingless 137
   4.2.6 Is a lateral break in the WG stripe required for anterior movement? 142
4.3 Discussion
   4.3.1 Oenocyte development – whorls, strings and clusters 151
   4.3.2 Escape from the posterior compartment 152
   4.3.3 A novel role for seven up in ventral cell migration 155
Chapter Five: Oenocyte Induction by abdA and the EGFR Pathway

5.1 Introduction

5.2 Results

5.2.1 abdA is necessary and sufficient for oenocyte formation

5.2.2 Oenocyte precursors are associated with the most dorsal primary COP

5.2.3 Oenocyte induction is regulated by EGFR activation

5.2.4 The degree of EGFR signalling controls cell-number but not cell-type

5.2.5 sal is sufficient to suppress COP recruitment and is required for oenocyte induction

5.2.6 Misexpression of the SPI inductive signal is sufficient to produce ectopic oenocytes in the thorax

5.3 Discussion

5.3.1 Oenocytes and secondary COPs are recruited by the same inducer

5.3.2 Fate mapping oenocyte and secondary COP induction

5.3.3 A prime-and-respond model

5.3.4 abdA and oenocyte specification

Chapter Six: General Discussion

6.1 From Hox genes to morphogenesis

6.2 The LNE is a target for multiple HOX proteins

6.3 Larval oenocytes - a new model system to study Hox specification at the single cell level

6.4 Oenocyte movement can be separated into AP and DV phases

6.5 Do oenocytes have serial homologues in other segments?

6.6 Multiple responses from one receptor: the role of prepatterns

6.7 Keeping an eye on oenocytes and COPs

6.8 What defines the oenocyte prepattern?

6.9 Does abdA function in the C1 COP, in the responding ectoderm or in both?

6.10 Is rho the principal target of ABDA?
Publications


# LIST OF FIGURES

## Chapter One

1.1 The genetic hierarchy for anteroposterior patterning in the *Drosophila* embryo  
1.2 *Hox* genes and their expression in the *Drosophila* embryo  
1.3 The regulation of midgut morphogenesis by *Hox* target genes  
1.4 Repression of wing genes by *Ubx* in the haltere  
1.5 Binding site selection and activity regulation models for HOX specificity

## Chapter Two

2.1 *S10^{boni}* P-element excision

## Chapter Three

3.1 *Hox* activation and repression of the LNE in *Drosophila*  
3.2 LNE-positive cells invaginate with the common salivary duct  
3.3 Cell movements during salivary gland development in *Drosophila*  
3.4 LNE sequence conservation and transgenesis constructs  
3.5 Reduced LNE activation resulting from mutation of HS1 or HS2  
3.6 Conserved regions outside of HS1/HS2 have a role in LNE function  
3.7 The LNE is almost fully exd-dependent  
3.8 LNE activity is increased in a weak *hth* allele but reduced in a strong allele  
3.9 A summary of LNE *cis* mutations and cofactor mutant backgrounds  
3.10 A model for HOX regulation of the LNE
Chapter Four

4.1 Oenocyte development during Drosophila embryogenesis 115
4.2 The down-and-out phenotype 118
4.3 A genomic map of the seven up locus 121
4.4 seven up mutants display dorsally misplaced oenocytes 124
4.5 Dynamics of en expression during oenocyte development 130
4.6 Oenocyte migration during embryonic development 133
4.7 Delaminated sickle-shaped oenocyte precursors down-regulate EN 135
4.8 Uncoupling the interdependence of wg and en 138
4.9 Uncoupling wg and en reveals an essential role for en in oenocyte formation 140
4.10 Persistent expression of en does not affect oenocyte migration 143
4.11 Oenocytes migrate anteriorly through a break in the WG stripe 146
4.12 Bridging the gap in the WG stripe 148

Table 4.1 A literature survey of genes expressed and/or displaying a function in oenocytes 110
Table 4.2 Oenocyte expressing lines 112
Table 4.3 The down-and-out (don) alleles generated 126

Chapter Five

5.1 The Hox gene abdA is essential for oenocyte development 162
5.2 ABDA is sufficient to promote oenocyte formation in the thorax 165
5.3 Oenocytes are associated with the Ich5 and form around C1 168
5.4 EGFR activation is required for oenocyte formation 172
5.5 EGFR hyperactivation results in recruitment of supernumerary oenocytes and chordotonal organs 176
5.6 sal is required to promote oenocyte induction but is necessary and sufficient to repress chordotonal recruitment 180
5.7 Misexpression of secreted spitz is sufficient to induce ectopic oenocytes 185
Chapter Six

6.1 A model for oenocyte induction by abdA and the EGFR pathway 213

Appendices

Appendix I Expression levels of LNE-lacZ constructs in stage 13 embryos 244

Appendix II The only abdominal chordotonal organs to derive from the en stripe are those in the Ich5 array 246
ABBREVIATIONS

A  Adenine
A1-A8  Abdominal segments 1 to 8
abdA  abdominal A
AbdB  Abdominal B
Aldh-III  Aldehyde dehydrogenase type III
ANT-C  Antennapedia complex
Antp  Antennapedia
AP  Anteroposterior
arm  armadillo
ato  atonal
β-gal  β-galactosidase
bcd  bicoid
bp  base-pair
BX-C  Bithorax complex
C  Cytosine
ci  cubitus interruptus
cnn  centrosomin
con  connectin
CR3  Conserved region 3 of the mouse Hoxb4 gene
C-terminal  Carboxy terminal
dap  dacapo
Dfd  Deformed
Dll  Distalless
DNA  deoxyribonucleic acid
don  down-and-out
dpp  decapentaplegic
DV  Dorsoventral
EGFR  Epidermal growth factor receptor
EGTA  ethyleneglycol-bis-(β-amino-ethyl ether) N,N'-tetranic acid
en  engrailed
esg  escargot
exd  extradenticle
fkh  forkhead
ftz  fushi tarazu
G    Guanine
hh   hedgehog
hnf4 hepatocyte nuclear factor 4
HRP  Horse radish peroxidase
hth  homothorax
IPCR Inverse polymerase chain reaction
lab  labial
lacZ The gene encoding β-galactosidase
LNE Late neural enhancer of the mouse Hoxb4 gene
Meis1 myeloid ecotropic insertion site 1
mRNA messenger ribonucleic acid
mrr  mirror
NES Nuclear export signal
NLS Nuclear localisation sequence
N-terminal Amino terminal
pb   proboscipedia
PBS Phosphate buffered saline
PCR Polymerase chain reaction
PIPES piperazine-N, N'-bis[2-ethane-sulphonic acid]
prd  paired
Prep1 Pbx regulating protein 1
ps   parasegment
r6/7 rhombomere 6/7 boundary
rho  rhomboid
sal  spalt
sca  scabrous
Scr  Sex combs reduced
svp  seven up
T    Thymine
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1-T3</td>
<td>Thoracic segments 1 to 3</td>
</tr>
<tr>
<td>TALE</td>
<td>Three amino acid loop extension</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
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<tr>
<td>tsh</td>
<td>teashirt</td>
</tr>
<tr>
<td>Ubx</td>
<td>Ultrabithorax</td>
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<tr>
<td>VM</td>
<td>Visceral mesoderm</td>
</tr>
<tr>
<td>vvl</td>
<td>ventral veinless also known as drifter (dfr)</td>
</tr>
<tr>
<td>wg</td>
<td>wingless</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-3-chloro-3-indolyl-β-D-galactoside</td>
</tr>
</tbody>
</table>
CHAPTER ONE

General Introduction
1.1 Hox gene function and segment identity in Drosophila

The main body axis in all animals is that which runs from head-to-tail, or anterior to posterior. In the Drosophila embryo this anteroposterior (AP) axis is divided into a series of repeated units or segments. A great deal is known about how the segmented body plan of Drosophila is established, including the genetic hierarchy that controls these events. The Hox/Homeotic genes lie at the bottom of this hierarchy and function to give each segment its individual identity and morphology. However, relatively little is known about the molecular mechanisms that link Hox genes to morphogenetic events. This gap in our knowledge of AP patterning is the focus of the work presented in this thesis.

1.2 Establishing the Drosophila body plan

The mechanisms by which the AP axis is set up in the Drosophila embryo have been described in great detail. It is only appropriate here to give a brief summary of these events (for excellent reviews see Wolpert et al., 1998; Carroll et al., 2001; Slack, 2001). Segmentation, and thus the basic Drosophila body plan, is established by the sequential expression of genes in overlapping domains along the AP axis. The first embryonic co-ordinates are set up by maternal mRNAs that are deposited into the egg by the mother. Several such mRNAs are localised to distinct regions of the embryo, and upon fertilisation they are translated. As the early embryo is a syncytium in which nuclei are present in a common cytoplasm, protein gradients can form along the AP axis. For example, bicoid (bcd) mRNA is localised to the anterior pole of the embryo, meanwhile BCD protein diffuses to form a concentration gradient from anterior to posterior. These maternally derived
factors, distributed in overlapping patterns, switch on the first zygotic genes, the gap genes (Figure 1.1). These genes are expressed in distinct broad regions in the embryo and encode transcription factors. Thus the maternal and gap genes divide the AP axis of the *Drosophila* embryo into regions containing different combinations of transcription factors.

The first sign of molecular segmentation in the *Drosophila* embryo occurs when the aperiodic patterns of maternal and gap proteins establish the periodic expression of pair-rule genes in the ectoderm (Figure 1.1). Also encoding transcription factors, the pair-rule genes control the initial expression of segment polarity genes (Figure 1.1). The segment polarity genes encode a mixed bag of proteins including transcription factors, secreted signalling molecules and membrane receptors, which serve to define 14 parasegmental units (Martinez-Arias, 1993). Although the mature *Drosophila* embryo is composed of segments, the parasegment has been identified as an earlier developmental unit of the same period but out-of-phase with the segments (Lawrence, 1992). More specifically, a parasegment is made up of the posterior compartment of one segment and the anterior compartment of the more caudal segment. A compartment comprises a non-intermingling set of lineage-restricted cells (Garcia-Bellido et al., 1973), and thus cell lineages in the anterior compartment are kept segregated from those in the posterior compartment. This results in a straight boundary between these two cell populations that is termed the compartment boundary (reviewed in Dahmann and Basler, 1999).

Genes of the maternal, gap, pair-rule and segment polarity classes coordinately regulate the transcription of the *Hox* genes (Figure 1.1) in precise
Figure 1.1 The genetic hierarchy for anteroposterior patterning in the

*Drosophila* embryo
domains of two or more parasegments. As discussed below, the Hox genes encode transcription factors that function to give each segment its identity.

1.3 The Hox/Homeotic genes

First described by Bateson in 1894, homeotic mutations cause the transformation of one body region into the “likeness of another”. In the Drosophila embryo/larva these mutations are generally characterised with respect to the patterning of denticle belts found in the ventral epidermis. Each segment has a characteristic pattern of denticles, or hairs, which serves to identify it. Loss-of-function mutations in Hox genes result in homeotic transformations of these denticle belts (Lewis, 1978; Wakimoto and Kaufman, 1981). In the adult fly, viable mutations altering Hox gene function produce more spectacular homeotic transformations. The gain-of-function mutations in the Antennapedia (Antp) gene provide a dramatic example where the antennal appendages of the head are transformed into legs (Kaufman et al., 1990). Another famous homeotic transformation is displayed in the four-winged fly, the result of loss-of-function mutations at the Ultrabithorax (Ubx) locus (Lewis, 1963). In this exquisite creature, the halteres, a pair of balancing appendages found on the third thoracic segment, are transformed into a pair of wings, usually associated with the second thoracic segment. The apparent complete segment transformations observed with Drosophila Hox mutations indicate a role for these genes in directing segment morphogenesis.

The Hox genes encode a conserved family of transcription factors that specify morphological differences along the AP axis in animals as diverse as vertebrates and arthropods. These genes are organised into clusters in the genome (reviewed in McGinnis and Krumlauf, 1992; Krumlauf, 1994). The
Drosophila genome contains a single Hox complex that is split into two clusters on the third chromosome (Figure 1.2), the Antennapedia complex (ANT-C) and the Bithorax complex (BX-C). In contrast, vertebrates including humans, mice (Figure 1.2) and chickens have four Hox complexes due to large-scale duplications of chromosomal segments, or even of entire genomes (tetraploidization), during evolution (for an excellent review see Carroll et al., 2001). One of the most intriguing features of all Hox gene complexes is colinearity, whereby the position of a gene in the complex correlates with its domain of expression along the AP axis (Figure 1.2 and Lewis, 1978; Duboule and Dolle, 1989; Graham et al., 1989). However, a discussion of colinearity is beyond the scope of this thesis.

In 1975 Garcia-Bellido proposed the “selector gene hypothesis”, whereby the expression of a Hox gene within a compartment directs the fate of all cells within that metamere (Garcia-Bellido, 1975). The model treated the compartment as an independent unit that is uniformly instructed by a single Hox gene to adopt a specific segment identity. It also suggested that continuous Hox expression is required to direct cells in this unit along a defined developmental pathway. In addition, Garcia-Bellido correctly predicted that, rather than directly specifying morphological differences along the AP axis, the Hox “selector” genes control a battery of target “realizator” genes. These target genes more directly promote segment-specific cellular properties such as growth, mitosis, adhesion and cell differentiation (Garcia-Bellido, 1975). Thus, according to this model, the complexities of segment-specific morphogenesis lay entirely downstream of the Hox selector genes.
Figure 1.2 Hox genes and their expression in the Drosophila embryo

(A) A schematic representation of Hox gene expression in the ectoderm of a Drosophila embryo at the extended germ-band stage. This figure depicts the general trend of Hox expression within any particular segment/parasegment as high (large block) or moderate (small block). However it should be noted that, due to intrasegmental modulation of Hox expression, levels within a block are not necessarily uniform.

(B) The single split Hox gene cluster of Drosophila comprises the ANT-C and BX-C complexes. Colours are as in panel (A) to show the relationship between the position of a Hox gene in the cluster and its domain of expression along the anteroposterior axis (termed colinearity).

(C) The mouse genome contains four different clusters of Hox genes (A, B, C and D) located on different chromosomes. Hox genes are coloured by paralogue group as in panels (A) and (B).
1.4 Systematic screens for Hox target identification

The *Drosophila* Hox genes are expressed in precise segmental domains (Figure 1.2), suggesting that their downstream targets would also be differentially expressed between segments. Based on this assumption, two studies were undertaken to look for genes specifically activated or repressed in response to ectopic expression of a *Hox* gene. In the first of these, the expression of enhancer traps was analysed in the developing antenna following misexpression of *Antp* (Wagner-Bernholz et al., 1991). Enhancer trap lines contain a single copy of a transposon that carries a fusion between *lacZ* and a basal promoter, which comes under the regulation of enhancer elements close to its random insertion site in the genomic DNA. Thus reporter expression generally reflects the whole, or part of the expression pattern of a neighbouring gene. Wagner-Bernholz and colleagues (1991) used enhancer traps to identify regulatory elements and the corresponding genes under the control of ANTP during the antenna-to-leg homeotic transformation. In a second approach, Feinstein et al. (1995) used subtractive hybridisation to enrich for genes transcribed following ectopic expression of UBX, and thus only isolated genes potentially activated by this HOX protein.

The above approaches do not distinguish between direct and indirect targets. To circumvent this problem, two molecular approaches have been used to isolate direct HOX targets based on HOX-DNA interactions. The first strategy used *in vivo* immunopurification to isolate UBX-bound chromatin fragments from embryonic nuclei (Gould et al., 1990). The second used a yeast one-hybrid screening assay that relied on UBX-mediated activation of a reporter gene, following binding to a *Drosophila* DNA fragment cloned upstream of the reporter
(Mastick et al., 1995). Although both approaches have the advantage of isolating potential direct targets of UBX, immunopurification has been the most successful. This strategy benefited from more stringent conditions, as targets were isolated from wild type *Drosophila* embryos. Therefore unlike the yeast assay, immunopurification combined the presence of physiological UBX levels with the proteins involved in co-operative DNA binding with UBX (see Section 1.8). Gould and colleagues isolated several targets, including *connectin (con)*, a gene involved in homophilic cell-cell adhesion (Gould and White, 1992; Nose et al., 1992). Using a similar immunopurification approach, Heuer et al. (1995) isolated the ANTP target *centrosomin (cnn)*, which encodes an essential centrosomal protein. As discussed in the next section, *cnn* has been implicated in the cell shape changes that occur during development of the second constriction of the midgut. In a third, closely related screen, this time incorporating DNA-protein cross-linking, *scabrous (sca)*, a gene encoding a secreted protein involved in cellular communication during neurogenesis, was identified as a putative direct target of UBX (Graba et al., 1992).

The above studies suggested that the number of *Hox* target genes is large, and that these genes are highly varied in nature and do not fall into any one particular family. Despite the identification of several *Hox* targets using such approaches, it has been very difficult to relate these to segment-specific morphological events. Thus we know very little about how *Hox* genes instruct segment identity.
1.5 Focussed candidate approaches to Hox target identification

Many of the *Drosophila Hox* target genes have been identified from previously characterised genes, known to be involved in morphogenetic processes, that are under *Hox* control. Studies on the formation of the central region of the midgut, during *Drosophila* embryogenesis, have provided one of the best examples of how *Hox* genes direct morphogenetic processes (reviewed in Graba et al., 1997). *Ubx* and *abdominal A* (*abdA*) expression in the visceral mesoderm (VM) controls the formation of the second midgut constriction and also cell differentiation events in the underlying endoderm (refer to Figure 1.3). *Ubx* is expressed in the VM adjacent to parasegment 7 (ps 7) where it controls the expression of *decapentaplegic* (*dpp*, Capovilla et al., 1994), a signalling molecule belonging to the TGF-β family. VM cells in parasegment 8 (ps 8) express *abdA*, which activates the transcription of *wingless* (*wg*, Reuter et al., 1990), a gene encoding a secreted factor of the WNT class. The combinatorial action of DPP and WG controls the expression of *teashirt* (*tsh*), a transcription factor required for formation of the second midgut constriction (Mathies et al., 1994). Previous studies revealed that the VM imposes the constriction on the underlying endoderm, and that VM cells close to the inner limits of the constriction contained dense bundles of microtubules (Reuter and Scott, 1990). *centrosomin*, isolated in one of the target gene screens described above, is involved in microtubule-dependent processes and has been implicated in these mechanical events. Isolated as a putative direct target of ANTP, *cnn* is positively regulated by *Ubx* in the VM, and mutants for *cnn* lack the second midgut constriction (Heuer et al., 1995). Li and Kaufman (1996) proposed that, based on
Figure 1.3 The regulation of midgut morphogenesis by Hox target genes

A summary of the genetic cascade initiated by $Ubx$ and $abdA$ that produces a central constriction of the visceral mesoderm and also endodermal cell differentiation. Note that by regulating target genes encoding secreted signalling molecules ($dpp$ and $wg$), a Hox gene expressed in one parasegment can mediate an effect in an adjacent parasegment. See text for details. This figure was compiled from references provided in section 1.5.
Constriction

Visceral mesoderm

Endoderm

Copper cells
Increased differentiation

Large flat cells

Parasegment 7

Parasegment 8

Ubx

dpp

abdA

wg

Cnn

DPP

Constriction

WG

DPP

WG

lab

High level

lab

Low level
its role in microtubule organisation, CNN participates directly in the cell shape
changes that occur during midgut constriction.

_Hox_ gene expression in the VM of parasegments 7 and 8 non-
autonomously patterns the underlying endoderm through the target genes _dpp_ and
_wg_ (refer to Figure 1.3). The integrated action of these signalling molecules results
in expression of the _Hox_ gene _labial (lab)_ in the endodermal cells of ps 7. The
closer endodermal cells are to the ps 8 WG source, the greater the transcription of
_lab_. Hence a gradient of LAB is observed, with high levels underlying posterior ps 7
and increasingly lower levels towards the anterior of this parasegment. _lab_ is both
necessary and sufficient for the specification of copper cells in the midgut
endoderm (Hoppler and Bienz, 1994). Interestingly, the level of LAB determines
the degree of differentiation of these highly specialised cuprophilic cells. Thus the
largest and most distinct copper cells are observed in the most posterior region of
ps 7 while poorly differentiated cells are found more anteriorly (Hoppler and Bienz,
1994). Although WG acts in ps 7 to promote _lab_ expression, the higher degree of
_wg_ signal received by the ps 8 endodermal cells results in the repression of _lab_ and
hence no copper cells (Hoppler and Bienz, 1995). Instead, these endodermal cells
differentiate into the so-called “large flat cells”.

To summarise, in the developing midgut _Ubx_ and _abdA_ lie at the top of
genetic hierarchies that result in the specification of structures in ps 7 and ps 8.
However, cross talk occurs between the downstream targets of _Ubx_ and _abdA_, and
this is essential for morphogenetic events in both parasegments. The identification
of intercompartmental signalling molecules as HOX targets is in direct contrast to
the “selector gene hypothesis”, in which the compartment was considered an
autonomous unit instructed to adopt a particular identity by a single _Hox_ gene.
As alluded to in section 1.3, it is remarkable that by altering the expression of a single Hox gene, the identity of a whole segment can be affected. Originally, based on the classical "selector gene" model proposed by Garcia-Bellido, it was though that a Hox gene acted to instruct all cells in a compartment to adopt a particular segmental identity. However, the results presented in recent studies strongly suggest that the Hox genes act at many levels in genetic hierarchies, independently regulating selected genes (Weatherbee et al., 1998). One good example is the development of wings and halteres, two serially homologous structures found on adjacent adult thoracic segments. Like most adult structures these develop from imaginal discs, the monolayer epithelial sacs set aside during embryogenesis. These proliferate during larval life and differentiate during metamorphosis to form the adult structures. Normally, no Hox genes are required or expressed in the developing wing-blade primordium of the wing disc while the haltere imaginal disc develops under the influence of Ubx. Removal of Ubx function from the developing haltere results in selection of the wing developmental pathway, and thus formation of a four-winged fly. Meanwhile, misexpression of Ubx can transform the identity of a structure as complex as a wing into that of a haltere. Weatherbee and colleagues (1998) dissected the function of UBX in the developing haltere through the examination of genes already known to be involved in wing patterning. They demonstrated that Ubx acts in the haltere imaginal disc to repress genes involved in wing development (Figure 1.4). Although direct regulation was not shown, their results suggest that Ubx negatively regulates target genes at several levels in the genetic hierarchy for wing patterning.
Figure 1.4 Repression of wing genes by *Ubx* in the haltere

The haltere and wing are serial homologues. The wing develops in the absence of any *Hox* input, whilst *Ubx* expression promotes haltere development. Shown here is the wing regulatory hierarchy and in red are those genes under the control of *Ubx* in the developing haltere. *Ubx* appears to repress genes at many levels in the wing genetic hierarchy. However, it should be noted that direct regulation has not been demonstrated for any of these *Ubx* target genes. From Weatherbee et al. (1998).
Anteroposterior Dorsoventral

en → hh → dpp → sal → vein positioning

en → hh → dpp → omb → wing cell growth and identity

en → hh → dpp → vg^B

vg^Q → DSRF

DSRF → intervein cell differentiation

Dorsoventral

ap → Ser → wg

wg → AS-C → bristle differentiation

POST → wg

POST → DSRF

POST → AS-C
1.6 Hox target genes – a summary

From the studies to date, Hox target genes encode a diverse range of molecules including transcription factors, growth factor-like molecules and membrane receptors (reviewed in Graba et al., 1997). Interestingly, most of these target genes have more than one HOX regulator and many have functions, often earlier in development, that are independent of Hox genes.

Based on expression patterns alone, thousands of genes are predicted to lie directly or indirectly downstream of the Hox genes (Bellen et al., 1989; Bier et al., 1989; Biggin and McGinnis, 1997). Given this number, it is surprising that less than 30 Hox target genes have been identified in Drosophila to date (reviewed in Pradel and White, 1998). In most instances, the direct nature of the target has not been demonstrated due to the laborious strategy that is necessary to prove this type of regulation in vivo (Schier and Gehring, 1992). In one case however, Capovilla et al. (1994) showed that UBX interacts directly with DNA binding sites in the dpp303 enhancer to regulate dpp expression in ps 7 of the VM. This was achieved by making corresponding changes to the binding specificity of UBX, and in its DNA binding sites within a dpp303-lacZ construct. LacZ expression was only observed from the mutant construct in ps 7 of the VM when the mutant UBX protein was also expressed in this tissue. This second site suppression strategy demonstrated that in the endogenous situation, UBX binds directly to the dpp303 element to regulate dpp expression in vivo.
1.7 Hox genes and the micromanagement of segment identity

The Hox genes are initially transcribed in broad domains consisting of two or more parasegments. However, later in development Hox gene expression becomes more complex, and at any one time each segment may contain a heterogeneous population of cells expressing and not expressing a specific Hox gene. This intrasegmental spatial and temporal regulation of Hox gene expression can have important effects on the final morphology of a segment. This was demonstrated for Ubx in preventing leg development in the first abdominal (A1) but not in the third thoracic (T3) segment, despite being expressed in both segments in the relevant cells (Castelli-Gair and Akam, 1995). These two distinct morphological outcomes result from the differential regulation of Distalless (Dll), a UBX target gene required for leg development. In T3, Dll is activated in the leg primordia through an early enhancer (Vachon et al., 1992), and expression is maintained through an autoregulatory loop involving a late enhancer element (Vachon et al., 1992; Castelli-Gair and Akam, 1995). The early enhancer is sensitive to UBX-mediated repression but is activated in T3 before Ubx expression is initiated. Meanwhile, Ubx is expressed earlier in the presumptive leg precursor cells of A1. Therefore, in this segment UBX can bind to the early enhancer, block the initial expression of Dll, and thus prevent the feedback loop and leg formation (Castelli-Gair and Akam, 1995). It has been proposed that although Ubx is expressed in the T3 leg following earlier specification, this Hox gene serves to modulate the structure of the leg, making it different from those on T1 and T2 (Castelli-Gair et al., 1994). Therefore slight differences in the timing of Ubx expression within a subset of cells can have enormous consequences on cell fate decisions. The importance of spatio-temporal
patterns of *Hox* expression has been extended to the non-segmented nematode *Caenorhabditis elegans*, where they are also critical for regulating several diverse developmental processes (Salser and Kenyon, 1996).

The work of Castelli-Gair and Akam (reviewed in Akam, 1998a; Castelli-Gair, 1998), together with that of Weatherbee et al. (1998), strongly suggests that, through the fine-grained spatio-temporal pattern of HOX target regulation, numerous cell-fate decisions are made on a cell-by-cell basis within a segment as a whole. This contrasts sharply with the original selector gene hypothesis, which predicted a uniform and continual requirement for *Hox* expression within a segment. Based on these studies, the present hypothesis is that *Hox* genes micromanage at the cellular level many diverse cell fates and behaviours, and it is the summation of all these cell fates that defines the identity of a segment (Akam, 1998b). These theories have led some researchers to change tack and begin to address how *Hox* genes specify the fate of a subset of cells within a segment, such as those in an organ. This is an altogether much simpler task than studying the segment as a whole unit as fewer cell types are involved.

Two excellent *Drosophila* organogenesis models that have been exploited recently are the salivary glands and the posterior spiracles. Contrary to haltere development, both of these structures are promoted rather than repressed by *Hox* genes. *Sex combs reduced* (*Scr*) is both necessary and sufficient to induce salivary gland development (Panzer et al., 1992), acting with the *Hox* cofactor Extradenticle (see next section) to specify both duct and gland cell fates in the salivary primordia (Henderson and Andrew, 2000). Input from the Epidermal Growth Factor Receptor (EGFR) receptor pathway makes the distinction between duct and gland precursors (Kuo et al., 1996). *Scr* acts in those cells receiving the signal to switch
on duct cell genes, whilst in those that do not see EGFR ligand, Scr modulates the expression of gland specific genes (Isaac and Andrew, 1996; Andrew, 1998; Andrew et al., 2000).

The posterior spiracles in *Drosophila* are distinct structures that form the posterior opening for the larval tracheal system. Similar to Scr-mediated specification of salivary glands, *Abdominal B* (*AbdB*) is both necessary and sufficient to direct posterior spiracle morphogenesis (Kuziora, 1993). Hu and Castelli-Gair (1999) showed that ABDB activates the transcription of different genes expressed in at least four distinct cell types that contribute to the posterior spiracle. However, even in such organ-based approaches, the exact number of distinct cell states and the mechanisms by which any single fate is specified by a *Hox* gene have not been elucidated. To address *Hox* gene function at the level of a single cell, I introduce a model cell type, the larval oenocyte, which is specified by a single *Hox* gene, *abdA*.

**1.8 Co-operative HOX-cofactor interactions in HOX target gene regulation**

The *Hox* protein products all contain a highly conserved 60 amino acid DNA binding domain termed the homeodomain, which is encoded by the homeobox. There is very little extended sequence conservation between the HOX proteins outside of the homeodomain. One notable exception is the hexapeptide (or YPWM) motif that is found at varied distances N-terminal to the HOX homeodomain (Acampora et al., 1989). The homeodomain was first described in genes of the ANT-C and BX-C complexes (McGinnis et al., 1984), and has subsequently been found in many other transcription factors (Gehring et al., 1994). *In vitro* studies have shown that divergent homeodomains can bind to similar DNA sequences
(Desplan et al., 1988; Hoey and Levine, 1988; Kalionis and O'Farrell, 1993). However, this does not appear to reflect the situation in vivo, where HOX proteins have distinct biological activities. The promiscuous binding of HOX proteins to DNA in vitro raises the question of how these transcription factors discriminate between their targets in vivo.

Through co-operative binding, Hox cofactors play a significant role in modulating the activity, affinity and specificity of the HOX proteins for their DNA target sites. The best characterised Hox cofactor in Drosophila is extradenticle (exd), a member of the PBC family that includes the vertebrate Pbx genes (Burglin and Ruvkun, 1992). This homeobox gene was isolated in a screen for mutations that disrupt cuticle patterning in the Drosophila larva (Wieschaus et al., 1984). Reduced levels of EXD result in homeotic transformations of the larval cuticle, a phenotype that is normally associated with the altered expression of Hox genes. However, Peifer and Wieschaus (1990) showed that this was not so in exd mutants, as most Hox genes were expressed normally. This suggested that EXD is not involved in regulating Hox expression but instead modulates HOX protein function. Based on these results, Peifer and Wieschaus proposed that EXD forms a complex with the homeotic gene products and alters their in vivo specificity of binding. More recent biochemical and genetic experiments have highlighted such a role and, as discussed in the following section, this forms the basis for the binding site selection model of HOX target recognition.

homothorax (hth, Jurgens et al., 1984) was identified in the same mutant screen as exd. This is a homeobox gene with homology to the vertebrate myeloid ecotropic insertion site 1 (Meis1) gene (Rieckhof et al., 1997; Kurant et al., 1998; Pai et al., 1998). As in exd mutants, hth loss-of-function mutations produce a
homeotic phenotype in the absence of alterations in the expression patterns of the trunk \textit{Hox} genes (Rieckhof et al., 1997). These observations, together with genetic interaction studies (Rieckhof et al., 1997), suggested that \textit{exd} and \textit{hth} act in the same pathway and that both genes are required for correct \textit{Hox} gene activity. As described below, it has subsequently been shown that \textit{hth} plays two roles; it controls the activity of \textit{EXD} and also acts in a complex containing \textit{HOX/EXD} proteins.

The functional activity of \textit{EXD} is regulated at the subcellular level (Mann and Abu-Shaar, 1996; Aspland and White, 1997). In \textit{Drosophila}, \textit{HTH} is necessary for the nuclear localisation and thus the activity of \textit{EXD} (Rieckhof et al., 1997; Kurant et al., 1998; Pai et al., 1998). In the absence of \textit{HTH}, \textit{EXD} is exported from the nucleus due to the presence of a nuclear export signal (NES, Abu-Shaar et al., 1999; Berthelsen et al., 1999). It has been proposed that on binding, \textit{HTH} induces a conformational change in \textit{EXD} that unmasks a nuclear localisation sequence (NLS) identified in the \textit{EXD} homeodomain (Abu-Shaar et al., 1999; Berthelsen et al., 1999). Thus, in the absence of \textit{HTH} the \textit{EXD} NES dominates, while interaction with \textit{HTH} alters the targeting activity in favour of the NLS. The protein product of the vertebrate homologue of \textit{exd}, \textit{Pbx1}, is similarly either nuclear or cytoplasmic in the mouse embryo (Gonzalez-Crespo et al., 1998). The nuclear translocation of \textit{PBX1} is presumably mediated via a similar mechanism, involving \textit{Meis1}, the vertebrate homologue of \textit{hth}. However, \textit{in vitro} experiments reported in Berthelsen et al. (1999) suggest that \textit{Pbx regulating protein 1 (Prep1)}, a \textit{TALE} family member with strong homology to \textit{Meis1}, may also play a role in the nuclear translocation of \textit{PBX1}.  

39
Once in the nucleus, PBC family members co-operatively bind to DNA with the HOX proteins. Structural analysis of HOX-PBC-DNA complexes shows that the HOX YPWM motif sits inside a hydrophobic pocket in the EXD/PBX homeodomain (Passner et al., 1999; Piper et al., 1999). This pocket is formed in part by a three amino acid loop extension (TALE) between helix 1 and helix 2 in the homeodomain. Recently HTH, also a TALE family member (Burglin, 1998), was shown to bind to DNA as part of a trimeric complex with LAB and EXD (Ryoo et al., 1999). Most importantly, this HOX protein complex was shown to be essential for the activation of a natural in vivo HOX target (Ryoo et al., 1999). A similar trimeric binding site has also been identified in a murine Hox target element and was shown to be essential for its in vivo activity (Jacobs et al., 1999; Ferretti et al., 2000).

1.9 Two models for achieving HOX functional specificity: binding site selection versus activity regulation

Following the dissection of a handful of Hox target genes, two models have been proposed to account for HOX specificity in vivo: the activity regulation model (also referred to as the widespread binding model) and the binding site selection model (see Figure 1.5A-B and Mann and Morata, 2000). According to the binding site selection model, through their interaction with cofactors, HOX proteins recognise and bind to specific DNA sequences. Therefore, a single HOX protein will control a specific subset of genes that contain the appropriate binding sites. In contrast, the activity regulation model predicts that different HOX-cofactor heterodimers can bind to a wide range of different sites. HOX-specific transcriptional regulation is
Figure 1.5 Binding site selection and activity regulation models for HOX specificity

(A) According to the binding site selection model all HOX monomers have a weak but observable affinity (dashed arrows) for most HOX binding sites (top). The bottom panel shows that EXD would raise the affinity (thin arrows) moderately for all HOX proteins, but would selectively raise the affinity even further (thick arrow) for one particular HOX/EXD heterodimer (in this example DFD/EXD).

(B) In the activity regulation model, multiple HOX/EXD heterodimers (in this example DFD/EXD and UBX/EXD) bind to the same bipartite site. Functional specificity is achieved post-binding through the abilities of a HOX/EXD dimer to recruit co-activators or co-repressors.
A Binding Site Selection

- EXD

B Activity Regulation

Able to recruit correct co-activators (or co-repressors)  Unable to recruit correct co-activators (or co-repressors)
then conferred after binding by the ability to recruit the correct co-activators and/or co-repressors.

The binding site selection model suggests that HOX proteins distinguish their target sites from other closely related sequences through co-operative binding with cofactors. Thus, in the presence of a cofactor, each HOX protein acts specifically on its battery of \textit{in vivo} target genes (Figure 1.5A). The characterisation of both \textit{Drosophila} and vertebrate Hox target elements has increased our understanding of how PBC family members might function to increase HOX specificity (Bergson and McGinnis, 1990; Tremml and Bienz, 1992; Zeng et al., 1994; Lou et al., 1995; Popperl et al., 1995; Gould et al., 1997; Ryoo et al., 1999). HOX and EXD/PBX bind to DNA as a heterodimer through their respective homeodomains. The bipartite consensus binding site is $^5\text{NNATNNATCA}^3$, where the HOX site is in bold and the overlapping EXD/PBX site is underlined. Although trimeric HTH/MEIS1-HOX-EXD/PBX1 complexes can regulate Hox targets \textit{in vivo} (Jacobs et al., 1999; Ryoo et al., 1999), as yet there is no evidence for HTH/Meis1 directly modulating HOX sequence specific binding. Therefore, focussing on HOX/EXD interactions, how does a single cofactor confer specificity upon numerous Hox genes in \textit{Drosophila}? It has been suggested that co-operative binding with EXD might induce conformational changes in HOX proteins, which could reveal latent specificity in the homeodomain (Mann and Chan, 1996; Mann and Morata, 2000). One region of the homeodomain already known to contribute to the specificity of HOX proteins is the N-terminal arm (Chang et al., 1996). This region, lying immediately N-terminal to helix 1 in the homeodomain, is less highly conserved than the rest of the homeodomain (Laughon, 1991). Interestingly, the homeodomains of SCR and ANTP differ in only four amino acids, all of which are
located in their N-terminal arms (Gibson et al., 1990). SCR-ANTP protein chimeras have been used to show that the N-terminal arms have a major influence on the functional specificity of these proteins in vivo (Furukubo-Tokunaga et al., 1993; Zeng et al., 1993). It has been predicted that HOX homeodomain specificity imparted by the N-terminal arm is through an interaction with the two variable central bases (NN) in the HOX/PBC recognition site (see above and Chan and Mann, 1996; Mann and Chan, 1996). Chan et al. (1997) demonstrated that changing these central nucleotides from CC to TA switched the in vivo specificity of a 20bp Hox-responsive element from lab to Deformed (Dfd). In another experiment, mutations were introduced in the two central basepairs of a HOX/EXD site found in an enhancer of the forkhead gene that is exclusively targeted by SCR (Ryoo and Mann, 1999). This simple change resulted in reduced specificity, such that this 37bp element was now activated by SCR, ANTP and UBX, and repressed by ABDA.

In summary, both the N-terminal arms and the central base-pairs in the bipartite site appear to contribute to the specificity of HOX target recognition. Crystal structures determined for two different HOX-PBC heterodimers bound to DNA reveal only a single non-specific contact between the HOX homeodomain N-terminal arm and either of the two variable central basepairs (Passner et al., 1999; Piper et al., 1999). However, more interactions are likely, as most of the HOX N-terminal arm was not visible in these structures (Mann and Morata, 2000). There also remains the possibility that factors other than EXD are required in vivo to uncover further cryptic DNA binding specificities in the HOX homeodomain. A good candidate for such a factor is HTH/MEIS1, the EXD/PBX1 cofactor that is known to bind to DNA close to the HOX/PBC recognition sequence (Jacobs et al., 1999;
Ryoo et al., 1999). *Prep1* is another candidate factor that could contribute to HOX specificity in vertebrates. *In vitro* studies have shown that PREP1 binds together with HOX and PBX1 proteins to MEIS1-HOX-PBX1 binding sites (Berthelsen et al., 1998a; Jacobs et al., 1999; Ferretti et al., 2000). Although a MEIS1/PREP1 binding site is necessary for activation of at least one HOX target element *in vivo*, it remains to be determined which protein (MEIS1 or PREP1) naturally forms a ternary complex with HOX and PBX1 on such sites.

Considering the affinity regulation (or widespread binding) model (Figure 1.5B), UV crosslinking studies have been used to demonstrate the widespread DNA binding of homeodomain proteins *in vivo* (Walter et al., 1994; Walter and Biggin, 1996). However, Li et al. (1999a) have shown that for at least one HOX protein, DFD, binding to a HOX recognition site is not sufficient for the transcriptional activation of reporter genes *in vivo*. This suggests that many of the homeoprotein-DNA interactions observed by UV crosslinking may be low affinity and/or functionally insignificant. Also, given the promiscuous *in vitro* binding of homeodomain monomers, it is not surprising that widespread binding is observed *in vivo*.

Bipartite HOX/PBC sites, rather than simple HOX recognition sequences, have been identified in most of the HOX target elements studied to date. According to the binding site selection model, HOX-EXD heterodimers bind to defined bipartite sites. In contrast, the activity regulation model suggests that a single HOX/EXD site can be occupied by most HOX-EXD heterodimers *in vivo* (Biggin and McGinnis, 1997; Mann and Morata, 2000). HOX functional specificity is then achieved post-binding by the subsequent recruitment of different co-activators/co-repressors by each HOX-EXD heterodimer (Figure 1.5B). Thus, the co-operative
binding of HOX and EXD to a target enhancer seeds the formation of a multiprotein complex, or enhanceosome. It has been suggested that components of this complex would be specific to certain HOX-EXD dimers (Mann and Morata, 2000), and restricted expression of these other factors could then further increase the specificity of HOX function.

Further support for the activity regulation model comes from in vivo experiments in which the potent transcriptional activation domain of the Herpes Simplex Virus VP16 protein was fused to UBX (Li and McGinnis, 1999). Misexpression of this UBX-VP16 hybrid in *Drosophila* embryos resulted in changes in segment identity similar to those conferred by ANTP rather than UBX. Thus changing the activity state of UBX altered the functional specificity of this molecule. This suggests that, in the endogenous situation, UBX must recruit the correct co-activators/co-repressors to function properly.

### 1.10 A composite binding site selection-activity regulation model

The binding site selection and activity regulation models are not mutually exclusive. As described above, changes in HOX functional specificity can be observed by altering either the HOX recognition sequence or the activity state of a HOX protein. Therefore a balance between the binding site selection and activity regulation models might account for the real in vivo specificity of Hox gene function.

Interestingly, further to its role in binding site selection, the cofactor EXD has also been implicated in regulating the activity of HOX proteins in *Drosophila*. Li et al. (1999a) observed that both DFD and a DFD-VP16 hybrid induced greater reporter gene expression from a DFD-EXD bipartite site than from a simple DFD binding site alone. *In vitro* analysis has also shown that DFD contains an activation
domain that is suppressed in a manner dependent on the presence of the homeodomain (Li et al., 1999a). The authors therefore proposed that, upon cooperative binding, EXD releases the transcriptional activation function of DFD. Thus, in addition to a role for EXD in increasing the specificity of binding site selection, this cofactor can also regulate HOX activity.

In a recent study using the homeodomain protein encoded by *Fushi tarazu* (*Ftz*), Nasiadka et al. (2000) provided further support for a composite binding site selection-activity regulation model. Similar to the HOX proteins, FTZ regulates a large number of genes either directly or indirectly (Liang and Biggin, 1998), and binds co-operatively to DNA with its cofactor FTZ-F1 (Guichet et al., 1997; Yu et al., 1997). Nasiadka et al. (2000) generated a chimeric protein containing FTZ fused to the strong transcriptional activator VP16. In misexpression experiments, FTZ-VP16 regulated previously identified FTZ target genes in different spatial and temporal patterns to those observed with FTZ alone. Interestingly, the addition of VP16 to FTZ resulted in the activation of two genes that this segmentation gene product would normally repress. Meanwhile genes suggested not to be direct targets of FTZ in earlier experiments (Nasiadka and Krause, 1999) were unaffected by FTZ-VP16. The results presented in this study suggest that co-operative binding with cofactors such as FTZ-F1 controls the specificity of FTZ target recognition (binding site selection). Meanwhile the recruitment of co-activators/co-repressors controls the exact nature, magnitude and temporal/spatial domains of the response (activity regulation). A similar balance between binding site selection and activity regulation may also control the specificity of HOX and other homeodomain protein functions.
1.11 Co-operative interactions with EXD do not account for all Hox-regulated morphological events

The nuclear distribution of EXD corresponds to the functional domains of this Hox cofactor. However, the regions of the Drosophila embryo affected by Hox mutations are more extensive and encompass areas where EXD remains cytoplasmic. One possible explanation is that other cofactor(s) operate in these areas. Another possibility is that HOX specificity and transcriptional activity, normally conferred by EXD, are not necessary in these regions. This appears to be the situation during development of both the leg and haltere imaginal discs. EXD is cytoplasmic in the pouch domain of the haltere imaginal discs (Aspland and White, 1997), where UBX mediates repression of the wing determining genes (Weatherbee et al., 1998). Ectopic expression of Ubx in the developing wing blade generates a contrabithorax wing-to-haltere transformation (Casanova et al., 1985), presumably due to the repression of wing determining genes (Weatherbee et al., 1998). Interestingly, ectopic expression of abdA or AbdB in the wing pouch produces a similar effect to that of Ubx in transforming wing into haltere (Casares et al., 1996). These experiments indicate that UBX functions in a less discriminatory and largely repressive manner in the distal portion of the haltere imaginal disc, where EXD is absent from the nucleus.

Dominant mutations that result in misexpression of Antp in the antennal imaginal disc lead to the transformation of the antenna into a leg (Kaufman et al., 1980). Meanwhile removing Antp from the leg discs, where it is normally expressed, causes the reciprocal leg-to-antenna transformation (Struhl, 1981). Based on these experiments it was proposed that Antp promotes leg
morphogenesis by repressing genes required for antennal development (Struhl, 1981; Struhl, 1982). Recently, Casares and Mann (1998) revealed that \textit{hth} and \textit{exd} are the antenna-determining genes. They showed that expression of \textit{hth}, and the resultant nuclear localisation of EXD, are both necessary and sufficient for the induction of antennae, even at such diverse sites as the anal plate. In the leg discs, antennal development is suppressed as \textit{Antp} represses \textit{hth} (Casares and Mann, 1998), thus explaining why EXD is cytoplasmic in the distal region of the leg imaginal discs (Aspland and White, 1997; Rieckhof et al., 1997). The ectopic expression of \textit{Antp} in the antennal imaginal disc causes the loss of \textit{hth} expression, and thus promotes an antenna-to-leg transformation. Interestingly an identical homeotic transformation is also observed upon misexpression of other \textit{Hox} genes in the antennal disc, including \textit{Ubx}, \textit{abdA}, \textit{AbdB} and even mouse \textit{Hoxd-10} (Casares et al., 1996; Azpiazu and Morata, 1998). Thus \textit{Hox} genes act in a rather indiscriminate manner to repress \textit{hth} (Morata and Sanchez-Herrero, 1998).

In summary, studies of two homeotic transformations have demonstrated that \textit{Hox} genes can function in the absence of EXD, albeit in a largely repressive manner.

1.12 Synopsis

Despite the description of homeotic mutations over 100 years ago and the molecular cloning of \textit{Hox} genes in \textit{Drosophila} over 20 years ago, how \textit{Hox} genes regulate morphological events at the cellular or DNA level remains largely a mystery. In this thesis, I will take both a molecular and a cellular approach to study \textit{HOX} protein function \textit{in vivo}. In the former approach, a conserved murine \textit{Hox} responsive element was dissected to investigate how \textit{HOX} proteins achieve their \textit{in}}
vivo specificity and activity status. Chapter 3 reports the results obtained from this study using a *Drosophila* transgenesis assay. In the cellular approach, I ask how is a single cell type, the larval oenocyte, specified by a single Hox gene, *abdA*? An initial study of larval oenocyte development is described in Chapter 4, whilst Chapter 5 focuses on the *abdA*-mediated induction of oenocytes.
CHAPTER TWO

Materials and Methods
2.1 *Drosophila* stocks and genetic manipulations

Flies were maintained in vials or bottles containing standard cornmeal/yeast/agar medium at 25°C. Genetic markers, balancer chromosomes and stocks not mentioned below are described in Lindsley and Zimm (1992) and Flybase (1999). Standard genetic crosses and techniques were performed according to Greenspan (1997) and Roberts (1998).

The following mutant strains were used:

<table>
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<th>Allele</th>
<th>Allele class</th>
<th>Reference</th>
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<td>Sanchez-Herrero et al. (1985)</td>
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<td>Amorph</td>
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<tr>
<td><em>ato</em>&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Amorph</td>
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<td><em>rho</em>&lt;sup&gt;7443&lt;/sup&gt;</td>
<td>Amorph</td>
<td>Jurgens et al. (1984) and Wasserman et al. (2000)</td>
</tr>
<tr>
<td><em>sal</em>&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Loss-of-function</td>
<td>Nusslein-Volhard et al. (1984)</td>
</tr>
<tr>
<td><em>sal</em>&lt;sup&gt;66&lt;/sup&gt;</td>
<td>Amorph</td>
<td>Kuhnlein et al. (1994)</td>
</tr>
<tr>
<td><em>sal</em>&lt;sup&gt;46&lt;/sup&gt;</td>
<td>Amorph</td>
<td>Jurgens (1988)</td>
</tr>
<tr>
<td><em>svp</em>&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Amorph</td>
<td>Gausz et al. (1981)</td>
</tr>
<tr>
<td><em>svp</em>&lt;sup&gt;H162&lt;/sup&gt;</td>
<td>Hypomorph</td>
<td>Mlodzik et al. (1990)</td>
</tr>
<tr>
<td>Df(Ubx)&lt;sup&gt; Abd100&lt;/sup&gt;</td>
<td>Df for <em>Ubx</em></td>
<td>Struhl (1984)</td>
</tr>
<tr>
<td>Df(Ubx)&lt;sup&gt;109&lt;/sup&gt;</td>
<td>Df for <em>Ubx</em> and</td>
<td>Lewis (1978)</td>
</tr>
<tr>
<td><em>Ubx</em>&lt;sup&gt;MX1&lt;/sup&gt; and <em>abdA</em>&lt;sup&gt;M1&lt;/sup&gt;</td>
<td>Above &amp; <em>Ubx</em>&lt;sup&gt;MX1&lt;/sup&gt; amorph</td>
<td>Sanchez-Herrero et al. (1985), recombined chromosome was a gift from D. Beuchle</td>
</tr>
<tr>
<td><em>AbdB</em>&lt;sup&gt;M1&lt;/sup&gt;</td>
<td>Amorph</td>
<td>Baker (1987)</td>
</tr>
</tbody>
</table>

For individual alleles see Sanchez-Herrero et al. (1985), recombined chromosome was a gift from D. Beuchle.
The FLP/ovoD method was used as per Rauskolb et al. (1993) to generate female germ-line clones to produce embryos lacking both maternal and zygotic exd contributions.

Following cloning, the $S10^{don1}$ enhancer trap (A gift from A. Gould and R. White) was submitted to Flybase as the allele $svp^{don1}$ (Flybase, 1999) and is also referred to as $svp$-$lacZ$ in the text. $rho$-$lacZ$ refers to the X81 line (Freeman et al., 1992a), an enhancer trap insertion approximately 100 nucleotides from the C1 specific insert $rhd^{act}$. $sal$-$lacZ$ refers to the $spalt$ enhancer trap allele A405 (Bellen et al., 1989). Heat-shock regimes for alleles $pb^{HSPB2-5}$ (Cribbs et al., 1995) and $abdA^{n7}^{PS}$ (Sampedro cited in Gonzalez-Reyes et al., 1992) were as described in Gould et al. (1997).

$EN^{on}$ embryos were created by crossing $wg^{CX4} en$-$GAL4/CyO x wg^{CX4} UAS$-$Arm^{S-10}/CyO$ (Gritzan et al., 1999). $WG^{on}$ embryos were formed by combining $en^{CX1}$, $wg$-$GAL4$ (Pfeiffer et al., 2000) and UAS-$Cl^{VP16}$ (A gift from C. Alexandre). The en-$GAL4$ UAS-$nlslacZ$ recombinant stock was a gift from JP Vincent. en-$GAL4$ (A. Brand, Wellcome CRC, Cambridge) was used to drive UAS-$en$ (Guillen et al., 1995) while prd-$GAL4$ (L. Fasano and C. Desplan cited in Yoffe et al., 1995) was used in combination with UAS-$wg$ (Lawrence et al., 1996). prd-$GAL4$ drives expression in alternate segments in the two en cells, the single wg cell and three cells anterior to this cell (see Figure 4.8 in section 4.2.5). It should be noted that in some segments this results in a three cell en stripe (C. Alexandre, unpublished data), presumably due to the expression of wg in the endogenous en cells.

For misexpression in the oenocyte and chordotonal organ precursors, en-$GAL4$ was used in combination with UAS-$EGFR^{DN}$ (O'Keefe et al., 1997), UAS-$sspi$ (Schweitzer et al., 1995) and UAS-$sal$ (Kuhnlein and Schuh, 1996).
The scheme used for P-element remobilization, to generate additional 
HS2mut lines, was essentially as described in (Roberts, 1998). The crossing 
scheme used to excise the lethal PlacZ transposon from S10dan1, and thus generate 
lethal excision lines, is given in Figure 2.1. All of these don excision alleles were 
balanced with a TM6,Tb balancer chromosome to reveal three classes of allele; 
viable, larval lethal and embryonic lethal. Embryonic lethal lines were analysed 
further by placing each don allele over a wildtype chromosome. These don/+ flies 
were intercrossed and embryos collected were allowed to develop for 48 hours at 
25°C. To determine the percentage lethality for each line, the number of empty 
and full chorions were counted.

2.2 Drosophila transformation

Transformation was carried out using standard procedures (Spradling, 1986) with 
the following modifications. The injection mix consisted of a transposase source, 
100ng/µl of pWC-TURBO helper DNA (A gift from JP. Vincent), and 300ng/µl of 
construct DNA (see next section), made up to volume with 0.1X PBS. Embryos 
were collected on yeasted grape juice-agar plates from yw hosts in a population 
cage (cn; ry hosts when injecting HZ50pL constructs). Following an approximately 
45 minute collection at 25°C, sodium hypochlorite (50 000 ppm) was poured onto 
the plate and embryos were dechorionated for 2 minutes. The contents of the 
plate were poured into a mesh basket and the embryos thoroughly washed with tap 
water. The mesh was removed from the basket and the embryos knocked from the 
mesh onto an agar plate. Here the embryos were aligned, all with the same AP 
orientation, and then transferred onto a line of dried glue on a slide. The glue was 
made by solubilising the adhesive from Scotch double-sided sticky tape in heptane.
Figure 2.1. S10\textsuperscript{don1} P-element excision

The crossing scheme used for excision of the S10\textsuperscript{don1} p[92] P-element insert and generation of stable lethal excision lines. The ry\textsuperscript{506} p[lacZ, ry] chromosome in the S10\textsuperscript{don1} stock was isogenised and females (always virgin in this scheme) crossed \textit{en masse} to males carrying the Δ2-3 transposase source (A). 200 male progeny were collected that had the Δ2-3 and the ry\textsuperscript{506} p[lacZ, ry] chromosomes, and used in single male crosses (B). The progeny of each cross were screened for ry males, the loss of the ry\textsuperscript{*} eye marker was used to detect an excision event and the presence of a ry\textsuperscript{506} Δp[lacZ, ry] chromosome (C). As ry progeny from a single cross in (B) might be derived from several independent excision events, single males were crossed to TM3/TM6 females in order to isolate each ry\textsuperscript{506} Δp[lacZ, ry] line (C). Non-TM3 progeny of (C) were crossed to the isogenised S10\textsuperscript{don1} stock (D), the presence of non-TM3/non-TM6 adult progeny indicated viable excision of the P-element. All lethal excision and a few viable excision lines, balanced over a TM3 chromosome, were crossed to TM3/TM6 females (E) and non-TM3 flies collected and inter-crossed (F). This created a TM6 balanced stock carrying the Tb larval marker to analyse the gross stage of lethality (see text).

In all of the recovered excision mutants, the ry gene in p[92] is disabled. In some lethal excision lines, both ry and lacZ were non-functional, suggesting the possible deletion of genomic DNA flanking both ends of the P-element insert. However, in many lethal lines (10/16) the lacZ gene remained functional. In such lines, if genomic DNA was deleted it would be flanking the ry\textsuperscript{*} end of the P-element insert.
The embryos, now aligned and fixed to a slide, were desiccated for approximately 10 minutes using a hairdryer (A ‘Philips Active Cool’ 1650 Watts on cool heat and low power settings) suspended 30 cm from the bench. Desiccated embryos were covered with 10S Voltalef oil (Elf Atochem) and injected. Injectants were incubated at 18°C in a moist box and upon hatching were transferred into a vial using a paintbrush (approximately 50 larvae per vial). Following isolation of transformants, X-gal stainings were performed on 10 lines per construct to establish the reproducible expression pattern and determine those lines to keep, balance and immunostain.

2.3 Generation of LNE cis mutations and transformation constructs

All DNA manipulations were performed according to Sambrook et al. (1989), except as described.

HS1mut, HS2mut, u/smut and d/smut versions of the mouse Hoxb4 LNE (see Figure 3.4A in section 3.2.3) were kindly provided by A. Gould and subsequently subcloned into EagI-Spel digested pBluescript II KS (pKS, Stratagene). Other directed mutations (see Figure 3.4A in section 3.2.3) were introduced into the LNE by inverse PCR (IPCR) mutagenesis, as described in McPherson et al. (1992). The IPCR template was the EagI-Spel LNE genomic fragment cloned into pKS (LNE-pKS). The following primer pairs were used; CC switch, GATGGTTTTCTGTATAATTCTCACATTTTTC and CATCACTTCTTTTCTTTAAATACGTATCC; PBCmut, CATTAATTTTTCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
polymerase (Stratagene) gave high fidelity IPCR amplification with blunt ends, abolishing the need for Klenow treatment of reaction products. To further increase the efficiency of the IPCRs, a Robocycler PCR machine (Stratagene) was used with a 60-71°C gradient of annealing temperatures across 12 reactions. The highest annealing temperature that produced significant product was chosen. IPCR bands were gel purified, ligated and transformed according to the published protocol (McPherson et al., 1992). Colonies were picked, cultured and DNA was purified by QIAprep spin Miniprep (Qiagen). Sdi-Dral diagnostic restriction enzyme digests were used to check the integrity of the IPCR-derived LNE mutant clones in pKS (LNE\textsuperscript{mut}-pKS). Digest positive clones were sequenced in both directions with the pKS primers KS and T7. Having not been sequenced previously, the u/s\textsuperscript{mut} and d/s\textsuperscript{mut} constructs were similarly sequenced from within pKS. Following a Perkin Elmer protocol, an ABI Prism Dye Terminator Cycle Sequencing kit was used. Sequencing was carried out on an ABI Prism 377 automated sequencer and data was analysed using the ABI Prism sequencer software.

Two copies of each mutated LNE sequence (LNE\textsuperscript{mut}) were sequentially cloned into pKS as a direct repeat. LNE\textsuperscript{mut}-pKS was Eagl-Spel digested, Klenow treated and the approximately 370bp insert fragment purified from a 1% agarose gel (Geneclean). The vector backbone was produced by SpeI-Sall digestion of LNE\textsuperscript{mut}-pKS followed by Klenow treatment. The blunt vector was purified from solution (Geneclean), Alkaline Phosphatase treated and purified from a 1% agarose gel (Geneclean). Vector and insert fragments were ligated and transformed, colonies picked, cultured and DNA isolated by QIAprep spin Miniprep.
Eagl-Spe1 digests determined those clones containing a direct repeat of the LNE\textsuperscript{mut} element (thus 2xLNE\textsuperscript{mut}-pKS).

Two copies of HS\textsuperscript{1mut} or HS\textsuperscript{2mut} were cloned from pKS into the Drosophila transformation vector HZ50pL, as described in Gould et al. (1997). All other cis mutations in the LNE, including a control wildtype version of the LNE, were cloned via a similar strategy into pWZ50pL (A gift from T. Gutjahr). pWZ50pL contains the polylinker sequence and hsp70 promoter-lacZ fusion of the HZ50pL HindIII-XbaI fragment (Hiromi et al., 1985), together with the hsp70 promoter-miniwhite fusion from the pW6 XbaI-HindIII fragment (Klemenz et al., 1987). DNA was prepared for injection using a QIAGEN Plasmid Maxi kit.

2.4 \textit{S10\textsuperscript{donf}} cloning strategy

Due to the absence of a plasmid origin of replication in the p[A92] P-element insert of \textit{S10\textsuperscript{donf}}, an IPCR strategy was used to clone the \textit{S10} locus. A phenol-chloroform/proteinase-K protocol (Roberts, 1998) was used for the isolation of Drosophila genomic DNA from \textit{yw} and isogenised \textit{S10\textsuperscript{donf}}/TM3 stocks. With the aim of IPCR amplifying sequences flanking both the 5' and 3' ends of the P-element insert, genomic DNA was digested with restriction enzymes which cut p[A92] close to the 5' end (Asel, Csp6I, EcoRI and Clal) or close to the 3' end (Asel, Csp6I, BglII and HindIII). 3.2\textmu g of genomic DNA was digested over night in a 140\mu l reaction with between 140-250 Units of enzyme, to ensure complete digestion. The digestion products were then purified from solution using a QIAquick Gel Extraction kit (Qiagen). Following resuspension in 100\mu l of dH\textsubscript{2}O, 7\mu l were run on a 1% agarose gel to confirm digestion. The remaining digest products were self-ligated in a large volume reaction (200\mu l) to prevent multimerisation of DNA.
fragments. These were incubated at 13°C overnight and ligation products subsequently purified from solution using the QIAGenous Gel Extraction kit. The ligated genomic DNA was added to a large 250μl IPCR mix that was aliquotted into 12 x 20μl PCR reactions. These 12 reactions were placed across an annealing temperature gradient of 56-67°C. The following PCR protocol was used: 95°C for 1 min, (95°C for 1 min, 56-67°C for 1 min, 72°C for 6 min) x 30 cycles, 72°C for 10 min. The 5'P primer ccttccctctcaacaagcctgcactggag was used in combination with; tggcaattttgctgcaagctgtgactggag (Asel); ctttaatgcattcgcagtggaaggctgcacc (Csp6I); ggtaccagaaatgctgagtttagagcagcag (EcoRI); or cttcgggtaacagttagttttctttattgcaagg (ClaI). The 3'P primer tcgcgtgcctcactcagactcaatcagacac was used in combination with: tcggattaaccctacctagctgccgtgag (Asel, Csp6I and HindIII); or cgacaaagccctttatgaggcacccaaag (BglII). Products from both S10don1 and YW control IPCRs were run on 1% agarose gels and band sizes compared to eliminate products formed by non-specific primer annealing. S10don1-specific IPCR products, amplified using the highest annealing temperature, were cut from gels and purified by Qiagenous Gel Extraction. In total four fragments were isolated; 5'P EcoRI (1.7 kb), 3'P Asel (3.1 kb), 3'P Asel (0.9 kb), 3'P HindIII (1.0 kb), and subcloned into Invitrogen's Zero Blunt TOPO vector. Following transformation and culturing of picked colonies, DNA was isolated using the Qiagenous spin Miniprep kit. Two clones per fragment were commercially sequenced (Oswel) using the T7 and SP6 primers. A sequence similarity search (BLASTN) was carried out for each clone sequence with those in the Berkeley Drosophila Genome Project (BDGP) data set (http://www.fruitfly.org/blast/).
2.5 Embryo immunolabelling and X-gal reactions

Embryos were collected at 25°C for 15 hours, aged for 2.5 hours and then dechorionated as previously described. Embryos were immunofixed for 30 min at room temperature in 4% paraformaldehyde in PEM buffer (1 mM MgSO₄, 2 mM EGTA, 0.1 M PIPES, pH 6.9) with heptane, and devitalised in methanol (protocol adapted from Mitchison and Sedat, 1983). Immunostaining was essentially as per Aspland and White (1997). Primary antibodies used were; rabbit anti-β-galactosidase (Cappell) at 1:6000 for fluorescent and 1:12000 for HRP detection, mouse anti-β-galactosidase (Promega) at 1:1000, anti-DFD (BabCo) at 1:400, anti-SCR (Glicksman and Brower, 1988) at 1:1000, rabbit anti-EN (DiNardo et al., 1985) at 1:100, 4D9 anti-EN (Developmental Studies Hybridoma Bank) ascites at 1:500, 4D4 anti-WG (Developmental Studies Hybridoma Bank) at 1:10, 22C10/anti-FUTSCH (Fujita et al., 1982; Hummel et al., 2000) at 1:50, anti-RHO (Sturtevant et al., 1996) at 1:600, anti-SAL (Kuhnlein et al., 1994) at 1:30 or anti-SAL (Barrio et al., 1999) at 1:500 on 15 min fixed embryos. In all non-fluorescent stainings, the Vectastain ABC kit (Vector Laboratories) was used according to manufacturers instructions for HRP immunochromistry. Stained embryos were photographed using a Zeiss Axiophot 2 with Nomarski DIC optics. Alexa 488/594 conjugated secondary antibodies (Molecular Probes) were used at 1:100 for immunofluorescence. Fluorescent embryos were mounted in Vectorshield medium (Vector Laboratories) and viewed by confocal microscopy (Leica TCS-NT microscope system), except in Figure 4.4 (section 4.2.3) where pictures were taken on the Zeiss Axiophot 2. To show true overlap (yellow) between two proteins Figures 4.1A and D-F, 4.5, 4.6B-F, 4.7 (main panels), 4.10, 4.12C and 5.3D are all
either single confocal sections or multiple sections through a z-axis of less than 1
cell diameter. The z-axis reconstruction of confocal data was obtained using the
Imaris 3.0 software on a Silicon Graphics Unit.

X-gal stainings were performed as per Gould (1990) with the modifications
described. Embryos were collected in vials following the timing given for
immunolabelling, and dechorionation was in vials as previously described.
Embryos were fixed for 25 minutes in heptane saturated with an equal volume of
2.5% glutaraldehyde in PBS. Stained embryos were mounted in 50% glycerol (in
PBS with 10mM sodium azide).

All photographic figures were compiled using Adobe PhotoShop 5.0.
Embryonic stages of development are according to Campos-Ortega and
Hartenstein (1997).
CHAPTER THREE

Molecular Mechanisms of *Hox* Gene Function
3.1 INTRODUCTION

Highly divergent homeodomain proteins display similar DNA binding properties *in vitro*, and yet somehow distinguish between different targets *in vivo*. This promiscuous *in vitro* DNA binding raises the question of how different HOX proteins naturally discriminate between their respective targets. Expanding on this question, how is the regulation of a single *Hox* target gene fine-tuned to produce a precise spatio-temporal expression pattern, restricted to a subset of cells expressing the HOX activator? The characterisation of *Hox* autoregulatory elements has provided great insight into the molecular mechanisms of *Hox* target gene regulation. Five autoregulatory elements have been analysed in detail: two *Hox* group-1 targets (Lab550, Grieder et al., 1997; repeat 3, Popperl et al., 1995 and Chan et al., 1997) and three *Hox* group-4 targets (NAE and EAE, Bergson and McGinnis, 1990 and Lou et al., 1995; CR3, Gould et al., 1997). Described in this chapter is the further analysis of conserved region 3 (CR3), which is contained in the late neural enhancer (LNE) of the murine *Hoxb4* gene (Gould et al., 1998).

Expression of *Hoxb4*, a Deformed orthologue (see Figure 1.2 in section 1.3), is initiated in the developing mouse hindbrain through activation of the early neural enhancer (ENE), a process which requires retinoid signalling (Gould et al., 1998). Subsequent activation of the LNE by HOXB4 itself (autoregulation), and other HOX proteins (cross-regulation), maintains *Hoxb4* expression and directs a sharp anterior limit of expression at the junction between rhombomeres 6 and 7 (r6/7 boundary, Gould et al., 1997; Gould et al., 1998; Stern and Foley, 1998).
Rhombomeres are the metameric units of the vertebrate hindbrain (Lumsden and Krumlauf, 1996), which bear some resemblance to compartments in *Drosophila*.

Homologous sequences to the mouse LNE of around 110bp are found in the *Hox* complexes of chick and pufferfish (Aparicio et al., 1995; Gould et al., 1997). Such extensive sequence conservation does not extend to *Drosophila* (A. Gould, unpublished). However, the neural (NAE) and epidermal (EAE) autoregulatory elements of *Dfd* could be considered functionally equivalent, possessing a similar *Hox* group-4 autoregulatory function. Interestingly, the LNE does contain a consensus 10bp HOX-PBC binding site (Gould et al., 1997 and see Figure 3.4 in section 3.2.3), which is almost identical to DFD-PBC sites located in the NAE and EAE (Chan et al., 1997). This HOX-PBC site in the LNE (termed HS2) lies 11bp from a second site (termed HS1), which consists of only the core TAAT HOX recognition sequence (Gould et al., 1997). Both HOX binding motifs must be mutated in order to lose all LNE-mediated expression from reporter constructs in the mouse (Gould et al., 1997). Hence, HS1 and HS2 are essential components of the LNE.

Previous experiments utilising *Drosophila* embryos transgenic for an LNE-minimal promoter-*lacZ* construct produced reporter expression in only a subset of cells within the *Dfd* domain. Expression is restricted to the most ventral and posterior cells of the maxillary segment (Figure 3.1A-B). LNE activation is also observed in anterior cells of the thoracic segments (T1-3), and is again ventrally restricted (Figure 3.1A-B). The enhancer is thus activated within three *Drosophila* *Hox* expression domains, *Dfd* in the maxillary segment, *Scr* in T1 and *Antp* in T2 and T3 (Figure 3.1A). Gould et al. (1997) used *Hox* loss-of-function and ectopic expression assays in the fly to show that the LNE is positively regulated by
Figure 3.1. *Hox* activation and repression of the LNE in *Drosophila*

In this and all subsequent figures anterior is to the left and dorsal upwards, unless otherwise stated.

(A) A diagram showing the restricted activation of the LNE (green) in a subset of cells which express the *Hox* genes *Dfd, Scr* and *Antp*.

(B-C) Stage 13 embryos transgenic for an LNE-*lacZ* construct, immunostained against β-gal. (B) A ventrolateral view of a wildtype embryo. (C) An embryo carrying a hs-*abdA* transgene showing residual LNE-*lacZ* activation in the maxillary segment (arrowhead) following heat-shock treatment.

The mandibular (Md), maxillary (Mx), labial (La) and thoracic (T1, T2, T3) segments are indicated.
group-4 (Dfd), group-5 (Scr) and group-6 (Antp) Hox genes. The LNE is not regulated by the group-1 Hox gene labial, whilst ectopic expression of Ubx, the group-7 Hox gene, resulted in repression of LNE-mediated reporter expression. In summary, the LNE acts as a Hox group-4 autoregulatory element in both the mouse and the fly and is under the control of both positive and negative Hox cross-regulatory interactions.

Detailed in this chapter is the continued dissection of the LNE to address how this model Hox target gene is regulated at the transcriptional level. This study was carried out in Drosophila rather than mouse for three reasons. Primarily Drosophila was chosen because of the sophisticated genetics that are available with this organism. Secondly, the mouse has nested Hox expression domains that overlap extensively in posterior regions of the embryo. In contrast, where Hox genes do overlap in Drosophila, only the more posteriorly expressed locus tends to be active. Thus, the distinct domains of Hox expression in the fly embryo provide a simple system for determining which Hox genes activate the LNE. Finally, Drosophila also benefits from having a single Hox cluster, hence the phenomenon of partial functional redundancy between Hox paralogues encountered in the mouse is not an issue (Gould et al., 1997).
3.2 RESULTS

3.2.1 LNE expression in the maxillary segment is associated with the common salivary duct

*Drosophila* embryos exhibit LNE-lacZ expression in ventral ectodermal cells of the maxillary and thoracic segments from stage 13 onwards (Figure 3.1B). The anterior cells of the thoracic segments continue to express lacZ and remain in the ectoderm, differentiating into epidermal cells (Figure 3.2A-C). However, LNE-lacZ positive ectodermal cells in the maxillary segment begin to invaginate during stage 14 and become associated with the common duct of the salivary glands. To understand this process a description of salivary morphogenesis is necessary (also refer to Figure 3.3). The salivary glands/ducts are formed from the salivary primordia by a progressive invagination process. In stage 11 embryos, the salivary primordia encompass most of the ventral domain of parasegment two (Kuo et al., 1996; Campos-Ortega and Hartenstein, 1997), and thus express Scr. The salivary placodes constitute the majority of the salivary primordium and form the glands themselves (Figure 3.3A and Panzer et al., 1992). Presumptive salivary duct cells are found ventrally (Kuo et al., 1996) and can be divided into common and individual duct precursors, respectively located anteriorly and posteriorly along the ventral midline (Figure 3.3A and Jones et al., 1998). The presumptive gland cells begin invaginating at late stage 11 from single positions on both sides of the embryo (Figure 3.3A-B and Panzer et al., 1992). The final gland precursors to invaginate remain in contact with cells that will form the distal ends of the individual ducts (Figure 3.3C). As the individual ducts lengthen, the two ventrolateral points of
Figure 3.2. LNE-positive cells invaginate with the common salivary duct

LNE-\textit{lacZ} immunostaining in wildtype (A-C and G) and \textit{pb Dfd} double mutant embryos (D-F).

(A-C) At late stage 13 (A) the maxillary LNE-\textit{lacZ} expression (closed arrowhead) is observed anterior to the invaginating common duct (red arrow) of the salivary glands. By late stage 14, head involution is almost complete, as is the invagination of the common salivary duct. The LNE-\textit{lacZ} positive cells are just beginning to invaginate at this stage (B). Following invagination (stage 16), LNE-\textit{lacZ} expressing cells are mainly located posterior to the common salivary duct (C and see G below).

(D-F) LNE-\textit{lacZ} expression is absent from the maxillary/duct region in \textit{pb Dfd} double mutants (compare with closed arrowheads in A-C), whilst thoracic \textit{lacZ} expression is still detected (open arrowheads). Early common duct development appears to be normal in \textit{pb Dfd} mutants (red arrows in D, E compared to A, B). At stage 16 it is difficult to locate the common duct and there is no obvious connection from the salivary glands to the pharynx (ph) in these double mutants.

(G) A high magnification ventral view of the common duct of the salivary glands.

Only the posterior cells surrounding the lumen of the common duct (lu) are LNE-\textit{lacZ} positive.
**Figure 3.3. Cell movements during salivary gland development in *Drosophila***

A ventral view of the progressive invagination process by which the salivary glands form.

(A) The most posterodorsal cells in the salivary placode begin the invagination process at the end of stage 11. As cells are internalised, the point of invagination moves anteriorly (black arrow).

(B-C) As the gland cells invaginate the ventrally located duct precursors rearrange by convergence and extension (red and blue arrows in B) to form two parallel rows of cells (C), the posterior of which form the individual ducts while the anterior row produces the common duct. The most proximal gland cells remain in contact with the individual duct precursors such that when these internalise a continuous lumen is formed between the individual duct and gland structures. The lateral points of invagination move ventrally (black arrows in C) as the individual ducts lengthen (see D).

(D-E) Finally the two points of invagination meet at the ventral midline and the two individual ducts fuse to form the common duct (D). Here a second convergence and extension event results in the formation of the common duct (E).

Compiled from Panzer et al. (1992), Kuo et al. (1996), Campos-Ortega and Hartenstein (1997), Jones et al. (1998).
Salivary gland precursors
Individual duct precursors
Common duct precursors
Point of invagination
invagination move towards the ventral midline and fuse (Figure 3.3C-D). Invagination continues as the common duct of the salivary glands forms (Figure 3.3E). At its most proximal end the common duct appears to remain in contact with ectodermal cells which subsequently form the floor of the pharynx.

Examination of the LNE-\textit{lacZ} expression pattern during salivary gland formation revealed that the \textit{lacZ}-positive cells in the maxillary segment are immediately anterior to the invaginating common duct (Figure 3.2A). These cells appear to be the last to invaginate, and become associated with the proximal end of the common duct, near to its junction with the pharynx (Figure 3.2B-C). Common duct invagination overlaps in time with the more complex morphogenetic movements of head involution. This is where tissues are internalised and the anterior of the embryo is reorganised, resulting in development of the larval head (Jurgens and Hartenstein, 1993). During this process the pharynx is formed, to which the common duct makes its connection (Kuo et al., 1996). Following these tissue movements, LNE-\textit{lacZ} cells are mainly found in a final position posterior to the lumen of the common duct (Figure 3.2 C, G). In cross-section, these duct-associated cells have a bottle shape that is reminiscent of the invaginating salivary gland precursors (Myat and Andrew, 2000). It has been proposed that the basal migration of nuclei, and subsequent constriction of the apical surface membrane, are prerequisites for the invagination of salivary gland cells. Similar morphological processes may be necessary for the internalisation of LNE-\textit{lacZ} positive duct cells, and would also explain their bottle shape. As these cells are not fully integrated into the epithelium of the common duct, this suggests that they may not be epithelial in nature. Given the similar shape of these LNE-\textit{lacZ} cells to the secreting gland cells, and as the constricted apical surface is inserted into the floor of the
pharynx (Figure 3.2C), it is possible that these LNE expressing cells are also secretory, emptying directly into the pharynx.

3.2.2 A role for Deformed during salivary duct development?

*Deformed* is essential for LNE activation in the maxillary segment of the *Drosophila* embryo (Gould et al., 1997). The group-2 *Hox* gene *proboscipedia* (*pb*) is also expressed in the maxillary segment, overlapping with the *Dfd* domain (Pultz et al., 1988). Thus, to further explore the requirement of *Hox* genes for maxillary LNE expression, *pb* was investigated. Embryos mutant for *pb* displayed normal expression of an LNE-*lacZ* construct (data not shown). Similarly there was no change in LNE activation following a ubiquitous pulse of PB from an Hsp70-*pb* transgene (data not shown). In conclusion, *pb* appears to have no input into the regulation of the LNE. Hence the loss of maxillary/duct associated LNE-*lacZ* expression observed in *pb Dfd* double mutants (Figure 3.2D-F), is likely to be due to the lack of DFD alone. Interestingly, duct development itself seems normal in these double mutants until stage 16, when the usual tubular connection between the common duct and the pharynx is not observed (Figure 3.2F). Two scenarios are compatible with these observations, either the common duct remains joined to the pharynx but its lumen is pinched closed near to the pharynx, or there is simply no connection between the duct and the pharynx. This raises the possibility that the LNE-positive cells normally play a critical role in connecting the common duct to the pharynx, and might be mis-specified or absent in *Dfd* mutants.

Previously *Scr* was shown to be necessary and sufficient for salivary gland/duct development (Panzer et al., 1992). Data presented here suggests that
this is not the full story and that there may be a novel role for *Dfd* in connecting the common duct to the pharynx.

### 3.2.3 HS1 and HS2 are targets for regulation by multiple *Hox* genes

Previous studies in *Drosophila* showed LNE autoregulation by *Dfd*, positive cross-regulation through *Scr* and *Antp*, and negative cross-regulation by the BX-C gene *Ubx* (Gould et al., 1997). To investigate the role of *abdA*, this abdominal *Hox* gene was ubiquitously expressed from a heat inducible promoter. LNE-*lacZ* expression was severely reduced in this experiment (Figure 3.1C), a similar result to that observed on misexpression of *UBX* (Gould et al., 1997). Taken together with results from the previous section, and those of Gould et al. (1997), the *Hox* regulation of the LNE can now be summarised as follows. There is no input from groups 1-2, activation by groups 4-6, and apparent repression by groups 7-8.

Genes from three different *Hox* groups (4, 5 and 6) activate the LNE, but do these inputs act via the same or different HOX binding sites? The LNE contains two essential HOX binding sites, HS1 and HS2. To assess which site(s) the HOX activators go through, the individual contributions of HS1 and HS2 were examined. Due to the non-overlapping domains of HOX activity, the loss of regulation by a *Hox* gene can be inferred from the disappearance of LNE expression within that specific *Hox* domain. To test the role of HS1 and HS2, the core TAAT HOX recognition sequences of these sites were individually mutated (see Figures 3.4 and 3.5). *Drosophila* embryos transgenic for an LNE-*lacZ* construct carrying only the HS1 mutation (HS1^mut^, Figure 3.4A) displayed normal maxillary but reduced thoracic activation of the LNE (Figure 3.5B). In contrast, mutation of HS2 alone (HS2^mut^, Figure 3.4A) abolished LNE-mediated *lacZ* expression in the maxillary
Figure 3.4 LNE sequence conservation and transgenesis constructs.

(A) Sequence alignment of a short section of the mouse LNE with homologous regions identified in chick and pufferfish (Fugu rubripes). Identical nucleotides are indicated with asterisks and the two highly conserved HOX binding sites (HS1 and HS2) are in bold. The mutation of HS1 (construct F) is predicted to abolish HOX binding to this site while the mutation incorporated into HS2 (construct G) was previously shown to prevent HOXB4 binding to this site in vitro (Gould et al., 1997). To assess the function of conserved regions outside of these HOX sites, mutations, deletions and additional bases were incorporated into the full length LNE (constructs M-T).

(B) The bipartite HOX-PBC consensus sequence is shown where the two central specificity determining bases are marked as NN. The HS2 site of the LNE, a consensus group-4 HOX-PBC site, is below. Similar sites with TA as their specificity determining nucleotides were identified in the Dfd NAE and EAE enhancers in Drosophila (Bergson and McGinnis, 1990; Lou et al., 1995).
Figure 3.5 Reduced LNE activation resulting from mutation of HS1 or HS2

Lateral views of stage 13 embryos carrying wildtype (A) or mutated (B-D) LNE-lacZ constructs and immunostained for β-gal.

(B) HS1\textsuperscript{mut} transgenic embryo with normal maxillary but reduced thoracic lacZ levels.

(C) Mutation of HS2 (HS2\textsuperscript{mut}) results in the absence of LNE activation in the maxillary segment and reduced thoracic expression. In addition, ectopic LNE-lacZ expression is observed in abdominal segments (asterisks).

(D) An embryo transgenic for the CC switch construct showing very weak residual LNE activation in the maxillary segment. Although the LNE contains a LAB-PBC site, it is not activated in the \textit{lab} expression domain which includes the anterior head region and the midgut (Diederich et al., 1989).

In this and subsequent figures in this chapter, filled black arrowheads and open arrowheads point out maxillary and thoracic lacZ expression respectively. See Appendix I for an expression summary for all LNE-lacZ constructs.
segment, and reduced the thoracic expression (Figure 3.5C). A similar complete loss of maxillary LNE expression was previously described in Dfd mutant embryos (Gould et al., 1997). Therefore, given the requirement of HS2 but not HS1 for the Dfd-mediated maxillary activation of the LNE, I can conclude that Dfd/group-4 Hox genes act solely through the HS2 site. This is consistent with the HOXB4 in vitro binding data of Gould et al. (1997), where strong specific in vitro binding of HOXB4 to HS2 was observed, but only very weak binding to HS1. Taken together, these results strongly suggest that, in the context of the mouse CNS, Hoxb4 autoregulation via the murine LNE is through HS2 but not HS1.

Gould and colleagues (1997) previously reported a total loss of thoracic LNE expression in embryos carrying mutations in both Scr and Antp. Here reduced reporter expression was observed in the thoracic segments in both HS1^{mut} and HS2^{mut} transgenic embryos. Taken together this suggests that Scr/group-5 and Antp/group-6 Hox genes similarly activate the LNE in thoracic segments through both HS1 and HS2. Interestingly, mutation of the HS2 site also produced ectopic lacZ expression in abdominal segments (Figure 3.5C). This is the region of the embryo where Ubx/abdA appear to act as LNE repressors, therefore it is likely that any repression by these two BX-C genes is mediated by HS2. In summary, the work presented here indicates that multiple Hox genes target the LNE through HS1 and HS2. Each site shows a qualitatively different Hox specificity, with DFD acting via the HS2 site only, whilst Scr and Antp inputs are mediated by both HS1 and HS2. In addition, Ubx and/or abdA appear to negatively cross-regulate the LNE in the abdomen through HS2.

Unlike HS1, the HS2 site is a consensus HOX-PBC binding site. Previous in vivo and in vitro studies suggested that the two central nucleotides in such sites
could contribute to HOX specificity (Chan and Mann, 1996; Chang et al., 1996; Lu and Kamps, 1996; Chan et al., 1997). In accordance with the NAE and EAE Drosophila Dfd autoregulatory elements (Bergson and McGinnis, 1990; Lou et al., 1995), these specificity determining bases in HS2 are TA. HOX-PBC sites with central nucleotides CC are preferentially bound by LAB (Chan et al., 1997; Grieder et al., 1997). Repeat 3, the 20bp minimal murine Hoxb1 autoregulatory element, contains such a LAB-PBC site (Popperl et al., 1995). In Drosophila, changing the two central bases from CC to TA switches the in vivo specificity of Repeat 3 from a LAB to a DFD responsive element (Chan et al., 1997). In the context of the LNE, I asked whether such a change could alter the specificity of this approximately 370bp enhancer, rather than that of a minimal HOX-PBC site. When the central two nucleotides within the HS2 site of the LNE were switched from TA to CC (CC switch, Figure 3.4A), dramatic reduction in reporter gene expression was observed in both maxillary and thoracic segments (Figure 3.5D). The surprising lack of ectopic lacZ in lab territory shows that LAB is unable to activate the CC switch construct, despite the presence of a LAB-PBC site. Furthermore, as a low level of residual maxillary expression is still observed, in contrast to HS2mut embryos, it seems likely that DFD-mediated activation of the LNE via HS2 has not been completely abolished. In conclusion, this switch experiment suggests a role for other factors, probably acting at sites remote from HS1 and HS2, in modulating the HOX target responses.

3.2.4 Remote sequences and the restriction of the HOX response

LNE activation by Dfd, Scr and Antp is restricted to a small subset of those cells that express these Hox genes. Even following ubiquitous expression of any one of
these genes, ectopic LNE activation is limited to small patches in the embryo (Gould et al., 1997). This poses the question of how LNE activity becomes restricted to only a subset of cells expressing the Hox activators? Given the high degree of sequence conservation in the LNE outside of HS1 and HS2 (Figure 3.4A), I screened these regions for mutations that disrupt restriction. Transgenic lines for the various mutated LNE-/lacZ constructs were generated and examined for alterations in the wildtype expression pattern.

To investigate the functional significance of the conserved region between the HS1 and HS2 sites, five of the eleven bases in this linker region were deleted (linker\textsuperscript{5}, Figure 3.4A). Embryos transgenic for a linker\textsuperscript{5}/lacZ construct displayed largely normal thoracic expression (Figure 3.6F). However, maxillary reporter expression was somewhat attenuated in these embryos, and in addition the LNE was ectopically activated in A1 (Figure 3.6F). Ectopic activation of the LNE in A1 suggested that the linker region is important for the AP restriction of this element. However, deletion of linker bases does not distinguish the potential requirement for linker sequence conservation from that for precise spacing between the two HOX binding sites. To clarify this issue, two new LNE-/lacZ transgenic lines were generated. The role of sequence conservation was investigated by mutating six bases within the linker (linker\textsuperscript{mut}, Figure 3.4A), while preserving its length. This resulted in a wider than normal T1 stripe (Figure 3.6D), while expression was as normal in the maxillary, T2 and T3 segments. To test the requirement for precise spacing between HS1 and HS2 five additional bases were incorporated into the linker region (linker\textsuperscript{r5}, Figure 3.4A). Embryos carrying this construct also displayed more thoracic expression than normal, but in different domains to that observed in the linker\textsuperscript{mut} transgenics. Firstly, extensive ectopic LNE activation was observed
Figure 3.6 Conserved regions outside of HS1/HS2 have a role in LNE function

Stage 13 embryos transgenic for wildtype (A) or mutated (B-F) LNE-lacZ constructs, all immunostained for β-gal.

(B) Mutation of a conserved region upstream of HS1 (u/s^mut^) results in greatly reduced maxillary and thoracic LNE-mediated lacZ expression.

(C) Embryos transgenic for the downstream mutant construct (d/s^mut^) display a total absence of thoracic expression and attenuated maxillary LNE activation.

(D) A ventrolateral view of an embryo carrying an LNE construct with a mutated linker region (linker^mut^) showing a posterior expansion in the T1 stripe of LNE expression. Also compare to the ventrolateral view of the wildtype LNE pattern in Figure 3.1B.

(E) An embryo transgenic for an extended linker construct (linker^ext^) showing three domains of ectopic LNE activation. Additional β-gal was detected in A1 (asterisk), in posterior cells of T1 and T2 (red arrowheads) and dorsally in anterior cells of T1 and T2 (marked by red dots). Compare to the ventrolateral view of the wildtype LNE pattern in Figure 3.1B.

(F) Deletion of five bases from the linker region (linker^del^) resulted in reduced maxillary but normal thoracic LNE-lacZ expression. Ectopic LNE activation was also observed in the first abdominal segment (asterisk).
dorsally in anterior T1 and T2, in stripes that almost fused with the normal ventral stripes of expression (Figure 3.6E). Thoracic activation was also observed in posterior T1 and T2. Additionally, LNE-mediated reporter expression was also observed in anterior A1 (Figure 3.6E), akin to linker\textsuperscript{a} transgenics. In summary, altering either the length or the sequence composition of the linker results in a loss of the normal DV and AP intrasegmental restriction of LNE activity. Therefore this sequence, remote to the HOX binding sites, imposes a negative influence on the LNE to restrict the extent of \textit{Hox} activation to subsets of the full \textit{Hox} expression domain.

In addition to the conservation of HS1, linker and HS2 sites in vertebrates, flanking sequences are also highly conserved (Figure 3.4A). To investigate whether these sequences contribute to the restricted activation of the LNE, mutations were introduced upstream (u/s\textsuperscript{mut}) and downstream (d/s\textsuperscript{mut}) of HS1/HS2. Embryos transgenic for the u/s\textsuperscript{mut} construct showed greatly reduced levels of both maxillary and thoracic \textit{lacZ} expression (Figure 3.6B). Mutation of the conserved region downstream of HS2 had an even more pronounced effect, completely abolishing thoracic activation of the LNE and leaving only weak residual maxillary expression (Figure 3.6C). This indicates that the downstream region modulates the HOX responses of both HS1 and HS2. Reduced LNE activity, resulting from either flanking mutation, shows that these conserved regions impose a positive influence on the LNE. This contrasts with the negative role of the linker region.

### 3.2.5 EXD is required for the \textit{in vivo} activation of the LNE

The HS2 site in the LNE, one of two sites essential for enhancer function, is a HOX/PBC consensus site (see Chapter Introduction). However, previous \textit{in vitro}
analysis failed to detect EXD or PBX-1 binding with HOXB4 on HS2-containing oligonucleotides (Gould et al., 1997). On other group-4 Hox sites, however, it has been shown that EXD can function in the absence of co-operative binding by stimulating DFD recruitment to DNA (Zeng et al., 1994; Chan et al., 1997). Thus to further investigate the in vivo requirement for cofactors in LNE regulation, embryos lacking maternal and zygotic exd contributions were generated using the FLP/ovoD germ-line clone method (see Materials and Methods). In these mutants, LNE activity was severely reduced, with only a small amount of residual lacZ expression remaining (Figure 3.7B). This result indicates that, as for other Hox autoregulatory elements, exd/Pbx are required for full activity.

To investigate any potential exd/Pbx independent component to LNE function, I decided to map the weak residual LNE expression remaining in exd mutants. As exd null embryos display very poor morphology and failure of head involution, it is difficult to clearly distinguish segment identity. For this reason, an antibody against SCR was used as a landmark for the labial and T1 segments. Residual LNE activity in exd mutants was found to be both within, anterior to and posterior to SCR territory (data not shown). Thus, it appears that the weak activity of the LNE in exd null embryos is within the normal maxillary and thoracic domains of LNE expression. Weak ectopic LNE-lacZ expression was also observed in abdominal segments of exd mutants (Figure 3.7B), similar to that observed when HS2 was mutated (Figure 3.5C), suggesting that exd-mediated repression via HS2 in these segments had been compromised. Residual expression in the maxillary segment in exd mutants was unexpected because DFD only activates the LNE through HS2, the DFD-EXD bipartite site. Thus these results suggest that Dfd can weakly activate the LNE through the HS2 site even in the absence of EXD.
Figure 3.7 The LNE is almost fully exd-dependent

Lateral views of germ band retracted embryos immunostained for β-gal.

(A, C) Embryos lacking only the maternal contribution of exd (m-z+) show normal activation of LNE (A) and HS1mut (C) reporter constructs.

(B, D) Very weak residual expression (black line) is observed from LNE (B) and HS1mut (D) constructs in exd null embryos (m-z-). Ectopic abdominal activation (asterisks) is also observed for the wildtype LNE construct in embryos deficient for exd.

(E) Mutation of an essential nucleotide in the EXD site in HS2, and that of a cryptic EXD site in HS1 (PBCmut) results in severely reduced maxillary and thoracic LNE activation.
To confirm this possibility, the individual HS2 site (HS1mut) was placed in an exd mutant background. As for the wild type LNE, the majority of lacZ expression was lost, but again residual weak expression remained in the maxillary and thoracic segments (Figure 3.7D and data not shown). In summary, the HS2 HOX-PBC site activates the LNE mainly through an exd/Pbx dependent pathway, although it also appears to be able to provide rather minor activity in the absence of exd. The true significance of the exd dependent expression is difficult to assess as exd mutants have such severe morphological abnormalities that they may lack many of the cell types where the LNE is expressed. For this reason I looked for another way of compromising EXD function.

A single GC base pair in the EXD binding portion of the bipartite HOX-EXD site has been shown to be essential for the in vitro and in vivo function of EXD (Chan and Mann, 1996; Chan et al., 1997; Grieder et al., 1997). Mutating this nucleotide pair thus abolishes EXD action at the DNA level, enabling examination of the role of exd in morphologically normal embryos. Such a mutation was introduced into the HS2 site and a similar mutation was also made in HS1, when more detailed sequence analysis revealed a possible cryptic EXD site. Transgenic lines were generated for an LNE reporter construct containing both point mutations (PBCmut, Figure 3.4A). Stage 13 embryos carrying this construct displayed greatly reduced LNE activity within the normal domain (Figure 3.7E), a very similar result to that obtained with exd mutants, although ectopic expression was not observed in the abdomen. Thus despite the lack of in vitro EXD binding (Gould et al., 1997), this result suggests that EXD binding is required in vivo for Hox-mediated activation of the LNE. It remains to be seen whether other proteins, absent from the in vitro assays, assist EXD binding to DNA.
The nuclear localisation and thus activation of EXD is controlled by hth (see General Introduction). In embryos homozygous for the hypomorphic allele hth<sup>5604</sup>, both the LNE (Figure 3.8B, D) and the HS1<sup>mut</sup> constructs (Figure 3.8F) were activated within their normal maxillary and thoracic domains. Interestingly, an ectopic domain of LNE-lacZ expression was also observed for both constructs. The ectopic expression was anterior to the normal domain of LNE activation, but still fell within Dfd territory in the posterior mandibular segment. Considering the interdependence of hth and exd, the presence of a normal expression pattern in hth<sup>5604</sup> mutants would seem to contradict the loss of expression in exd null embryos (see Figure 3.7). To try to resolve this discrepancy, a stronger allele of hth (hth<sup>64-1</sup>) was tested. In this mutant background expression was severely reduced, but in agreement with the exd null result, some weak residual activation remained. However, unlike the exd mutants, no ectopic LNE expression was ever observed in the abdomen. Taken together, the results in this section show that the major mode of LNE activation is via an exd/Pbx and hth/Meis dependent pathway. Meanwhile a minor independent pathway, possibly using an as yet unidentified cofactor, also appears capable of weakly activating this enhancer.
Figure 3.8 LNE activity is increased in a weak \textit{hth} allele but reduced in a strong allele

Wildtype (A, C, E) and \textit{hth}^{SE04} (B, D, F) or \textit{hth}^{64-1} (G) mutant embryos at stage 13 immunolabelled against $\beta$-gal.

(A-D) In embryos carrying a weak \textit{hth} allele (\textit{hth}^{SE04}) the LNE is activated normally in the maxillary and thoracic segments (B, D). Ectopic $\textit{lacZ}$ expression was observed anterior to the maxillary domain of expression, in the posterior mandibular segment (red arrow in B and D). The ventral views (C, D) illustrate the gnathal phenotype used to identify \textit{hth}^{SE04} mutant embryos. The labial segment (L) is enlarged in \textit{hth}^{SE04} embryos whilst the maxillary segment (M) is reduced in size.

(E-F) The \textit{hth}^{SE04} mutant background also had no effect on the ability of HOX proteins to activate the HS1^mut construct. Again an ectopic region of reporter expression is observed, in posterior cells of the mandibular segment (red arrow in F).

(G) Use of the strong hypomorph \textit{hth}^{64-1} (Kurant et al., 1998) results in a near loss of LNE-$\textit{lacZ}$ expression. Weak residual activation is marked with black lines.
3.3 DISCUSSION

Due to the limited number of \textit{Hox} target genes identified to date, \textit{Hox} autoregulatory elements have been used to address questions concerning the molecular mechanisms of \textit{Hox} target gene regulation. The approach taken here was to use the mouse \textit{Hoxb4} LNE, an autoregulatory element that is conserved in other vertebrate species. This model \textit{Hox} target gene was dissected in \textit{Drosophila} at the molecular level to ask how HOX proteins discriminate between their targets \textit{in vivo}. Similarly, the LNE was also used to investigate the general question of how target gene transcription is restricted to only a subset of the cells expressing the \textit{Hox} activator. In both studies the regulation of mutated LNE constructs was assayed \textit{in vivo}, thus the nature of proteins directly binding to sites in this element is not yet known. However, in the case of HS2, previous \textit{in vitro} experiments have indicated that HOX proteins of the group 4 class can bind to this site.

The major findings of the \textit{in vivo} study reported here are discussed below and are summarised in Figure 3.9. A model for the \textit{Hox} regulation of the LNE through the HS1 and HS2 sites, and other cis regulatory regions, is shown in Figure 3.10. In this model, direct binding of HOX proteins to HS1 and HS2 is inferred from \textit{in vitro} binding studies reported in (Gould et al., 1997) and (Chan et al., 1997).

3.3.1 HS1 and HS2 have different Hox specificities in \textit{Drosophila}

Previous studies showed that the LNE behaves as a group-4 \textit{Hox} autoregulatory element in both mouse and fly (Gould et al., 1997). This work also revealed that the LNE is under the control of positive cross-regulation, by \textit{Scr}/group-5 and
Figure 3.9 A summary of LNE cis mutations and cofactor mutant backgrounds

An overview of LNE activation/repression within the relevant Drosophila Hox expression domains following the incorporation of cis mutations and/or loss of EXD/HTH function.

(✓) indicates activation or repression.

(x) shows a large reduction or complete loss of activation, or a loss of repression.

(LOR) refers to the Loss Of DV or AP intrasegmental Restriction.
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Figure 3.10 A model for HOX regulation of the LNE

In view of the LNE cis mutations and expression in cofactor mutant backgrounds the following model is proposed for LNE activation and repression by Hox gene products in *Drosophila*. Five regulatory modules have been identified in the LNE; the HOX sites (HS1 and HS2), a linker region between these sites, and two flanking regions located upstream (u/s) and downstream (d/s) of HS1/HS2.

(A) Activation of the LNE in the Dfd expression domain occurs solely through HS2, a DFD-PBC bipartite site. Previous in vitro assays have shown that DFD binds to this site and that binding is enhanced by EXD (Gould et al., 1997; Chan et al., 1997). Here, in vivo analysis showed that LNE activation by DFD is almost fully EXD dependent and is also influenced by the u/s and d/s regulatory modules. In addition, the linker region appears to weakly contribute to repression of the LNE in the maxillary domain.

(B) Scr and Antp can activate the LNE in the thorax through either HS1 or HS2 in an almost fully exd dependent manner. The d/s sequence is essential for SCR and ANTP mediated LNE expression while the u/s sequence has less of an influence on enhancer activation. The linker region has a major role in the intrasegmental restriction of LNE activity in the thorax.

(C) Ubx and abdA mediated repression of the LNE appears to be through the HS2 site and under the influence of the linker region.

HOX/EXD proteins are shown as a dashed circle where direct binding is inferred from the studies of Gould et al. (1997) and Chan et al. (1997).
A Maxillary (DFD Expressing).

B Thorax (SCR Expressing) *

C Abdomen (UBX Expressing) *

* Same for ANTP

* Same for ABDA
Antp/group-6 genes, and suggested negative cross-regulation by Ubx/group-7 Hox genes. Here, using an overexpression approach, I have shown that the abdA/group-8 Hox genes also have a repressive effect on LNE function. To confirm that Ubx/abdA endogenously repress LNE expression in the abdomen, enhancer activity would have to be examined in mutants for either Hox gene. However, the available data suggests that in the mouse the LNE is positively cross-regulated by Hox genes expressed immediately posterior to Hoxb4, whilst those expressed even more posteriorly provide negative cross-regulation. In contrast, where cross-regulations exist in the Drosophila embryo they tend to be negative in nature, and result in Hox domains that do not extensively overlap. Therefore, positive rather than negative cross-regulation by some posteriorly expressed Hox genes begins to explain why mouse, and not fly, Hox domains overlap extensively. Meanwhile negative cross-regulation of the LNE, by the most posteriorly expressed Hox genes, probably accounts for the weak caudal-most expression of Hoxb4 (Gould et al., 1997). Hence the LNE activity observed in Drosophila is characteristic of the mouse Hoxb4 gene, and not the fly group 4 Hox gene, Dfd.

Assessing the individual contributions of HS1 and HS2 in Drosophila (Figure 3.9) revealed that HS1 mediates a response to Scr and Antp, whilst all three LNE Hox activators (Dfd, Scr and Antp) function through the HS2 site (Figure 3.10). These results are comparable to those reported in the mouse for identical HS1 and HS2 mutations (Gould et al., 1997). With HS2 alone, the rostral limit of LNE activation was preserved at the r6/7 boundary in the mouse hindbrain. If HS2 solely mediates the autoregulatory response to HOXB4, as inferred from experiments in the fly, then HS1 alone would be expected to display an anterior boundary of expression more posterior than r6/7. As in the fly, much weaker

1 See section 3.2.3 and Gould et al. (1997)
expression was observed with HS1 alone and the anterior limit, although not clearly defined, may well be posterior to r6/7. Mutation of the HS2 site also resulted in derepression of the LNE in abdominal segments of the *Drosophila* embryo. This suggests that *Ubx/abdA* mediated repression of the LNE is modulated through the HS2 site. However, mouse embryos transgenic for an LNE construct containing an identical mutation did not display derepression of the LNE in the posterior CNS (Gould et al., 1997).

Changing the two specificity-determining bases in an HS2-containing oligonucleotide from TA to CC is sufficient to switch the *in vivo Hox* response from *Dfd* to *lab* (Chan et al., 1997). However, I have shown here that an identical change in the context of the full LNE element does not alter *Hox* specificity (Figure 3.9). This result is consistent with that obtained in a reciprocal experiment when CC was exchanged for TA in a LAB-PBC site within a 550bp *lab* autoregulatory element (Grieder et al., 1997). This mutation in the LAB550 element resulted in reduced enhancer activation in *lab* territory. Although DFD binds to the TA-containing site *in vitro*, no ectopic expression was observed in the *Dfd* domain. Thus, *Dfd* is incapable of activating this enhancer *in vivo*. These experiments demonstrate the complexity of an endogenous *Hox* target element, and reveal the importance of sequences remote to the HOX recognition site.

In summary, the *in vivo* studies in *Drosophila* have revealed that both HS1 and HS2 modulate the response to more than one *Hox* regulator, and that each site has a different *Hox* specificity. Interestingly, changing the specificity of the HS2 site does not alter the specificity of the full enhancer.
3.3.2 Conserved sequences remote to HS1 and HS2 modulate Hox activation of the LNE

During mouse embryogenesis Hoxb4 is expressed in the neural tube, the somites, and in the mesodermal components of many internal organs (Graham et al., 1988; Whiting et al., 1991). Meanwhile the LNE, a HOXB4 response element, is only activated in the neural tube. Similarly, group-4 autoregulatory enhancers of the Drosophila Dfd gene (EAE and NAE) exhibit tissue-restricted activation. More specifically, the EAE can be split into several modules, each of which is expressed in a distinct epidermal subset of the full Dfd expression domain (Zeng et al., 1994). Furthermore, NAE activity is restricted to a subset of Dfd expressing cells, this time in the embryonic CNS. LNE activation in the fly embryo is also restricted to a subset of those cells expressing the Hox activators, including Dfd. In contrast, an oligonucleotide simply containing a HS2 DFD-PBC site is expressed in the fly almost throughout Dfd territory (Chan et al., 1997). This raises the possibility that sequences outside of HS2 are important for limiting Dfd-mediated activation.

Mutations introduced into the conserved linker reveal a role for this region in restricting the Hox response. Both the sequence and length of the linker are critical for imposing the full negative influence seen with the wild type enhancer (see Figure 3.9). Mutating six base pairs in this region resulted in increased LNE activation within Scr territory (Figure 3.9), suggesting that repressors normally act through this region (Figure 3.10). Moving HS1 and HS2 further apart by 5 bp resulted in an increase in Scr/Antp mediated enhancer activation (Figure 3.9). Meanwhile, addition of bases into, or deletion of bases from, the linker region both resulted in ectopic LNE expression in abdominal segment A1, implying a loss of
Ubx mediated repression (Figure 3.9). Precise spacing between HS1 and HS2 could be required to achieve repression if, for example, steric hindrance prevents an activator from binding to an HS site or if cross-talk between molecules bound to HS1 and HS2 is required for repressor recruitment and/or function.

In this study I have also identified two further conserved sequences, remote to the linker and HOX sites, that are necessary for normal activation of the LNE. The first element is located downstream of HS2 and is essential for activation of the LNE by Scr and Antp (see Figures 3.9 and 3.10). A second flanking module, found in a region upstream of the HS sites, is also critical for full Hox-mediated activation of the LNE (see Figures 3.9 and 3.10). In the LNE, Scr and Antp both act through the HS2 site and yet an oligonucleotide containing this site only activates gene expression in Dfd territory. It is therefore possible that other factors acting through the u/s and d/s sites modulate the specificity of HS2, enabling activation by Scr and Antp. These results are consistent with those of Li et al. (1999b), who have shown that sequences remote to HOX-PBC sites can dictate the specificity of the bipartite site. However, future work will be required to elucidate the identity of the factors that bind to the flanking regulatory regions identified in this study.

3.3.3 A major role for cofactors in Hox activation of the LNE

The LNE is almost fully dependent on the PBC family cofactor exd for activation in the fly embryo. This was demonstrated by a near complete loss of LNE expression in both exd null embryos and mutants for hth, a gene required for EXD function. I have shown that the EXD site in HS2 is extremely important for LNE activation in vivo by Dfd, Scr and Antp (PBCmut in Figure 3.7E). The residual expression in PBCmut transgenics and exd or hth mutants (Figure 3.9) did suggest the existence
of a minor exd independent pathway for LNE activation. As this pathway only appears to be weakly active, it has not been analysed further.

Whether exd contributes to Ubx/abdA driven repression of LNE activity is less clear. Abdominal activation of the LNE in exd mutants (Figure 3.9) suggests a possible role for this cofactor, however, continued abdominal repression was observed in hth mutants and when the EXD site in HS2 was mutated (PBC$^{mut}$, Figure 3.9). Interestingly, mutating the HS2 site (HS2$^{mut}$) resulted in derepression of the LNE in abdominal segments, while changing only the two variable bases in this site (CC switch) had no effect (Figure 3.9). This suggests that UBX/ABDA do not mediate repression of the LNE through precise recognition of the HS2 site. Previous studies have shown that in the absence of EXD, HOX proteins appear to have equivalent biological activities and function in a largely repressive manner (Casares and Mann, 1998; Weatherbee et al., 1998). It is therefore possible that UBX/ABDA repress LNE activity without any input from EXD in the abdominal segments (Figure 3.10). However, the reasons for the discrepancies outlined above are not fully understood and further experiments would be required to completely eliminate a role for exd in Ubx/abdA mediated repression of the LNE.

3.3.4 Perspectives

An important conclusion to be drawn from this study of a model Hox target gene is the sheer complexity of Hox target function at the molecular level (see Figure 3.10). In Drosophila, the LNE integrates the action of three Hox activators and their cofactors, together with two potential Hox repressors. In addition to two previously described HOX sites, I have identified three additional regulatory modules: a linker region with a negative influence as well as upstream and
downstream flanking sequences, both of which exert a positive influence on LNE activity. All of these sites are required for the precisely regulated expression of this model $Hox$ target gene, hence full LNE function depends on much more than minimal HOX-PBC binding sites. In conclusion, in order to understand the complexities of $Hox$ target gene activation, such enhancers must be studied as a whole unit.
CHAPTER FOUR

The oenocyte: a model for studying cellular mechanisms of Hox gene function
4.1 INTRODUCTION

In the previous chapter I described the molecular dissection of a model Hox target gene, revealing regulation by more than one Hox gene and influences from a number of DNA sites. As each Hox target gene represents only a small proportion of the overall target complexity, individually they make only a limited contribution to what we recognise as segment identity. Thus, detailed analysis of single Hox target genes can tell us a great deal about how Hox genes control transcription, but rarely helps us to understand how morphological segment diversity is generated (Akam, 1998b).

Studying segment identity is a complex process due to the large number of different cell types (muscle, epidermal and neuronal) specified by a single Hox gene. Here I have deconstructed the segment down to its simplest unit, the cell, to ask how a single Hox gene specifies a single cell identity. The Drosophila larval oenocytes were chosen as the model cell type for this study because, as I will show in Chapter 5, a single abdominal Hox gene, abdA, is both necessary and sufficient to promote the formation of these cells.

Oenocytes are a secretory cell type found in many arthropods including diptera, arachnids, cockroaches and flour beetles (see oenocyte function references cited below). In Drosophila melanogaster there are two generations of oenocytes: larval and imaginal (Bodenstein, 1950). Larval oenocytes are ectodermal in origin and, following delamination, form bilateral clusters in abdominal segments A1 to A7. Each cluster contains between 4 and 9 larval oenocytes, 6 on average, and occupies a final position sandwiched between the
epidermis and the lateral chordotonal organs of the PNS (Hartenstein and Jan, 1992). During larval life, oenocytes undergo endoreplication, reaching a staggering 80 μm in diameter by the end of the third larval instar (Bodenstein, 1950). These large conspicuous cells have a dense granular cytoplasm consisting of an abundance of smooth endoplasmic reticulum (Bodenstein, 1950), characteristic of a secretory cell type (Rinterknecht and Matz, 1983). Larval oenocytes degenerate during metamorphosis at which stage the imaginal oenocytes develop from the imaginal histoblast nests that generate the adult abdominal hypoderm. Despite sharing a name, there is very little functional or morphological evidence that imaginal oenocytes are the adult equivalents of larval oenocytes.

Although oenocytes are conserved across arthropod species, and have been studied for over 100 years, the function of these cells is unclear. They have been implicated in the production of cuticle components (Wigglesworth, 1970; Rinterknecht and Matz, 1983; Baikova et al., 1993). In addition, it has been demonstrated that oenocytes synthesise ecdysones in vitro (Romer et al., 1974; Romer and Gnatzy, 1981). The proposed functions of oenocytes in cuticle and ecdysteroid production are both compatible with a role for these cells during the process of moulting (Locke, 1969; Wigglesworth, 1970; Romer, 1971; Romer, 1974; Dorn and Romer, 1976).

This study focuses on the *Drosophila* larval oenocytes (hereafter referred to as oenocytes), asking how a single *Hox* gene (*abdA*) acts to specify this segmentally repeated cell type. Given that the above is a summary of essentially all that is known about the development of *Drosophila* oenocytes, I initially undertook a study of the developmental origin of this cell type. An enhancer trap screen for genes expressed in oenocytes provided the resources to follow
oenocyte development from induction through to programmed cell death. One of the PlacZ lines identified in the screen, $S10^{don1}$, displayed an oenocyte phenotype and hence was investigated further. $S10^{don1}$ provided a wealth of information about oenocytes and initially highlighted the fact that these cells move across the parasegment boundary. In this chapter, I describe a preliminary investigation of the role of segment polarity genes in this migratory process.
4.2 RESULTS

4.2.1 A screen for new oenocyte genes

Very few studies have been carried out on Drosophila oenocytes, but a chapter by Bodenstein in ‘The Biology of Drosophila’ (1950) provides a reasonable review of these conspicuous cells. In recent years, 12 genes have been shown to be expressed and/or have a function in oenocytes (Table 4.1). To redress the limited number of oenocyte resources, I have been involved in an ongoing in silico and subsequent expression screen of PlacZ and GAL4 lines, with the long-term aim of identifying new oenocyte genes. The primary screen involved collecting together putative oenocyte expressing lines identified in Drosophila database searches (Flybase, 1999; Flyview, 2000). However, an expression screen was necessary, as many of the primary lines were not actually expressed in oenocytes. This was probably due to the difficulty in identifying this rather mysterious cell type. In total, 24 oenocyte expressing lines were recovered as positives from the secondary screen (Table 4.2). Of the seven known genes identified in this screen, three were previously reported to be expressed in oenocytes (Hnf4, sal and svp in Table 4.1). There are no reports of the other 4 known genes (mrr, Aldh-lll, dap, esg) being expressed and/or having a function in larval oenocytes. The remaining 16 lines in the collection represent new genes involved in oenocyte formation.

4.2.2 The embryonic development of oenocytes

The S10^{don1} PlacZ enhancer trap, recovered from the preceding screen, has proved particularly useful as it labels oenocytes throughout their embryonic life. S10^{don1} is
Table 4.1 A literature survey of genes expressed and/or displaying a function in oenocytes
<table>
<thead>
<tr>
<th>Gene</th>
<th>Oenocyte expression</th>
<th>Function in oenocytes</th>
<th>References</th>
</tr>
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<td>Notch (<em>N</em>)</td>
<td>-</td>
<td>Yes</td>
<td>Hartenstein et al. (1992)</td>
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<td><em>big brain</em> (<em>bib</em>)</td>
<td>-</td>
<td>Yes</td>
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<td><em>mastermind</em> (<em>mam</em>)</td>
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<td><em>hepatocyte nuclear factor 4</em> (<em>Hnf4</em>)</td>
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<td><em>seven up</em> (<em>svp</em>)</td>
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<td>?</td>
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<td><em>spalt</em> (<em>sal</em>)</td>
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<td><em>Delta-aminolevulinate synthase</em> (<em>alas</em>)</td>
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<td>?</td>
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<td><em>dFKBP59</em></td>
<td>Yes</td>
<td>?</td>
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Table 4.2 Oenocyte expressing lines

Data compiled from an ongoing *in silico* screen for oenocyte expressing lines using the *Drosophila* databases Flybase (1999), Flyview (2000) and private enhancer trap collections of Cahir O’Kane and Andrea Brand. GAL4 lines were crossed to flies carrying a UAS-*nlslacZ* transgene. Oenocyte expression was assessed for each line either by immunostaining against β-gal or by fluorescent microscopy in living embryos for GFP lines.

The stock names are given according to the source of the line. Homozygous phenotypes are listed where E and L refer to lethality in the embryonic and larval stages, respectively. The presence (+) or absence (-) of oenocyte expression in embryos (Emb) and third instar larvae (L3) is shown. High-level reporter expression in oenocytes is indicated by ++. A question mark is given if future double labelling experiments, with an oenocyte specific marker, are necessary to confirm or rule out oenocyte expression. st.x> refers to expression from stage x onwards during embryonic development.

This information was collected in collaboration with Shilpa Mahadevaiah, Véronique Brodu and Alex Gould.
<table>
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<th>GAL4 Drivers</th>
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<td>56B</td>
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<td>P1785</td>
<td>III (72D)</td>
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<td>R. Schuh</td>
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<td>++(st.10&gt;)</td>
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<td>A. Gould and R. White</td>
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first expressed at early stage 11 in dorsal stripes of cells in abdominal segments A1 to A8. Double-labelling with an anti-EN antibody revealed that these cells lie in the posterior compartment (Figure 4.1A). During embryonic stage 11, a subset of these cells delaminate from each dorsal stripe and maintain $S10^{don^I}$ expression (data not shown). In A1 to A7, the $S10^{don^I}$ positive cells adopt a characteristic shape and time-course experiments provide strong evidence that they will later become oenocytes (see below). However, in A8 oenocytes are not formed. Instead, $S10^{don^I}$ positive cells delaminate and migrate in a posterodorsal direction to become associated with the posterior spiracle (data not shown). In A1 to A7, delaminated oenocyte precursors are EN-negative (Figure 4.1E), while the residual non-delaminated epidermal cells in the $S10^{don^I}$ stripe remain EN-positive (Figure 4.1D). By stage 15, oenocytes are found clustered in their final position (Figure 4.1F), and it is evident that these cells have migrated ventrally with respect to the residual $S10^{don^I}$/EN double-positive epidermal stripe. Also, despite having arisen in en territory, mature oenocytes are located anterior to the EN stripe. This suggested the possibility of both anterior and ventral components of cell migration. Both of these movements will be discussed further within this chapter.

*spalt (sal)* was one of the genes previously reported to be expressed in oenocytes (see table 4.1), and two enhancer trap inserts in sal were isolated in the screen for oenocyte expressing lines (see Table 4.2). Here I use an antibody against the zinc-finger protein encoded by sal to follow the early stages of oenocyte development. Anti-SAL moderately labels a dorsal domain (referred to as the dorsal SAL domain) that straddles abdominal and thoracic segments from embryonic stage 9 onwards (data not shown). By early stage 11, SAL is upregulated in characteristic sickle-shaped nuclei (Figure 4.1B) which form whorls
Figure 4.1 Oenocyte development during *Drosophila* embryogenesis

Embryos carrying the PlacZ enhancer trap $S10^{don1}$ are shown in panels A and D-F where β-gal is in green and EN in red. Panels B and C display wildtype embryos immunostained with anti-SAL (red).

(A) An early stage 11 embryo showing $S10^{don1}$ expression overlaps (yellow) with EN in the most dorsal cells of the EN stripe. $S10^{don1}$ is also expressed in the embryonic CNS from this stage onwards (not shown).

(B) A dorsolateral view of two parasegments on the curve of the extended germband in a stage 11 embryo. Low level SAL is observed in a dorsal domain, with a sharp boundary between expressing and non-expressing cells. SAL is upregulated in sickle-shaped oenocyte precursor nuclei that form a whorl, positioned at the ventral extent of the dorsal SAL domain.

(C) Following delamination the oenocyte nuclei become rounded and during stage 12 oenocytes are observed migrating ventrally as strings of SAL-positive nuclei.

(D-E) Two confocal planes through the same stage 13 embryo. Cells remaining in the $S10^{don1}$ stripe are observed in the plane of the epidermis, and they continued to show an overlap with EN (D). Beneath the epidermis the oenocytes are beginning to cluster and have switched off *en* (E).

(F) By stage 15, oenocytes are clustered in their final position and lie ventral to the remains of the $S10^{don1}$ epidermal stripe (yellow overlap) and anterior to the EN stripe.

Panels A and D-F were kindly provided by Alex Gould.
at the ventral extent of the dorsal SAL domain. These sickle-shaped nuclei also
display upregulated $S10^{don}$ expression (data not shown). Both pointed ($pnt$) and
ventral veinless ($vvi$) are also expressed in cells of the whorl prior to their
expression in mature oenocytes (Elstob et al., 2001; Rusten et al., 2001 and data
not shown). These expression data strongly suggest that the sickle-shaped cells
are oenocyte precursors, and further evidence is provided in a later section where
lineage-labelling experiments are described. By stage 12, the delaminated
oenocyte nuclei have rounded up and are observed in strings migrating ventrally
(Figure 4.1C). Anti-SAL continues to label oenocytes throughout the rest of their
embryonic development, and also labels some components of the lateral
chordotonal organs of the PNS, with which oenocytes are associated in the mature
embryo (see below).

4.2.3 Ventral migration of oenocytes: a role for seven up

Out of more than 10 unknown P-element inserts displaying oenocyte expression,
$S10^{don}$ was selected for investigation because homozygous embryos displayed an
oenocyte phenotype (Figure 4.2B). This was a variable phenotype, observed in
50% of homozygotes, with single misplaced clusters in primarily the DV, but also
the AP and z-axis (Figure 4.2A-B and data not shown), through to the most severe
embryos in which oenocytes were completely absent in some segments whilst

1 A copy of Elstob et al. (2001) is included in the back sleeve of this thesis.
Figure 4.2 The down-and-out phenotype

Panels A and B show $S10^{don1}$ homozygous embryos immunostained for β-gal. All other panels display X-gal stains of $S10^{don1}$ imprecise excision lines, in which the lacZ reporter gene remains active. Unless otherwise stated all embryos are at stage 16. In all panels closed arrowheads mark oenocyte clusters whilst open arrowheads label the $S10^{don1}$ positive cells which arise in A8 and become associated with the posterior spiracles.

(A-B) $S10^{don1}$ embryos are homozygous lethal and display a range of oenocyte phenotypes. In some embryos oenocytes develop and cluster in approximately their normal position (A). In other homozygous embryos the clusters form normally but are misplaced in the AP, DV and/or z-axis (data not shown but see panels C and F for examples of this phenotype). In the most severe class of phenotype only a few oenocytes formed and most of these were not in clusters (B).

(C) A don2 homozygote showing misplaced oenocyte clusters in the AP and DV axis.

(D) In don3 heterozygotes, lacZ is absent from oenocytes, but not the CNS or A8.

(E) A DV misplacement phenotype is visible in this don6 homozygote despite weak oenocyte lacZ expression.

(F) A don13 homozygous embryo with misplaced oenocytes, mainly in the AP axis.

(G-H) don18 heterozygous embryos show very strong X-gal staining. At stage 11, oenocyte precursor whorls and A8 precursors are easily recognised (G). The ectodermal stripe of lacZ is also stronger relative to $S10^{don1}$. The residual epidermal stripe is still visible following delamination in a stage 13 embryo (H).
clusters were misplaced in others (Figure 4.2B). In all cases, where oenocytes did not form they had delaminated from the ectoderm. Hence \textit{S10} was given the allele name \textit{down-and-out 1 (don1)} based on an apparently normal delamination event followed by mis-migration and out-of-place oenocytes.

Before investigating the \textit{S10}^{don1} allele further, the P-element was excised from an isogenised stock to determine whether homozygous lethality was due to the \textit{p(A92)} insert (see Materials and Methods). Upon excision, homozygous viable flies were obtained, proving that the P-element caused the lethality of \textit{S10}^{don1}. With this knowledge, I decided to clone the \textit{don1} locus. An inverse PCR based protocol was used to amplify DNA flanking the P-element insert (see Materials and Methods) and this was subsequently sequenced. Using a BLASTN search, sequences flanking the 5'P end were found to be 98% identical to the extreme 5' end of a cDNA encoding the orphan nuclear receptor protein \textit{seven up (svp)}. For further analysis, a BAC contig containing \textit{svp} genomic sequences was used (NCBI Genbank accession number AC017240). Sequences flanking the 3'P end showed homology to the non-coding region upstream of the \textit{svp} transcription start site. Submitted cDNA sequences for two alternative splice forms of \textit{svp} (Flybase, 1999), together with BAC AC017240, were used to generate a genomic map for the \textit{svp} locus (Figure 4.3). A second BAC containing sequences up to 40kb upstream of the transcription start site was also identified (NCBI Genbank accession number AC017307) and included in the map (Figure 4.3).

To verify that the P-element insert was a lethal \textit{svp} allele, complementation tests were carried out with \textit{S10}^{don1}. \textit{S10}^{don1} failed to complement the two alleles \textit{svp}^{e22} and \textit{svp}^{H162}. Thus in conclusion, \textit{S10}^{don1} is an insertion of \textit{p(A92)} into the
Figure 4.3 A genomic map of the seven up locus

A map drawn approximately to scale illustrating the insertion of the p{A92} don1 allele at the 5' end of the svp coding region. BAC AC017240 contains the svp genomic region while BAC AC017307 contains upstream regulatory sequences. Given are the NCBI Genbank accession numbers for both BACs.
3'P

__don1__

5'P

ry

lacZ

BAG AC017307

BAC AC017240

svp type I

svp type II

1kb
very 5' end of seven up, a gene encoding a nuclear receptor protein. For this reason S10^don1 will now be referred to as an allele of svp, svp^don1.

Embryos homozygous for svp^don1 showed an oenocyte phenotype that was of variable expressivity and penetrance, factors that might be attributed to the svp^don1 allele being a hypomorph. Oenocytes were thus analysed in embryos homozygous for the amorphic allele svp^a22, using an antibody against SAL. Surprisingly, these svp^a22 mutants showed a less severe oenocyte misplacement phenotype than svp^don1, such that a subset of clusters were dorsally misplaced (Figure 4.4B, D). Although cells within these clusters do migrate ventrally, this is somewhat delayed and clusters appear unable to reach their usual ventral extent. Although all svp^a22 homozygotes examined displayed some misplaced clusters, not every segment was affected in these embryos. This suggests either a partial requirement for svp in the ventral migration of oenocytes or the possibility of redundancy (see Discussion section 4.3.3).

In the svp^don1 excision experiment a number of lethal svp^don alleles were generated (Table 4.3). Each excision event was identified by the loss of the ry+ eye marker and not the lacZ gene (see Materials and Methods). Indeed lacZ was still expressed in many of these svp alleles tested (10/16), resulting in some informative homozygote and heterozygote expression patterns (Table 4.3). Presumably where lacZ remained active, svp upstream genomic DNA flanking the 3'P/ry+ end of the P-element was deleted. Interestingly, svp^don3 heterozygotes showed no oenocyte staining whilst CNS and posterior spiracle associated lacZ expression remained normal (Figure 4.2D). This might be due to deletion of an oenocyte-specific enhancer from the svp locus. Embryos homozygous for svp^don6 and svp^don18 had reduced and increased lacZ levels respectively, and both
Figure 4.4 *seven up* mutants display dorsally misplaced oenocytes

Heterozygous (A, C) and homozygous (B, D) svp<sup>h02</sup> embryos immunolabelled with anti-SAL. The dorsal trunk of the trachea (DT) serves as a good landmark for the DV position of oenocyte delamination.

(A-B) By stage 13, oenocytes have delaminated and are in the process of migrating ventrally (A). In *svp* mutants a subset of clusters fail to migrate (arrowheads) and remain at the DV position of delamination (B).

(D) A stage 14 *svp* mutant shows that abnormal clusters never reach the full ventral extent of migration assumed by clusters in adjacent segments.

It should be noted that despite panels (B) and (D) both showing misplaced clusters in A5 and A6, any abdominal hemisegment can be affected.
Table 4.3 The *down-and-out (don)* alleles generated

Listed are the lethal alleles produced by imprecise excision of *don1*. Embryonic lethality (Emb) is generally given with a bracketed number, which displays the percentage of dead embryos observed in a lethality test. According to Mendelian genetics, 25% of embryos should die as a result of a homozygous lethal allele (see Materials and Methods). Asterisks indicate that the percentage is an average from two independent lethality tests. Emb (?) refers to those alleles where the percentage embryonic lethality was not determined. A question mark against the presence of *lacZ* indicates a difficulty in distinguishing background staining from real expression. The *lacZ* expression pattern and/or non-embryonic lethal phenotypes are described. Dorsal vessel expression is indicated by “dor ves” and L3 denotes the third larval instar.
<table>
<thead>
<tr>
<th>don allele</th>
<th>Lethality (%)</th>
<th>lacZ</th>
<th>lacZ expression pattern</th>
<th>Homozygous Phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (S10)</td>
<td>Emb (26.5°)</td>
<td>+</td>
<td>Oenos, CNS and A8</td>
<td>Missing or mis-placed oenocytes in AP, DV and z axes.</td>
</tr>
<tr>
<td>2</td>
<td>Emb (?)</td>
<td>+</td>
<td>As for S10</td>
<td>As for S10 but more frequent oenocyte mis-placement.</td>
</tr>
<tr>
<td>3</td>
<td>Emb (24)</td>
<td>+</td>
<td>CNS, A8 but NOT in oenos</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Emb (27)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Emb (26.5)</td>
<td>?</td>
<td>Weak ubiquitous</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Emb (25)</td>
<td>+</td>
<td>As for S10 plus fat body</td>
<td>As for S10. Slight decrease in no. oenocytes per cluster.</td>
</tr>
<tr>
<td>7</td>
<td>Emb (25)</td>
<td>+</td>
<td>Weak ubiquitous</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>L3 or pupal</td>
<td>-</td>
<td>-</td>
<td>Homozygous L3 and pupae observed</td>
</tr>
<tr>
<td>9</td>
<td>Emb (26)</td>
<td>+</td>
<td>Strong S10 pattern</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>L3 (2.3°)</td>
<td>-</td>
<td>-</td>
<td>Homozygous L3 but not pupae observed</td>
</tr>
<tr>
<td>11</td>
<td>Emb (26.5°)</td>
<td>?</td>
<td>Weak ubiquitous</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>Pupal</td>
<td>-</td>
<td>-</td>
<td>Homozygous pupae observed but die in pupal cases</td>
</tr>
<tr>
<td>13</td>
<td>Emb (32.5°)</td>
<td>+</td>
<td>As for S10 plus fat body</td>
<td>Oenocyte phenotype similar to S10 but more severe</td>
</tr>
<tr>
<td>14</td>
<td>Emb (?)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>Emb (29.5)</td>
<td>+</td>
<td>Weak ubiquitous</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>Emb (32°)</td>
<td>+</td>
<td>Weak ubiquitous</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>L3</td>
<td>+</td>
<td>Strong S10 pattern</td>
<td>Homozygous L3 found.</td>
</tr>
<tr>
<td>18</td>
<td>L3/pupal</td>
<td>+</td>
<td>Strong S10, Dor Ves, PNS</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>Semi- pupal-lethal</td>
<td>-</td>
<td>-</td>
<td>Some homozygous flies eclose others die in pupal cases</td>
</tr>
</tbody>
</table>
displayed a similar misplaced cluster phenotype to that observed for $svp^{don^1}$ (Figure 4.2 E, G-H and data not shown). Both $svp^{don^2}$ and $svp^{don^13}$ homozygotes displayed a more frequent DV and AP misplacement of oenocytes than the original $svp^{don^1}$ insert (Figure 4.2C, F). The various $don$ alleles form an allelic series that should prove useful for analysing $svp$ function and regulation.

Taken together with the analysis of oenocytes in embryos homozygous for the $svp^{a22}$ amorphic allele, these results demonstrate that $svp$ plays a role in the ventral migration of oenocytes.

### 4.2.4 Anterior movement of oenocyte precursors

In addition to their ventral migration, oenocytes also appear to migrate anteriorly, arising in EN territory and yet clustering in a final position anterior to the EN stripe (see Figure 4.1A, F). This aspect of oenocyte development was intriguing as the anterior edge of the EN stripe has previously been described as a boundary of lineage restriction (Vincent and O'Farrell, 1992). That is, cells in the EN stripe, and their progeny, should be unable to migrate anteriorly. Oenocytes fail to respect this boundary, which also corresponds to the compartment or parasegment boundary. The compartment boundary is required for cell segregation, preventing mixing between populations in adjacent parasegments (Reviewed in Vincent, 1998; Dahmann and Basler, 1999). So why are oenocytes not subject to this restriction and how do they move out of the posterior compartment? Here I present the results from an initial study to address these questions, investigating the role(s) of $en$ and $wg$, two genes expressed in cells on opposite sides of the parasegment boundary.
Firstly, the anterior movement of oenocytes was studied in more detail using UAS-nlslacZ driven by en-GAL4, which reproduces the EN distribution pattern in the dorsolateral ectoderm, albeit slightly delayed (see Figure 4.5). The nuclear targeted β-gal is stable and persists in all those nuclei where nlslacZ was once transcribed (Pfeiffer et al., 2000). Therefore, en-GAL4 UAS-nlslacZ serves as a crude lineage label of all nuclei that at some stage expressed the en gene. As oenocytes are born in EN territory, their development was followed at fixed time-points using this marker, together with anti-EN, or anti-SAL, a more specific oenocyte label. At stage 10, en-GAL4 drives UAS-nlslacZ expression in a 3 to 4 cell stripe, with a straight anterior edge that correlates with the anterior border of the 2 cell-wide EN stripe (Figure 4.5A). The additional 1 to 2 posterior rows of UAS-nlslacZ nuclei are composed of cells that once expressed en, and upon switching it off were able to escape posteriorly from the EN domain (Vincent and O’Farrell, 1992). These nuclei are only found posterior to the EN stripe and never anteriorly due to the lineage restriction at the parasegmental boundary.

By early stage 11, an anterior bulge is visible from the dorsolateral portion of the en-GAL4 UAS-nlslacZ stripe (Figure 4.5B). All of the cells within this structure are EN-positive and have rounded nuclei. Given these characteristics, and the inclusion of epidermal cells just dorsal and ventral to the bulge within the single confocal section of Figure 4.5B, it is likely that all cells in the early stage 11 bulge are still in the epidermal layer. Lineage studies using caged fluorescent dyes have shown that cells in the bulge originate from the 2-cell wide EN stripe (unpublished data from JP Vincent, NIMR, London), and are therefore not switching on en de novo.
**Figure 4.5 Dynamics of *en* expression during oenocyte development**

In all panels embryos carry *en-GAL4* and UAS-*nslacZ* and are immunostained with anti-β-gal (green) and anti-EN (red). For each embryo (A-E) the third panel displays the merge (yellow). Where not obvious, refer to Figure 4.6 for the approximate positions of oenocytes in the *en-GAL4* UAS-*nslacZ* stripe.

(A) At stage 10 the EN stripe is only 2 cells wide in comparison to the 3-to-4 cell wide *en-GAL4* UAS-*nslacZ* stripe. No cells break the anterior border of the EN stripe whilst EN-negative/β-gal positive cells do migrate posteriorly.

(B) All cells within the *en-GAL4* UAS-*nslacZ* bulge are EN-positive at early stage 11, including the oenocyte precursors that are located in the bulge.

(C) Following delamination *en* is switched off in the oenocytes and the late stage 12 embryo pictured shows EN-negative oenocytes in the anterior-ventral portion of the bulge. Also note that a single cell in the middle of each segment (white chevrons) becomes EN positive. As the expression of *nslacZ* in this cell is delayed (see panel E) this cell is switching on *en de novo*.

(D-E) Two confocal planes through the same embryo where the more external EN-negative oenocytes are anterior to the EN/β-gal double positive stripe (D). Just internal to the oenocytes, all components of the five lateral chordotonal organs are β-gal positive (ligament cell, l; neuron, n; scolopale cell, s; cap cell, c) while only the neurons appear to weakly label with anti-EN (E). Note that overlap is now observed with EN and β-gal in the single cell at the centre of each segment (white chevrons).
Later during stage 11, sickle-shaped oenocyte nuclei are observed in the bulge (Figure 4.6B) and these down-regulate EN (Figure 4.7A-B). Data obtained from multiple confocal sections was used to serially reconstruct transverse and sagittal sections through the cells of the whorl at late stage 11 (Figure 4.7A-B). This revealed that the EN-negative sickle-shaped oenocyte precursors lie just beneath the ectoderm and, at this stage, the bulge comprises ectodermal and subectodermal cells (Figure 4.7A-B). At late stage 11, generally one nucleus of the whorl is located within the AP domain of the original EN stripe (Figure 4.6B) but by stage 12 the rounded oenocyte nuclei are all found at the anterior edge of the bulge (Figures 4.5C and 4.6C). Furthermore, most of the cells in the bulge are EN-negative at this stage, and following their ventral migration during stage 13, the oenocytes are positioned at the ventral-most part of the bulge (Figure 4.6D). It appears that all of the cells within the bulge have delaminated by stage 13 (data not shown). Although EN is no longer seen in the oenocytes, β-gal is detected in these cells throughout the rest of embryonic development (Figures 4.5D and 4.6E). Interestingly, the remaining anterior cells that are labelled by this en lineage marker correspond to the five lateral chordotonal organs, which lie just internal to the mature oenocyte cluster (Figures 4.5E and 4.6F). EN protein itself however is only detected at very low levels in one of the four cell types that comprise these PNS components; the neuron (Figure 4.5E).

In summary, oenocyte precursors comprise a subset of cells in the dorsolateral bulge that occurs when cells from the EN stripe move anteriorly. As the en-GAL4 UAS-nlslacZ lineage marker also labels the five lateral chordotonal organs, this suggests that the non-oenocyte cells in the bulge correspond to lateral chordotonal organ precursors (COPs) and their progeny (see Chapter 5).
Figure 4.6 Oenocyte migration during embryonic development

All panels show embryos carrying en-GAL4 and UAS-nls lacZ transgenes (see Materials and Methods) immunostained against β-gal (green) and SAL (red).

(A) At stage 10, a low-level dorsal domain of SAL is observed and en-GAL4 is expressed in a DV stripe that is approximately 3 cells wide.

(B) During stage 11, a lateral bulge is observed in the en-GAL4 stripe. This includes the sickle-shaped nuclei of the delaminating oenocyte precursors.

(C, D) Following delamination, the rounded oenocyte nuclei, often the most anterior cells in the bulge, begin to migrate ventrally (C). By stage 13, oenocytes begin to cluster and are now observed at the ventral extent of the en-GAL4 UAS-nls lacZ bulge (D).

(E-F) Two confocal planes in the same stage 15 embryo display the more external oenocytes which are observed anterior to the main en-GAL4 UAS-nls lacZ stripe (E). Just internal to, and closely associated with, the oenocytes are the lateral chordotonal organs (F). Shown here is lacZ expression in the ligament (l), neuron (n) and scolopale (s) cells that, together with the cap cells (not shown), make up each of the five chordotonal organs. SAL is found in all of the chordotonal support cells but not the neuron (see Chapter 5).
Figure 4.7 Delaminated sickle-shaped oenocyte precursors down-regulate EN

Panels A and B each show the same two parasegments from a single late stage 11 en-GAL4 UAS-nlslacZ embryo where β-gal is in green and EN is in red. Anterior is to the left and dorsal is down. Concentrating on the posterior (right) parasegment, this contains four sickle-shaped oenocyte precursors (1 to 4) that are EN negative/β-gal positive. A single EN positive, β-gal negative, cell lies at the centre of the whorl (asterisk). This is probably the C1 lateral chordotonal organ precursor (COP, see next chapter). Optical reconstructions of transverse and sagittal sections are shown to the right and below the main panel respectively. The transverse and sagittal sections are taken at the DV and AP positions of the white lines, respectively, and thus (A) and (B) display different sections through the same segment. In these INT and EXT refer to internal and external respectively.

(A) The transverse section slices through whorl cell number 3 and shows that this EN negative cell lies beneath β-gal/EN double-positive ectodermal cells. The sagittal section through 1 and 2 similarly shows these oenocyte precursors are subectodermal.

(B) The transverse and sagittal sections respectively show that the sickle-shaped oenocyte precursors 1, 3 and 4 are found at the same subectodermal level as the presumptive C1 cell (asterisk).
Formation of the bulge is surely part of the mechanism by which oenocytes and lateral COPs move out of the en stripe.

4.2.5 A requirement for engrailed but not wingless

Before exploring the complexities of anterior cell movement, I addressed the question of whether en and/or wg are required during oenocyte development. Due to the interdependence of wg and en (reviewed in Perrimon, 1994) it is not trivial to uncouple the inputs from each gene. WG, a secreted glycoprotein, acts to promote en autoregulation (DiNardo et al., 1988; Heemskerk et al., 1991), while HH, a secreted factor produced by the EN cells, has been proposed to maintain wg transcription (Ingham et al., 1991; Ingham, 1993). Thus, functional WG is required for the continued expression of en, and vice versa (see Figure 4.8B), and embryos singly mutant for either gene will eventually lack the products of both segment polarity genes (Martizez Arias et al., 1988). Hence the loss of oenocytes observed in a wg mutant (Figure 4.9B) would also be expected in an en mutant background, and suggests that at least one of these two genes is essential for oenocyte formation.

Two genetic backgrounds were established to uncouple the mutual requirement of en and wg: WG\textsuperscript{on} and EN\textsuperscript{on}. In WG\textsuperscript{on} embryos, wg expression is maintained in the absence of functional EN (see Materials and Methods and Figure 4.8C). Oenocytes were missing in WG\textsuperscript{on} embryos (Figure 4.9D), indicating an absolute requirement for en in the formation of these cells. In the complementary EN\textsuperscript{on} experiment, en expression is maintained in the absence of wg (see Materials and Methods, Figure 4.8D and Grützan et al., 1999), although this does not work very efficiently in dorsal regions (C. Alexandre and P.R.E., unpublished).
Figure 4.8 Uncoupling the interdependence of wg and en

(A) A stage 11 embryo with parasegment boundaries marked by thick black lines. The wg-GAL4, en-GAL4 and prd-GAL4 expression domains are marked, note that prd-GAL4 is only expressed in alternate parasegments/segments.

(B) In the wildtype embryo as of stage 7 secreted Wingless (WG) acts on cells in the posterior compartment to promote engrailed (en) expression. These cells in turn secrete their own signalling molecule Hedgehog (HH) which acts on the most posterior row of cells in the anterior compartment to induce wg transcription.

(C) WG<sup>on</sup> embryos carry the en<sup>CXr</sup> allele where EN is cytoplasmic and non-functional. The hh signalling pathway is artificially provided in the wg cells by expressing a constitutively active form of the HH signal transducer ci (ci<sup>W P16</sup>).

(D) In EN<sup>on</sup> embryos the WG signalling molecule is absent (wg<sup>CXr</sup>) and the wg signalling pathway is maintained in the en cells through the expression of a constitutively active version of the WG signal transducer armadillo (arm<sup>S10</sup>).
A. Stage 11

B. Wild type

C. \text{WG}^{\text{on}}
\begin{align*}
\text{wg} & \quad \text{arm} \\
\text{ci}^{\text{VPi6}} & \quad \text{en}^{\text{CX1}}
\end{align*}

\text{hh signalling pathway maintained in wg cells}

D. \text{EN}^{\text{on}}
\begin{align*}
\text{wg}^{\text{CX4}} & \quad \text{arm}^{S-10} \\
\text{ci} & \quad \text{en}^{\text{CX1}}
\end{align*}

\text{wg signalling pathway maintained in en cells}
Figure 4.9 Uncoupling *wg* and *en* reveals an essential role for *en* in oenocyte formation

Immunolabelling of stage 14/15 embryos with anti-β-gal (A-B) and anti-SAL (C-F).

(A-B) Embryos carrying the *svp-lacZ* enhancer trap *svp^{don1}* either wildtype (A), or mutant for *wg* (B). The *wg* mutants display a complete lack of oenocytes while other *svp-lacZ* expression remains in the CNS.

(C) SAL distribution in a wildtype stage 14 embryo.

(D) Oenocytes are not formed in WG^{on} embryos, where *wg* is added back into an embryo in which EN is non-functional (see Materials and Methods).

(E-F) In EN^{on} embryos, where *en* is added back to *wg* mutant embryos (see Materials and Methods), oenocytes (arrowheads) are formed in a subset of hemisegments (see text for discussion).
Remarkably in EN° embryos, oenocyte clusters were found in a subset of segments (Figure 4.9E-F). The fact that the artificially generated EN stripe rarely extends dorsally into the SAL domain probably explains why oenocyte rescue is only partial. These experiments indicate that en is essential for oenocyte formation and strongly suggest that wg is not required for this process.

en is required for oenocyte formation but is down-regulated during the whorl stage of development, and hence during the process of oenocyte delamination. Is the clearance of EN from oenocytes important for the subsequent development of these cells, for example by providing the trigger for delamination or ventral migration? To address this issue the en-GAL4 driver was used to prolong en expression in oenocyte precursors. Using this system, largely normal oenocyte clusters were produced (Figure 4.10). Thus, although en is essential for oenocyte formation, switching off en during delamination does not seem to be required for subsequent development. However, one unexplored possibility is that the absence of EN is necessary for the expression of genes more downstream than sal, the marker used in this experiment.

4.2.6 Is a lateral break in the WG stripe required for anterior movement?

wg is expressed anterior to the compartment boundary in a one-cell-wide stripe that juxtaposes the more posterior two-cell-wide EN stripe. Previous studies of wg RNA distribution revealed that by stage 11 there is a lateral break in the wg stripe (Ingham, 1993; Ingham and Hidalgo, 1993). It is plausible that this gap in wg expression could allow cells from the EN stripe to migrate anteriorly, resulting in the dorsolateral bulge described above. To map the location of this break with reference to the bulge, WG distribution was examined with respect to that of β-gal
Figure 4.10 Persistent expression of en does not affect oenocyte migration

Embryos overexpressing UAS-en driven by en-GAL4 and immunolabelled with anti-SAL (red) and anti-EN (green).

(A) Despite the continued expression of en, oenocytes have delaminated and remain amongst the most anterior cells in the bulge whilst they migrate ventrally as a string of nuclei during stage 12 (compare to Figure 4.6C).

(B) Following migration, oenocytes cluster anterior to the EN stripe. Using this misexpression system, within a single segment some oenocytes have high levels of EN, while en expression is almost absent in others. However, all oenocytes behave similarly and cluster together in their correct dorsoventral position anterior to the EN stripe.
in en-GAL4 UAS-nls lacZ embryos (Figure 4.11). Lower levels of WG were observed in the lateral region of the stripe during stage 10 (Figure 4.11B). By early stage 11 the break is clear but remains partially bridged by WG containing vesicles (Figure 4.11C). At this stage, it is evident that cells are no longer restricted to the en-GAL4 UAS-nls lacZ stripe and are beginning to move anteriorly (arrow in Figure 4.11C). This migration into the gap in the WG domain results in formation of the bulge (Figure 4.11D), which contains oenocyte precursors and probably precursors of the lateral chordotonal organs (see section 4.2.4 and Chapter 5). Interestingly, although WG regresses even further ventrally, no additional ventral en-GAL4 UAS-nls lacZ positive cells migrated anteriorly (Figure 4.11E-F). In summary, the dorsolateral cells begin to bulge from the EN stripe exactly when and where the break occurs in the adjacent row of cells expressing wg.

Is the formation of the bulge, or more importantly the anterior movement of oenocytes, a consequence of the lateral regression of WG? This theory was tested using prd-GAL4 UAS-wg to prevent the wg break from occurring. At stage 11, a full and expanded stripe of WG was observed in alternate segments, where GAL4 is expressed under the control of the prd promoter (see Figure 4.8A and Materials and Methods). Disappointingly, anterior movement of oenocytes was still normal in the prd-GAL4 UAS-wg segments (Figure 4.12). The even AP spacing between neighbouring mature clusters in wildtype and prd-GAL4 UAS-wg segments also suggests that the extent of this anterior movement is normal (Figure 4.12C). Therefore even in the absence of a break in the WG stripe, oenocytes continue to migrate anteriorly. Perhaps the most interesting phenotype observed in the prd-GAL4 UAS-wg segments is a dorsal misplacement of clusters (Figure
Figure 4.11 Oenocytes migrate anteriorly through a break in the WG stripe

Panel (A) shows a wildtype embryo immunolabelled with anti-WG (red). In all other panels embryos carry en-GAL4 and UAS-nlslacZ and are immunostained against β-gal (green) and WG (red).

(A) A stage 8 embryo showing a full stripe of WG. The posterior boundary of this stripe marks the presumptive parasegmental boundary.

(B-C) The WG stripe breaks during stage 10, however WG-containing vesicles bridge this gap (B). By early stage 11 the break becomes more apparent and the en-GAL4 UAS-nlslacZ stripe just begins to bulge into this gap (arrowheads in C).

(D-F) At stage 12 the bulge from the en-GAL4 UAS-nlslacZ stripe, which contains the oenocyte precursors, extends into the gap in the WG stripe (D). During stages 13 and 14 (E and F respectively) WG regresses further dorsally and ventrally, thus increasing the width of the break.
Figure 4.12 Bridging the gap in the WG stripe

Three segments from embryos misexpressing UAS-wg driven by prd-GAL4, the middle segment in each panel is “wildtype” as prd-GAL4 is expressed in a pair-rule fashion in alternate segments (see Figure 4.8 and Materials and Methods). All three embryos are immunostained against SAL (red) and WG (green).

(A) At stage 11 the DV position of oenocyte precursor whorls (asterisks) is as wild type. The fact that oenocyte whorls are observed in the most posterior domain of the prd-GAL4/UAS-wg stripe is consistent with these cells continuing to form within territory where en is still expressed (C. Alexandre, unpublished).

(B) A stage 14 embryo showing the normal ventral migration in the middle “wildtype” segment compared to the more dorsal location of oenocyte clusters in segments where wg was misexpressed. Segment boundaries are marked by dashed lines. In those segments with a full WG stripe, oenocytes still migrate anteriorly out of the en domain.

(C) In stage 16 embryos the dorsal misplacement of oenocyte clusters remains obvious, but less pronounced, as if the oenocytes have migrated to a certain degree but have failed to reach their final position. High levels of WG are observed in some, but not all, oenocytes in the segments where wg is misexpressed.
4.12C). This suggests that although the break in WG does not play a role in the anterior movement of oenocytes, wg signalling, or the lack of it, may hold the key to understanding the ventral migration of these cells.
4.3 DISCUSSION

In this chapter I have introduced the larval oenocytes of *Drosophila melanogaster* as a relatively new model system in which to study how Hox genes regulate the specification of a single cell type. To facilitate this study I undertook a detailed investigation of oenocyte formation. Oenocyte morphogenesis was followed from cell induction to the end of embryogenesis using a range of markers obtained from an ongoing P-element screen. Twenty-four oenocyte-expressing lines were identified in this screen. These have provided the raw material for adding a further 4 known genes to the 12 published "oenocyte genes" and promise the identification of many new oenocyte genes. I focused on one PlacZ insert into *seven up* and revealed a novel role for this gene and *wg* in oenocyte migration. In addition, I presented initial studies aimed at elucidating the mechanism of anterior oenocyte cell movements.

4.3.1 Oenocyte development – whorls, strings and clusters

This study has revealed that following oenocyte induction (see Chapter 5) there are three main phases to oenocyte morphogenesis. These are delamination, anterior movement and ventral migration. Preliminary data suggests that there may also be a fourth phase with respect to the subepidermal positioning of oenocytes, close to the lateral chordotonal organs (P.R.E. and A. Gould unpublished). Oenocytes are ectodermal in origin and delaminate from this germ layer sometime during early stage 11. They arise in the *en* stripe and this segment polarity gene is essential for their formation. Oenocytes are first distinguished during stage 11 as sickle-shaped nuclei labelled with anti-SAL, *vvl-lacZ*, *svp-lacZ* or *pnt-lacZ* (Figure 4.1B; P.R.E.
unpublished data; Elstob et al., 2001; Rusten et al., 2001). A stable NLS targeted β-gal was used as a lineage tracer for cells originating in the en domain in embryos carrying en-GAL4 UAS-nlslacZ. Both cells in the whorl and mature oenocytes expressed lacZ, strongly suggesting that the sickle-shaped cells correspond to the oenocyte precursors. The anterior movement of oenocyte precursors is discussed in detail in the next section. The final phase of oenocyte movement is their ventral migration. This occurs during stage 12 when a dorsoventral string of oenocyte nuclei is observed migrating ventrally away from their point of delamination close to the dorsal tracheal trunk. Following their migration, mature oenocytes cluster in a final lateral position by the end of stage 13.

4.3.2 Escape from the posterior compartment

Delamination and anterior movement are two early phases of oenocyte development. From the results presented in this study I am unable to confirm which of these two events occurs first. To address this question, a detailed time-course of oenocyte delamination with respect to the anterior movement of cells out of the en domain is required. This is an important issue that will have a bearing on the proposed mechanism for the anterior movement of oenocytes. The anterior edge of the en domain is considered to be a boundary of lineage restriction that keeps ectodermal cells in the posterior compartment segregated from those in the anterior compartment (Vincent and O'Farrell, 1992). There are two possible ways in which oenocytes could move out of the posterior compartment. The first is that cells delaminate first and then migrate anteriorly, thus burrowing under the

1 A copy of Elstob et al. (2001) is included in the back sleeve of this thesis.
undisturbed parasegment boundary. This would not involve violating a lineage restriction within the plane of the ectoderm. However, results presented in this report would argue against this model. Two pieces of data favour the second possibility, namely that oenocyte precursors move anteriorly, while still in the ectodermal layer, and then delaminate afterwards. Firstly, the initial bulge at early stage 11 contains no sickle-shaped cells (Figure 4.5B), the distinctive delaminating oenocyte precursors. Secondly, confocal cross-sections reveal that some of the cells in the bulge, having moved anteriorly, are in the plane of the ectoderm (Figure 4.7A-B).

If it is true that oenocyte precursors move anteriorly while still in the plane of the ectoderm, how do they escape the normal confines of the posterior compartment? One possibility is that the parasegment boundary is not present when the bulge forms. Interestingly it is known that the parasegmental grooves disappear during stage 11 (Martinez-Arias, 1993). However, the en-GAL4 UAS-nlslacZ lineage trace results demonstrate that, in the late embryo, oenocytes and lateral chordotonal organs (see Chapter 5) are the only two cell types that have moved anteriorly out of the en domain. This strongly suggests that, at earlier stages, all of the cells in the bulge are either oenocyte or lateral chordotonal organ precursors. Therefore, the parasegmental lineage restriction would only be violated by two cell types, and only during a very narrow time window. Another possible explanation for the formation of the bulge is that the parasegmental boundary of lineage restriction is maintained but its position is displaced anteriorly to follow the contours of the bulge. This would seem to require a change in the properties of cells anterior to the parasegmental boundary. One attractive hypothesis to explain this was that a break in the WG stripe enables anterior movement. However this
appears not to be the case, as bridging the gap in the WG stripe using a combination of prd-GAL4 and UAS-wg does not block anterior movement. With hindsight this was probably not the best test of the theory as, using the prd-GAL4 driver, wg is also abnormally expressed within the oenocyte precursors and other cells both anterior and posterior to the wg domain. A cleaner experiment would be to misexpress a membrane-tethered version of wg (Pfeiffer et al., 2000), that is resistant to degradation, using wg-GAL4.

Unfortunately there have been very few studies of cell segregation with respect to compartment boundaries in the Drosophila embryo. Most investigations have been carried out on the epithelial monolayer of the wing imaginal disc, where it has been shown that mutant clones of cells lacking en function no longer respect the AP compartment boundary and move anteriorly across it (Morata and Lawrence, 1975). However, loss of en alone can not be the mode of oenocyte movement in the embryo because I have shown that en continues to be expressed in the anterior migrating cells of the bulge. Additionally, even when expressing en at a high level from stage 9 throughout embryogenesis (en-GAL4 UAS-en, Figure 4.10), oenocytes continue to migrate anteriorly.

When cells in the anterior wing compartment lose the ability to respond to HH, the signalling molecule secreted by the EN cells, they invade the posterior compartment (Blair and Ralston, 1997; Rodriguez and Basler, 1997). Dahmann and Basler (2000) recently suggested that cubitus interruptus (ci) and en function in the anterior and posterior compartments, respectively, to regulate cell adhesion molecules and thus control cell segregation. Oenocyte precursors provide evidence that in the embryo the situation may be somewhat different as they continue to express en but can still move out of the posterior compartment.
4.3.3 A novel role for *seven up* in cell migration

*seven up* was identified in the oenocyte screen and is a member of the nuclear receptor gene superfamily of zinc-finger DNA binding proteins. This *Drosophila* molecule contains domains similar to those found in other family members: the glucocorticoid receptor, retinoic acid receptor (RAR) and thyroid hormone receptor (Mlodzik et al., 1990). *seven up* (*svp*) has two alternative splice variants that form type I and type II proteins with an identical amino terminal DNA binding domain, but a divergent carboxy terminus (Mlodzik et al., 1990). The type I *svp* protein is the *Drosophila* homologue of the vertebrate COUP (*chicken ovalbumin upstream promoter*) transcription factor (Mlodzik et al., 1990). These two proteins share 94% identity over 66 amino acids in the DNA binding domain, whilst the putative C-terminal ligand binding domains are 93% identical over 222 amino acids. However, the nature of any ligand for these homologous receptors is unclear. Interestingly both *svp* type I, with its ligand binding domain, and *svp* type II, with no consensus ligand binding domain, have produced similar effects *in vitro* (Zelhof et al., 1995) and *in vivo* (Hiromi et al., 1993) assays.

The name *seven up* derives from a phenotype observed in the fly eye. The compound eye in an adult *Drosophila* is composed of approximately 800 units, or ommatidia. Each ommatidium contains six outer (R1-R6) and two inner (R7, R8) photoreceptors. *svp* mutant ommatidia contain an excess of R7 type photoreceptors, hence the name *seven up* (Mlodzik et al., 1990). This nuclear receptor is required in photoreceptors R1, R3, R4 and R6, and in its absence these neurons assume an R7 fate. Hiromi et al. (1993) used a misexpression assay to show that *svp* acted in these four photoreceptors by preventing R7 differentiation.
svp is also involved in the development of the *Drosophila* malpighian tubules, the structures with a function analogous to the vertebrate kidneys. In this context, *svp* controls the expression of cell cycle regulators (Kerber et al., 1998), and thus cell division (see section 5.3.1).

The phenotype of *svp* mutants reveals a novel role for this *Drosophila* nuclear receptor in cell migration. In mutant embryos, oenocyte clusters were misplaced dorsally but only in a subset of segments. One possible explanation for this non-penetrant migratory phenotype could be redundancy with another nuclear receptor gene. *Hnf4* would be a good candidate as expression of this closely-related nuclear receptor gene has previously been observed in oenocytes (Hoshizaki et al., 1994). Interestingly mutations in the mouse mCOUP-TFI gene, one of two *svp* homologues, display abnormal axonal guidance and arborization in a subset of neurons in the PNS (Qiu et al., 1997). This phenotype may be attributable to defects in growth cone guidance and thus a role in cell migration may be conserved for *svp* and COUP genes.

In addition to the role in oenocyte migration discussed above, I would like to speculate that embryonic *svp* expression might also be involved in oenocyte differentiation. Using a yeast-two-hybrid assay, Zelhof et al. (1995) demonstrated an interaction between SVP and the ecdysone receptor (EcR). Perhaps by sequestering EcR (Zelhof et al., 1995), embryonic SVP could prevent a premature response to the pulse of ecdysone observed during mid-embryogenesis (Riddiford, 1993). If *svp* were subsequently switched off during larval life this would then enable oenocytes to respond to the ecdysone pulses in each of the three larval instars (Riddiford, 1993), and in turn secrete gene products required for moulting. To begin to test this model, an analysis of the temporal expression of *svp* would be
necessary. Although the model predicts that $svp$ would not be expressed around the time of larval moulting, the larval expression of $svp^{don1}$ may not recapitulate the expression of the endogenous gene. In summary, a future study of the role of $svp$ in oenocytes might begin to address the proposed function of oenocytes during moulting.
CHAPTER FIVE

Oenocyte Induction by \textit{abdA} and the EGFR Pathway
5.1 INTRODUCTION

The complex question of how Hox genes direct morphological diversity along the AP axis remains largely unanswered. Unravelling the Hox-mediated regulation of segment identity has proved difficult, due to the multitude of different cell types within a single segment. In a novel approach, I have simplified the question, asking how a single Hox gene specifies the identity of just one cell type. The larval oenocytes of Drosophila were chosen as the model cell type for this study because, as demonstrated in this chapter, a single Hox gene (abdA) is both necessary and sufficient for their specification. In the preceding chapter I focused on the genetic mechanisms controlling oenocyte migration. In this chapter I have concentrated on the embryonic origin of oenocytes and find that it is closely linked to that of the lateral chordotonal organs.

Chordotonal organs are components of the Drosophila peripheral nervous system (PNS) that sense stretch in the larva and adult fly. During embryogenesis, a total of eight chordotonal organs form in each abdominal hemisegment (Ghysen et al., 1986; Campos-Ortega and Hartenstein, 1997); five grouped laterally (Ich5), one dorsally (v'ch1) and two ventrally (vchA and vchB) (see Figure 5.3A). Abdominal chordotonal organs are generated in a two-step process. Five primary chordotonal organ precursors (primary COPs) are selected from proneural clusters of equipotent cells in the lateral ectoderm, that express the proneural gene atonal (ato, Jarman et al., 1993; Jarman et al., 1995). The selected primary COPs then signal, via lateral inhibition (reviewed in Bray, 1998 and Lewis, 1998), to prevent surrounding cells from assuming the same fate (Goriely et al., 1991).
Subsequently, the five primary precursors induce three secondary COPs (two Ich’s and one of the vchA/B class), resulting in eight COPs per abdominal hemisegment (Okabe and Okano, 1997; zur Lage et al., 1997). The signalling event for secondary recruitment is initiated when sustained ato expression in the primary COPs switches on rhomboid (rho) in these cells. RHO is an integral membrane protein that is required for the correct presentation of SPITZ ligand (SPI) to the EGF receptor (EGFR, Bang and Kintner, 2000). Activation of the EGF in ectodermal cells overlying the primary COP results in their differentiation and recruitment as secondary COPs. The recruitment of secondary COPs is under tight negative regulation, as the secreted EGFR inhibitor ARGOS is concomitantly produced in the EGFR activated cells (Okabe and Okano, 1997; zur Lage et al., 1997). Thus ARGOS essentially provides a second round of lateral inhibition, limiting the spread of COP recruitment.

In this chapter I show that oenocytes, like secondary COPs, are induced in abdominal segments in response to EGFR signalling. I provide evidence that the recruitment of oenocytes, rather than COPs, occurs due to molecular differences that prime ectodermal cells for an oenocyte fate. Finally, having established the mode of oenocyte induction, and hence part of the gene hierarchy for oenocyte formation, an initial link is made between the roles of abdA and EGFR signalling.
5.2 RESULTS

5.2.1 *abdA* is necessary and sufficient for oenocyte formation

The precursors of the larval oenocytes arise in the posterior compartment of abdominal segments A1 to A7 during embryonic stage 11, and subsequently move anteriorly (see Chapter 4). The class 7 *Hox* gene *Ubx*, and the group 8 *Hox* gene *abdA* are both expressed within this region but at high levels in the anterior and posterior compartments, respectively (see Figure 1.2 in the General Introduction). Consistent with their origin in the posterior compartment, ABDA is observed in the sickle-shaped oenocyte precursors (V. Brod and A. Gould, unpublished). However, this *Hox* gene is rapidly switched off in these cells as ABDA is never observed from stage 12 onwards. As shown in the preceding chapter, following their anterior movement, oenocytes are located in the anterior compartment. Thus oenocytes are born in *abdA* territory and move into *Ubx* territory, although they do not express *Ubx* (A. Gould, unpublished). To test the possible requirements for *abdA* and *Ubx*, I examined individual and multiple *Hox* mutants for the presence of oenocytes using anti-SPALT as a marker for these cells (see Chapter 4). Removing the single abdominal *Hox* gene *abdA* resulted in the complete loss of oenocytes (Figure 5.1D), whilst clusters are still formed in *Antp, Ubx* and *AbdB* single mutants (Figure 5.1A-C). Therefore *abdA* is the sole *Hox* gene that is essential for oenocyte formation and *Ubx* appears to have no role in the early specification of this cell type.

To test whether *abdA* is sufficient to promote ectopic oenocyte formation, this abdominal *Hox* gene was ubiquitously expressed using a heat inducible
Figure 5.1 The Hox gene abdA is essential for oenocyte development

All panels show embryos between stages 14 and 16 immunostained with anti-SAL (in red).

(A-C) Oenocytes form normally in Antp\textsuperscript{w10} (A), DlUbx\textsuperscript{bx100} (B) and AbdB\textsuperscript{Mt} (C) mutant embryos.

(D-F) Removing abdA function alone results in the loss of oenocytes (D). Oenocytes are also absent when the embryo is deficient for both abdA and Ubx (E), and when all three members of the BX-C are mutated (F).
promoter. A dual heat-shock regime was applied as per Gould et al. (1997) in an attempt to obtain a strong phenotype. Using svp-lacZ as a marker, a range of ectopic oenocyte phenotypes was revealed. The weakest phenotype was a single ectopic oenocyte in T3 (Figure 5.2B) whilst the strongest was that of ectopic clusters in all three thoracic segments (Figure 5.2D). Ectopic clusters were only ever observed in the following groups of thoracic segments: T3; T2 and T3; and T1, T2 and T3 (Figure 5.2B-D). No obvious ectopic oenocytes were formed anterior of T1, even though ubiquitous ABDA has previously been shown to influence the embryonic cuticle in this region (Sanchez-Herrero et al., 1994). Abdominal oenocytes remained wildtype in number following overexpression of abdA (4 to 9 cells per cluster), while ectopic clusters in the thorax contained slightly fewer cells (between 1 and 6). Otherwise the thoracic clusters were identical in appearance and in their dorsoventral position to those found in abdominal segments. These results show that ectodermal cells in the thoracic segments are competent to form oenocytes, and that providing ABDA in these segments is sufficient to trigger this cell fate.

As discussed in earlier chapters, Hox genes often function co-operatively with cofactors belonging to the PBC family. The only member of this family identified in Drosophila is exd, a homeobox gene with both maternal and zygotic embryonic contributions. To assess the requirement for exd during oenocyte development, exd null embryos were generated by the FLP/ovoD germline clone method (see Materials and Methods). Using the svp-lacZ marker revealed that oenocytes were completely absent in these embryos (Figure 5.2E), indicating an essential role for exd in the formation of oenocytes. Unfortunately, from the data presented here, I am unable to draw any firm conclusions regarding the
Figure 5.2 ABDA is sufficient to promote oenocyte formation in the thorax

All embryos are at stage 14 or 15, carry the svp-lacZ marker and are immunostained with anti-βgal. Panels (A-D) are following heatshock treatment (see Materials and Methods) and the white arrowhead marks abdominal segment A1.


(B-D) Ubiquitous expression of abdA, from an hs-abdA transgene, resulted in the formation of ectopic oenocytes in thoracic segments. A range of phenotypes was observed from a single ectopic oenocyte in T3 (red arrowhead in B), to ectopic clusters in all three thoracic segments (D). Panel (C) shows an intermediary phenotype of clusters in T2 and T3.

(E) An embryo lacking maternal and zygotic contributions of exd. Oenocytes are absent although svp-lacZ expression remains in the CNS, out of focus and indicated by a white line.
co-operative function of \textit{exd} with \textit{abdA}. This is because functional \textit{exd} is required for the continued expression of \textit{en} in at least some regions of the embryo after stage 9 (Peifer and Wieschaus, 1990; Rieckhof et al., 1997), and I have already shown that \textit{en} is required for oenocyte formation (see section 4.2.5). To ensure that oenocytes are not lost in an \textit{exd} background due to a lack of \textit{en}, \textit{en} expression would need to be analysed within the dorsal domain of these mutant embryos.

In summary, the single \textit{Hox} gene \textit{abdA} is both necessary and sufficient for the development of oenocytes. Hence this cell type is an excellent model system in which to study how a single \textit{Hox} gene can trigger all of the complexities of cell identity, including induction, delamination, migration and differentiation.

\textbf{5.2.2 Oenocyte precursors are associated with the most dorsal primary COP}

Following their delamination and migration, mature oenocytes coalesce to form tight clusters. These clusters are located just beneath the epidermis and sit in a nest-like structure formed by the five lateral chordotonal organs (Figure 5.3B). This close association between oenocyte clusters and the Ich5 arrays suggested that their formation might be linked. To investigate this idea further, markers for oenocyte precursors (anti-SAL, \textit{sal-lacZ} and \textit{svp-lacZ}) were used together with those for chordotonal organ precursors (anti-RHO and \textit{rho-lacZ}) to follow the development of these two structures. Of the eight COPs formed per hemisegment, only the five primary COPs (named C1 to C5) express \textit{rho}. This is a prerequisite for the EGFR-mediated recruitment of the three extra secondary COPs (Okabe and Okano, 1997; zur Lage et al., 1997). During late stage 10/early stage 11 \textit{RHO} is localised in membrane plaques in all five primary COPs (Figure 5.3C and zur Lage et al., 1997). As described in the preceding chapter, oenocyte precursors are
Figure 5.3 Oenocytes are associated with the Ich5 and form around C1

(A-B) Oenocytes are closely associated with the Ich5 array of the PNS. Abdominal segments from stage 16 embryos immunostained with antibody 22C10 (green in A and brown in B), which labels most neuronal cells of the PNS. Marked in (A) are the three chordotonal arrays observed in each abdominal hemisegment: v’ch1, Ich5 and the vchA/B. Arrowheads in (B) indicate the Ich5 dendrites. Oenocytes, here expressing svp-lacZ (black), lie between the Ich5 and the epidermis.

(C) During late stage 10/early stage 11 RHO is observed in all primary COPs (C1-5). C1/C2 appears to lie under the dorsal SAL domain and sickle-shaped sal-lacZ nuclei are just beginning to form around these PNS precursors. ²

(D) rho-lacZ labels the most anterior lateral chordotonal organ, Ich5a. In addition to labelling oenocytes (asterisks in panel D), SAL is found in the ligament (l), scolopale (s) and cap (c) cells of the lateral chordotonal organs. rho-lacZ overlaps with SAL in these non-neuronal components of Ich5a (not Ich5b-e) and also labels the neuron (n) and one extra cell (open arrowhead, see text). v’ indicates the scolopale cell of the v’ch1.

(E-G) C1 and its daughters express rho and lie at the centre of the oenocyte precursor whorl. (E) Weak rho-lacZ expression is first observed during late stage 10 in the C1 COP, which underlies the dorsal epidermal SAL domain. During stage 11, both SAL (F) and svp-lacZ (G) are upregulated in the sickle-shaped oenocyte precursor nuclei. These form a whorl around C1 and its progeny, which express rho-lacZ (F) and display a localised distribution of RHO in membrane plaques (G).

Panel B was kindly provided by A. Gould.

1 Anti-RHO staining in cells of the tracheal pit/placode is marked “T”.
2 White arrowheads mark two sickle-shaped sal-lacZ positive nuclei that are just beginning to form around C1/C2.
observed at early stage 11 as sickle-shaped nuclei with upregulated levels of the zinc-finger protein SPALT. These sickle-shaped nuclei form characteristic whorl structures at the ventral extent of the dorsal SAL domain (see Chapter 4). Using a sal enhancer trap line, it can be seen that the most dorsal RHO-positive primary COP, C1, lies within the dorsal SAL domain (Figure 5.3C). Believed to be positioned immediately ventral to and overlapping with C1, C2 may also lie within this domain (Figure 5.3C and Okabe and Okano, 1997; zur Lage et al., 1997). The sal-lacZ positive sickle-shaped oenocyte precursors form around C1/C2 (arrowheads in Figure 5.3C).

Each COP divides asymmetrically to produce the ligament, neuron, scolopale and cap cells that constitute a single chordotonal organ (Bodmer et al., 1989; Brewster and Bodmer, 1995; Brewster and Bodmer, 1996). The only chordotonal organ that has previously been mapped to its precursor is the most anterior of the lch5 array (Ich5a) which is formed by the progeny of C1: zur Lage et al. (1997) reported that the C1-specific enhancer trap rho\textsuperscript{lacZ} only labelled Ich5a. Similarly I show that a second PlacZ insert into rho\textsuperscript{x81}, hereafter called rho\textsuperscript{-lacZ} also specifically labels Ich5a (Figure 5.3D). Interestingly, five rather than four cells are labelled, with the additional cell lying just dorsal to the cap cell (open arrowhead in Figure 5.2D). The extra cell is probably one of the two Ich5 attachment cells, thought to function as epidermal anchors for this stretch receptor array (Matthews et al., 1990; Brewster and Bodmer, 1995). Having established that rho-lacZ is C1 specific, this insert was used to follow the C1 lineage simultaneously with the oenocyte precursors, labelled with anti-SAL. C1 was first visualised with rho-lacZ post-delamination, as a SAL-negative cell lying beneath a plane of SAL-positive epidermal cells (Figure 5.3E). By stage 11, oenocyte
precursors can be distinguished by their sickle-shaped nuclei and upregulated levels of SAL. The whorls formed by these cells surround C1 and its progeny (Figure 5.3F). By late stage 11, RHO is absent from C2-C5 (data not shown) but remains in C1, which is surrounded by oenocyte precursors (Figure 5.3G). Unfortunately, due to the absence of any C2 specific markers, I have been unable to confirm that C2 lies at the centre of the whorl. In conclusion, however, C1 clearly lies at the centre of the whorl of oenocyte precursors.

5.2.3 Oenocyte induction is regulated by EGFR activation.

As oenocyte precursor whorls form around the RHO-positive C1 cell during stage 11, presumably they are exposed to secreted SPI ligand (sSPI). In fact, rho is expressed in the C1 cell before oenocyte precursors can be visualised (see Figure 5.3E), therefore it is likely that cells in the dorsal ectoderm see EGFR ligand just before the induction of oenocyte precursor whorls. So is sSPI the signalling molecule for oenocyte induction? To investigate this, I looked in mutants for rho or spi. Sickle-shaped oenocyte precursors and SAL upregulation were absent in both mutant backgrounds (Figure 5.4A-C) but importantly, the moderate dorsal SAL domain remained (Figure 5.4B-C and see Discussion section 5.3.3), suggesting that SPI is not required for this early expression pattern. The lack of oenocyte whorls at stage 11 correlated with the complete absence of mature oenocytes in rho<sup>As5</sup> (Figure 5.4D) and spi<sup>+</sup> (Figure 5.4E) embryos. Additionally, an Ich3 phenotype was observed in both mutants, in agreement with previously published data and the fact that two lateral chordotonal organs are recruited via EGFR signalling (Rutledge et al., 1992; Okabe and Okano, 1997; Wappner et al., 1997; zur Lage et al., 1997). Active SPI is therefore required for oenocyte induction, as
Figure 5.4 EGFR activation is required for oenocyte formation

In all panels anti-SAL is in red and antibody 22C10 in green. Arrowheads indicate the position of each chordotonal organ in (D-F).

(A-C) During stage 11, SAL is upregulated in sickle-shaped nuclei that form a whorl (asterisks in A). These distinctive nuclei and SAL upregulation are both absent in rho (B) and spi (C) mutants. However, the dorsal domain of moderate SAL levels remains in both mutants.

(D-E) Stage 16 embryos mutant for either rho (D) or spi (E) display a fully penetrant Ich3 phenotype and lack all oenocytes.

(F) A stage 15 embryo reared at 25°C and misexpressing UAS-EGFR\textsuperscript{DN} driven by en-GAL4. At this temperature EGFR activity is partially blocked, indicated by the reduced number of lateral chordotonal (Ich4), however a complete absence of oenocytes is also observed.
well as secondary COP recruitment. Given the position of C1 at the centre of the whorl, its requirement for oenocyte formation (see Elstob et al., 2001)\(^1\) and the prolonged expression of \(\text{rho}\) in this cell (zur Lage et al., 1997), I conclude that C1 is the source of the SPI signal.

As active SPI is a ligand for the EGFR, it seems most likely that oenocytes are induced via the activation of this particular receptor. To test this, a dominant-negative form of the EGFR, which lacks the cytoplasmic kinase domain (Freeman, 1996; O’Keefe et al., 1997), was misexpressed in the oenocyte precursors. In the previous chapter I showed that oenocyte precursors originate in \(en\) territory. Therefore an \(en\)-GAL4 driver was used to drive UAS-\(\text{EGFR}^{\text{DN}}\) from stage 9 in the dorsal ectoderm, and subsequently in the sickle-shaped oenocyte precursors themselves. Reducing EGFR activity in this manner resulted in the complete loss of oenocytes (Figure 5.4F).

In addition to oenocyte precursors, all of the lateral chordotonal organ precursors, but not the \(v'\chi1\) or \(v\chi A/B\) COPs, delaminate from the \(en\) stripe (see Appendix II). Using \(en\)-GAL4 to misexpress UAS-\(\text{EGFR}^{\text{DN}}\) (at 25°C) in these PNS precursors resulted in an Ich4 phenotype (Figure 5.4F). The Ich3 phenotype predicted from the complete loss of EGFR activation in \(spi\) and \(\text{rho}\) mutants, was only observed at a higher temperature (29°C, data not shown) when more GAL4 activity, and thus dominant-negative EGFR was produced. The total suppression of oenocyte induction at 25°C, compared to the partial repression of COP recruitment, suggests that oenocytes require more EGFR signalling for their induction. One

\[^{1}\] A copy of Elstob et al. (2001) is included in the back sleeve of this thesis.
possible explanation for this observation could be that the signalling threshold for oenocyte induction is higher than that for COP recruitment.

In summary, the C1 COP appears to be the signalling centre for the recruitment of oenocyte precursors. C1 expresses rho and thus produces active SPI ligand, which in turn activates the EGFR in overlying cells to trigger their induction as oenocytes. This is essentially the same mechanism as that for secondary COP recruitment by the primary COPs (Okabe and Okano, 1997; zur Lage et al., 1997).

5.2.4 The degree of EGFR signalling controls cell-number but not cell-type

Oenocyte precursors only form around C1, and earlier results strongly suggested that this primary COP is the source of the oenocyte inducing signal, SPI. Reduction of EGFR activity using a dominant-negative form of this receptor suggested that a higher level of signalling is required for oenocyte induction than COP recruitment. This correlates well with the rho expression pattern in the five primary COPs, as C1 appears to express rho at a higher level and for longer than the other primary COPs (data not shown and zur Lage et al., 1997). This difference in thresholds might be how the decision is made between oenocyte and secondary COP cell fates. To test this hypothesis, the EGFR pathway was hyperactivated around the more ventral primary COPs (C2-C5), to see if ectopic oenocyte whorls could be formed around these PNS precursors. Firstly, I examined mutants for the secreted EGFR inhibitor argos, in which EGFR signalling should be increased around all primary COPs. These embryos displayed an Ich6 phenotype (Figure 5.5C) as previously described by Okabe et al. (1996). In addition, I also observed enlarged oenocyte precursor whorls with a radius of two sickle-shaped cells (Figure 5.5A),
Figure 5.5 EGFR hyperactivation results in recruitment of supernumerary oenocytes and chordotonal organs

(A-C) Stage 11 argos mutants have additional oenocyte precursors in the whorl, indicated by upregulated SAL (A). By stage 16, these mutants display oenocyte clusters of 15-27 cells (B) while the lateral chordotonal arrays contain an extra organ, resulting in an Ich6 phenotype (C).

(D-F) Embryos carrying en-GAL4 and UAS-sspi show an enlarged whorl (D) during stage 11, similar to argos mutants. SAL upregulation is also observed in a dorsal stripe, where UAS-sspi was misexpressed in the dorsal SAL domain (D). However, sickle-shaped nuclei were not found outside of the expanded whorl structure. At stage 16, mature oenocyte clusters of 21-39 cells were observed (E) in association with disorganised Ich6-7 chordotonal arrays (F).

Arrowheads in (C, F) mark the dendrites of chordotonal neurons.

Mean mature oenocyte numbers are given in Elstob et al. (2001), a copy of which is included in the back sleeve in this thesis.
rather than the normal one cell radius (see figure 5.4A). Consistent with an increase in the number of precursors, large mature oenocyte clusters containing 15-27 cells were observed (Figure 5.5B). Although additional oenocytes were produced in argos mutants, these all derive from a single expanded whorl observed in its normal DV position within the dorsal SAL domain. Why are ectopic whorls not formed ventrally? One possible explanation for this was that EGFR signalling levels were not sufficiently high, or produced for long enough, in argos embryos to induce oenocytes ventrally. To address this issue the UAS/GAL4 system was used to overexpress a constitutively active form of the EGFR ligand, secreted SPI (sSPI, Schweitzer et al., 1995). Overproduction of constitutively active SPI, using en-GAL4, resulted in an increase in the recruitment of both oenocyte and chordotonal precursors. In the mature embryo, lateral chordotonal arrays of 6 or 7 organs were found associated with giant oenocyte clusters of 21-39 cells (Figure 5.5E-F). The increase in oenocyte numbers could be traced back to stage 11 when expanded whorls of precursors were seen (Figure 5.5D). Interestingly, SAL upregulation was not only observed in sickle-shaped nuclei but also in rounded nuclei dorsal to the whorl (Figure 5.5D). Due to the absence of the sickle-shape normally associated with oenocyte precursors, the fate of these dorsal cells is unknown. Despite the dramatic increase in oenocyte numbers following overexpression of active SPI, whorls were never observed outside of the dorsal SAL domain. As no ectopic whorls were produced, I conclude that increased EGFR signalling is not the deciding factor for the induction of oenocytes rather than secondary COPs. Thus, the EGF signalling pathway regulates the number rather than the fate of precursors recruited.
5.2.5 *sal* is sufficient to suppress COP recruitment and is required for oenocyte induction

Results presented in the previous chapter indicate that *en* is responsible for restricting the AP extent of oenocyte induction. As described above, along the DV axis, oenocyte precursors are restricted to the dorsal SAL domain, even in the presence of excess inducing signal. This suggests that some factor(s) restrict oenocyte development to dorsal regions. Given that oenocyte precursors are always recruited from within the dorsal SAL domain, *sal* was a prime candidate for such a factor. Initially, oenocytes were examined in a hypomorphic *sal* mutant, *sal*<sup>1</sup>, using the *svp-lacZ* marker. A severe reduction in oenocyte numbers was observed in these embryos (Figure 5.6A) together with an Ich6 phenotype (Figure 5.6B). This decrease in mature oenocytes was consistent with the loss of sickle-shaped precursors at stage 11 (data not shown). Using the null alleles *sal*<sup>16</sup> or *sal*<sup>145</sup> (Kuhnlein et al., 1994) a more severe phenotype was observed (see Elstob et al., 2001). Mutants for either allele displayed an almost complete loss of oenocytes and a mixed Ich6/Ich7 phenotype. To ensure that the formation of additional chordotonal organs and loss of oenocytes in *sal* null embryos was not a result of aberrant *rho* expression, RHO distribution was examined in these embryos and found to be normal (Figure 5.6C, compare with Figure 5.3C). This provides evidence that *rho* expression in C1 is *sal*-independent. Given that the signalling C1 cell does not express SAL (Figure 5.3E-F), the data suggest that *sal* functions in the responding ectoderm to promote oenocytes and to suppress secondary COPs produced by EGFR signalling.
Figure 5.6 sal is required to promote oenocyte induction but is necessary and sufficient to repress chordotonal recruitment

All panels except (C) show stage 16 embryos. In (B, E) antibody 22C10 is in green and arrowheads label the lateral chordotonal organ neurons.

(A-B) Embryos homozygous for the hypomorphic allele sal' display a severe reduction in oenocyte numbers (A). Oenocytes are labelled with svp-lacZ and arrowheads mark the remaining cells. An Ich6 phenotype was also observed in sal' embryos (B). The asterisk marks a sensory organ of uncertain identity.

(C) The protein distribution of RHO (in red) appears normal in late stage 10/early stage 11 sal'Δ mutants (compare to Figure 5.3C). RHO is located in membrane plaques in the five primary COPs (labelled 1-5) and in cells of the tracheal pit/placode (marked by T).

(D-E) Misexpression of UAS-sal driven by en-GAL4. The expression of svp-lacZ (in red) revealed a modest reduction in the number of oenocytes per cluster (D), while either an Ich3 or Ich4 chordotonal phenotype was observed (E). Mean mature oenocyte numbers are given in Elstob et al. (2001).
Is sal sufficient to repress COP and to promote oenocyte induction when misexpressed ventrally? To explore these possibilities, en-GAL4 was used to drive UAS-sal expression in the ectoderm overlying lateral COPs C1, C2 and C3 (see Appendix II). Rather than generating extra oenocytes, overexpressing sal caused a slight reduction in the number of cells per cluster to between 3 and 6 (Figure 5.6D). Earlier experiments suggested that a higher EGFR signalling threshold exists for oenocyte induction in comparison to COP recruitment (see section 5.2.3). Given this finding, and that sal is expressed in the oenocyte precursors but not the signalling COP, sal itself might be responsible for increasing the signalling threshold for oenocyte induction. In support of this hypothesis, the reduction in oenocyte number upon sal overexpression was more than rescued by the co-expression of an excess of activated EGFR ligand (see Figure 5C in Elstob et al., 2001).

In addition to a weak oenocyte phenotype, en-GAL4 UAS-sal embryos also displayed a consistent Ich3 array (Figure 5.6E), similar to that obtained when EGFR signalling was compromised (see section 5.2.4). This was not rescued to an Ich5 array by co-expressing sSPI (Elstob et al., 2001), indicating that SAL is sufficient to repress EGFR-mediated recruitment of secondary COPs. In conclusion, this suggests that in the endogenous situation, moderate sal expression in the dorsal ectoderm above C1 is sufficient to suppress secondary COP recruitment and is essential to promote oenocyte induction.
5.2.6 Misexpression of the SPI inductive signal is sufficient to produce ectopic oenocytes in the thorax

I have identified five factors that are essential for the development of abdominal oenocytes: an EGFR signalling source, the homeotic gene abdA, its cofactor exd, the zinc-finger transcription factor SAL, and the segment polarity gene en. How do these factors integrate during normal development? In particular, how is oenocyte induction restricted to abdominal segments? Interestingly, the early dorsal SAL domain is not restricted to the abdomen, but also extends into the thoracic segments (data not shown). Similarly, en is expressed in the posterior compartment of all trunk segments (data not shown). However, abdA is restricted to the abdomen and expressing it in the thorax was sufficient to induce ectopic oenocyte clusters. Therefore cells in the thorax have the potential to become oenocytes, and express both competence factors sal and en. Interestingly zur Lage et al. (1997) reported that rho, the activator of the EGFR ligand SPI, is expressed at a lower level in thoracic COPs in comparison to their abdominal counterparts. They suggested that, for this reason, the recruitment of secondary COPs does not occur in the thorax. In agreement with this, thoracic chordotonal arrays are normal in mutants for the EGFR pathway, while overexpression of rho in the thorax was sufficient to promote secondary COP recruitment (zur Lage et al., 1997). Data presented here and in Elstob et al. (2001) suggests that the threshold of EGFR activation required for oenocyte induction is higher than that for COPs, therefore the endogenous EGFR signalling in the thorax is probably insufficient for oenocyte induction. For these reasons, I asked whether supplying a high level of inducing signal in the thorax might be sufficient to generate ectopic oenocytes in the
absence of ABDA. To test this, I used en-GAL4 system to drive secreted SPI in thoracic segments and looked for oenocyte induction with two markers, anti-SAL and svp-lacZ. Remarkably, stage 11 oenocyte precursor whorls were discernible in the thorax with an additional dorsal stripe of cells with upregulated SAL (Figure 5.7A), as seen in abdominal segments (also see Figure 5.5D). Consistent with the presence of ectopic precursors, oenocyte clusters were observed at later stages with both anti-SAL (Figure 5.7B-C) and svp-lacZ (Figure 5.7D-E). Mature thoracic clusters often displayed slight misplacement dorsally with respect to the enlarged abdominal clusters (Figure 5.7C-D). This displacement was also apparent at stage 13 (Figure 5.7B) suggesting a possible delay in the ventral migration of these cells, similar to that observed in svp mutants, as described in Chapter 4.

In summary, misexpression of either the Hox specifier (abdA) or the inducing signal (active SPI ligand) is sufficient to promote the oenocyte developmental cascade in thoracic segments. It is amazing that simply by supplying a high enough level of the inducing signal, oenocytes can be formed ectopically.
Figure 5.7 Misexpression of secreted spitz is sufficient to induce ectopic oenocytes

All embryos misexpress UAS-sspi using en-GAL4 and are immunolabelled against SAL (green) or, if also carrying svp-lacZ, against βgal (red).

(A) At stage 11, SAL upregulation marks ectopic oenocyte precursor whorls in thoracic segments T2 and T3. SAL upregulation was also observed in thoracic segments dorsal to the ectopic whorl, roughly within the en-GAL4 domain. This is also observed in abdominal segments (compare T2/T3 to A1 and also see Figure 5.5D).

(B-C) SAL also labels the mature clusters found in T2 and T3. These ectopic thoracic clusters are generally located dorsal to, and are smaller than, the giant clusters found in abdominal segments. In (B), DT labels the dorsal trunk of the trachea, a good landmark for the DV position of oenocyte delamination.

(D-E) Ectopic oenocyte clusters are also observed in thoracic segments using a second marker, svp-lacZ. Again thoracic clusters are generally smaller, and often found in a more dorsal position than abdominal clusters (D). However, clustering is not always normal and this results in oenocytes being scattered laterally (E).
5.3 DISCUSSION

In this chapter I have identified abdA as the abdominal Hox gene that regulates oenocyte cell fate in the Drosophila embryo. As misexpression of abdA is sufficient to specify ectopic oenocytes, this is an ideal model in which to study how a single Hox gene controls the identity of a single cell type. Presumably abdA lies towards the top of a genetic hierarchy for oenocyte development, which would control cascades of events sequentially resulting in cell induction, delamination, migration and differentiation. In addressing the nature of oenocyte induction I have begun to dissect this genetic hierarchy. Here I discuss how oenocytes are induced by the PNS, in a way that is analogous to the secondary recruitment of chordotonal organ precursors. In both instances the inducing signal is the EGFR ligand SPI, and its source is a primary COP. A prime-and-respond model is presented to explain how two distinct cell types are formed in response to an identical signal.

5.3.1 Oenocytes and secondary COPs are recruited by the same inducer

Where is EGFR activation required during oenocyte and chordotonal organ induction? Compromising EGFR activity by a number of means (rho, spi, en-GAL4 UAS-EGFR) results in the loss of oenocytes. However, given that both C1 and the oenocyte precursors express a lacZ enhancer trap in pointed (see Figure 1G in Elstob et al., 2001)\(^1\), a transcription factor acting downstream of the EGFR (O'Neill et al., 1994), this could be an indirect effect of reducing EGFR activity in C1. Two pieces of evidence indicate that this is not the case. Firstly, activated Rolled Map

\(^1\) A copy of Elstob et al. (2001) is included in the back sleeve of this thesis.
kinase, a downstream component of the EGFR signalling pathway, is only observed in the sickle-shaped oenocyte precursors and not in C1 (Rusten et al., 2001). This suggests that the EGFR pathway is not active in C1 at the time of oenocyte recruitment. Secondly, neutralising EGFR activity in the presumptive oenocytes, but not C1, was sufficient to prevent oenocyte induction (sal-GAL4 UAS-EGFR\textsuperscript{ON} in Elstob et al., 2001). Therefore, like secondary COP recruitment (Rusten et al., 2001), EGFR activation is required in overlying ectodermal cells to trigger oenocyte induction. In both cases the signalling centres are the primary COPs that produce active SPI ligand under the control of \textit{ato} and \textit{rho} (zur Lage et al., 1997).

The maximum number of sickle shaped oenocyte precursors observed in wild type embryos is four, while oenocytes clusters contain four to nine mature cells. Two possible explanations for this irregularity are that the oenocyte precursors divide or that there is more than one round of recruitment. Considering the former possibility, it is interesting that EGFR signalling regulates cell division in the developing malpighian tubules (Baumann and Skaer, 1993). It has been shown that \textit{svp}, a gene also expressed in oenocytes, acts downstream of the EGFR pathway to control the expression of cell cycle regulators \textit{string} and \textit{cyclin E} (Kerber et al., 1998). However, oenocytes do not appear to be labelled when BrdU experiments were carried out at the approximate time when we would expect oenocytes to be dividing (Bodmer et al., 1989). I therefore favour the second model to explain the anomaly between the number of oenocyte precursors and the number of mature cells, whereby more than one round of oenocyte recruitment occurs.
Increased EGFR signalling resulted in a huge excess of oenocytes but only a limited number of extra lateral chordotonal organs. This suggests that COP recruitment has an additional level of negative control relative to oenocyte induction. Additional negative regulation might also account for the exact number of secondary COPs in the wild type embryo (three) in comparison to the variable size of an oenocyte cluster (four to nine cells). Notch-mediated lateral inhibition is a good candidate for the negative regulator as it has been shown to antagonise EGFR signalling in a number of developmental contexts (de Celis et al., 1997; zur Lage and Jarman, 1999; Culi et al., 2001). To take the closest example, the reiterative recruitment of sensory organ precursors (SOPs) of the adult *Drosophila* chordotonal organs is controlled by Notch-EGFR antagonism (zur Lage and Jarman, 1999). Here SOPs are progressively recruited from a persistent *ato* positive proneural cluster, which is maintained by EGFR signalling. The EGFR pathway also induces SOP commitment, similar to its role in the recruitment of secondary COPs in the embryo, while Notch signalling inhibits commitment. Thus, in addition to control at the level of *spilargos*, antagonism by N could account for the precise number of secondary COPs recruited in the wild type situation, and also for the limited number of extra chordotonal organs recruited upon EGFR hyperactivation.

**5.3.2 Fate mapping oenocyte and secondary COP induction**

Within an abdominal hemisegment, five primary COPs (C1-C5) induce three secondary COPs and approximately six oenocytes. Which of these signalling centres induce oenocytes and which recruit chordotonal precursors? The continued formation of oenocytes in *ato* mutant segments, where only C1 remains,
demonstrated that C1 is likely to recruit these precursors (see Elstob et al., 2001). This is in agreement with C1 lying at the centre of oenocyte precursor whorls, positioned at the ventral extent of a dorsal ectodermal SAL domain (Figure 5.8). Further support for C1 recruiting oenocytes from the SAL domain is given in Elstob et al. (2001), where expression of a dominant-negative form of the EGFR driven by sal-GAL4 had no effect on chordotonal numbers, while oenocytes were lost. Even when hyperactivating the EGFR all along the DV axis, oenocytes are only ever induced dorsally due to an absolute requirement for SAL. So the role of C1 seems clear, but the experiments presented in this thesis are less conclusive regarding the position of C2 (Figure 5.8). If C2 does lie in the dorsal SAL domain (see Figure 5.3C and section 5.2.2), it is also possible that it does not produce a sufficiently high enough level of RHO to promote oenocyte induction (zur Lage et al., 1997).

Although sal is essential for oenocyte formation, misexpression of this gene is insufficient to promote oenocyte recruitment around the more ventral sources of EGFR ligand. In contrast, driving sal expression ventrally is sufficient to inhibit the recruitment of secondary COPs. Therefore, in the endogenous situation, sal must prevent the recruitment of secondary COPs by C1.

Lineage labelling studies in Chapter 4 showed that oenocytes and all five lateral chordotonal organs derive from en territory, meanwhile data presented in Appendix II demonstrates that the v'ch1 and vchA/B organs do not. Given that C1 is the Ich5a precursor (see section 5.2.2 and zur Lage et al., 1997), this primary COP must delaminate from the en domain. C2 and C3 lie in a line directly ventral to C1 (Figure 5.8), thus by inference these two primary COPs also delaminate from the posterior compartment, and must therefore contribute to the Ich5. In addition to

190
Figure 5.8 A fate map for oenocytes and abdominal chordotonal organs

Each panel represents a single abdominal hemisegment. Primary COPs and the oenocytes and secondary COPs they recruit are shown in (A), which encompasses embryonic stages 10 and 11. The mature oenocyte and chordotonal arrays are shown in (B) at stage 15. It should be noted that in the embryo there is overlap between the oenocyte cluster and the lch5 and v'ch1 arrays, which have been drawn apart in this cartoon for clarity.

Each abdominal hemisegment contains five primary COPs (C1 to C5 in A). C1-C3 are located in a DV column in the EN domain (brown stripes) while C4 and C4 are slightly anterior and lie outside of the EN domain (see Appendix II). EGFR signalling (arrows in A) from C1 results in the induction and delamination of 4 to 9 (6 on average) sickle-shaped oenocyte precursors (dark red) from the ectodermal dorsal SAL domain (light red). Ventral to the SAL domain, EGFR signalling from primary COPs results in the recruitment of secondary COPs. C1, C2, and C3 (light green), together with the two secondary COPs (dark green) recruited by C3, contribute to the lch5. C4 (yellow) does not induce any secondary precursors and produces the single v'ch1 array. EGFR signalling from the C5 COP (light blue) results in the recruitment of a single secondary COP (dark blue) and together these generate the ventral vchA/B pair of chordotonal organs, as shown. See text for more details.
Oenocyte induction

A

C1

C2

C3

C4

C5

B

Oenocytes

C4

C1 C2 C3

Ich5

C5

v'ch1

vchA

vchB
C1-C3, two extra secondary COPs need to be recruited from the en domain in order to form the complete Ich5. As C2 is probably not a significant signalling centre, by elimination, C3 must recruit both of the secondary lateral COPs (Figure 5.8). This conclusion differs from that of zur Lage and colleagues (1997) who thought that C1 and C3 each recruited a secondary COP.

Chordotonal precursors C4 and C5 are located more anteriorly than C1-C3, and presumably lie outside of the en stripe. This suggests that C4 and C5 contribute to the v'ch1 and vchA/B organs, which do not label with en-GAL4 UAS-nls lacZ. C4 expresses rho at a low level, suggesting that this is the progenitor of the v'ch1 while C5, with a higher level of RHO, probably recruits one extra COP to form the vchA/B doublet (Figure 5.8 and zur Lage et al., 1997). Interestingly, there appears to be a correlation between the duration and/or level of rho expression and the number of cells recruited by a primary COP: C1 induces 4-9 oenocytes, C3 recruits two COPs and C5 recruits one COP, while C2 and C4 do not appear to recruit at all. With respect to rho, C1 maintains expression at the highest level for the longest period, followed by C3 and C5. C2 and C4 express low levels of rho and switch off expression first (zur Lage et al., 1997). Hence there is a correlation, in that the longer the duration, and higher the level of rho expression, the more cells are recruited.

I have been able to use data presented in this thesis and that from zur Lage et al. (1997) to construct a fate map for all chordotonal organs and oenocytes in the abdomen (Figure 5.8). This is in broad agreement with the origin of the chordotonal organs inferred by zur Lage et al. (1997), who followed the primary COPs and their progeny in embryos carrying an ase-lacZ transgene.
5.3.3 A prime-and-respond model

The sal mutant phenotype is a loss of oenocytes and an increased number of lateral chordotonal organs. Epistasis tests have confirmed that the additional chordotonal organs in these embryos are derived from secondary COPs (Elstob et al., 2001; Rusten et al., 2001). These experiments also showed that sal acts downstream or in parallel to EGFR signalling to both repress the recruitment of secondary COPs and induce oenocytes. Data presented here indicates that sal must function in both a parallel pathway and downstream of the EGF signal. The early moderate dorsal expression of sal continues in both rho and spi mutants and is thus EGFR-independent. Hence the dorsal SAL domain is established via a parallel pathway to EGFR signalling. There are two pieces of evidence for sal acting downstream of EGFR signalling. Firstly, in both rho and spi mutants, the SAL upregulation normally seen in whorls was absent. Secondly, an activated secreted form of SPI driven in the en domain resulted in SAL upregulation wherever misexpression overlapped with the dorsal SAL domain. This also implies that a moderate level of sal expression is a prerequisite in a cell for the upregulation of SAL upon receipt of the SPI signal.

How do ectodermal cells decide on an oenocyte versus a COP cell fate following receipt of the EGFR induction signal? Although sal is not sufficient to direct a cell fate decision it is an essential factor for oenocyte formation, required both in parallel and downstream to EGFR activation. These two distinct phases of sal function can be explained in a prime-and-respond model for oenocyte induction (Figure 5.9). According to this model, moderate sal expression, independent of EGFR signalling, primes cells in the dorsal ectoderm to become oenocytes, rather
Figure 5.9 The prime-and-respond model

The central panel represents a single abdominal hemisegment and illustrates the positions of five ato-expressing primary COPs (C1-C5) relative to the dorsal domain of low sal expression (light red). C1 is the signalling centre for the induction of oenocytes (left panel) whilst C3 is the signalling centre for the recruitment of two secondary COPs (right panel), which contribute to the Ich5 array (see Figure 5.8 for further details). Strong and persistent SPI signalling from C1 (large yellow arrows in the left panel) activates the EGFR pathway in overlying ectodermal cells which results in the induction of oenocytes (O). Low level SAL is one component of a prepatter that primes cells to become oenocytes. One of the earliest events in response to EGFR signalling is the upregulation of SAL itself. The right panel shows the induction of two secondary COPs (2°) via moderate SPI signalling from C3 (small yellow arrows). This results in activation of the EGFR in overlying SAL-negative ectodermal cells and the recruitment COPs. SAL expression in the dorsal ectoderm also acts to suppress secondary COP recruitment by C1.
oenocyte induction → primary COPs → chordotonal induction

- **Dorsal**
  - C1
  - C2
  - C3
  - C4
  - C5

- **Ventral**
  - 2°

Legend:
- **ATO**
- low/high **RHO**
- **EGFR activation**
- low/high **SAL**

The diagram illustrates the progression from oenocyte induction to chordotonal induction through the intermediation of primary COPs.
than adopting the default chordotonal organ fate upon receipt of the inducing signal. Molecular differences that prime subsets of cells to adopt particular fates upon induction are termed prepatterns. Hence, the prime-and-respond model proposes that sal prepatterns dorsal ectodermal cells to become oenocytes. However, it can be assumed that other dorsal competence factors are also required, given that sal expression alone is insufficient to promote oenocyte induction upon EGFR activation. In addition to its role as an oenocyte prepattern gene, the results from experiments varying the concentration of sal or EGFR activity suggest that sal also increases the apparent threshold of EGFR signalling required to promote a response. This correlates with the high and prolonged expression of rho that is seen in C1 in comparison to the other abdominal primary COPs.

One of the earliest events in response to EGFR signalling is the upregulation of SAL, observed in the committed sickle-shaped oenocyte precursors. As previously discussed, a moderate level of SAL appears necessary for this subsequent upregulation. Therefore sal provides a molecular link between the oenocyte prepattern and the response to EGFR signalling. This may be similar to the relationship between sal and EGFR signalling during the development of the dorsal tracheal trunk. In this system, it has been reported that the initial transcription of sal is EGFR-independent, while EGFR signalling is required for the maintenance of sal expression (Chen et al., 1998).

The absence of svp and vvl expression in sal mutants (Figure 5.6A in this chapter; Figure 4B in Elstob et al., 2001; Figure 7H in Rusten et al., 2001) indicates that both genes lie downstream of sal in the genetic hierarchy for oenocyte differentiation. Both of these genes are upregulated in cells of the whorl, but not in

197
other cells within *sal* territory. This suggests that high levels of SAL positively regulate the transcription of *svp* and *vvl*, while the moderate level of *sal* that primes the dorsal ectoderm has no input. However, the dorsal SAL domain does appear to repress secondary COP recruitment and thus low level SAL might negatively regulate the expression of genes specific to these neuronal precursors. A concentration dependent role has also been implied for SAL in the transcriptional regulation of *knirps*, during the formation of the L2 wing vein (de Celis and Barrio, 2000).

In summary, the prime-and-respond model has been proposed to explain the dual role of SAL during oenocyte induction. This model predicts that SAL functions in the dorsal ectoderm to prime cells with an oenocyte prepattern. An early EGFR response appears to be the upregulation of SAL itself, which is required for the expression of downstream oenocyte markers.

5.3.4 *abdA* and oenocyte specification

The abdominal *Hox* gene *abdA* is necessary for the formation of oenocytes and is sufficient to promote ectopic clusters in the thoracic segments. In this latter experiment, *abdA* was ubiquitously expressed using a heat-inducible promoter and a dual heat-shock protocol (see Materials and Methods). The variable thoracic phenotype produced (see Figure 5.2) suggests that there is a critical time window during extended germband stages when ABDA can specify the oenocyte developmental pathway. A more precise single heat-shock regime would be necessary to determine this competence period more precisely. A similar approach could be used to establish the time at which endogenous *abdA* function is required for the formation of abdominal oenocytes. Using the heat-shock system, ABDA
could be added back into abdA mutants at specific developmental time points, and the embryos examined for the presence of oenocytes. Although diagrams often depict uniform expression of a Hox gene within a single segment this is generally not the case. Rather a Hox gene is expressed in specific cells within a segment at different times and levels (Castelli-Gair and Akam, 1995). Salser and Kenyon (1996) showed the importance of this mosaic of Hox expression in Caenorhabditis elegans, where the precise expression of a single Hox gene in a single lineage controls four different cell fate decisions. Similarly, spatiotemporally regulated expression of abdA might be required to promote oenocyte induction in the abdominal segments.

Oenocytes are absent in abdA mutants, and the abdominal IchS is replaced by a dorsal chordotonal array consisting of three organs (dch3), which is characteristic of thoracic segments (Heuer and Kaufman, 1992). Therefore, with respect to both oenocytes and chordotonal organs, in abdA mutants, the abdomen is transformed towards the identity of the thorax. In the wild type situation, the thoracic primary COPs express rho at a low level (zur Lage et al., 1997), probably explaining the lack of oenocyte induction and secondary COP recruitment in this region. I have shown that misexpressing a constitutively active form of the EGFR ligand (sSPI) mimicks the misexpression of abdA, as in both cases it is sufficient to promote ectopic oenocyte formation in thoracic segments. This suggests that abdA could promote oenocyte formation by influencing the duration and/or level of EGFR ligand production. In this scenario, abdA would play a non-autonomous role in the formation of oenocytes by controlling the induction signal generated in a single cell, the C1 primary COP.
Interestingly, misexpression of the active EGFR ligand (sspi) resulted in giant oenocyte clusters, while overexpression of abdA had no effect on abdominal oenocyte numbers. This would seem inconsistent with the speculative role of abdA in regulating the degree of EGFR signalling. This discrepancy could be explained by an additional level of control, other than abdA, that restricts the upper limit of EGFR signalling. However, it is equally likely that this is a cell number versus levels issue. That is, it is possible that the degree of EGFR signalling from C1 is almost saturated in the wildtype situation, which would explain why adding more abdA has no effect. Meanwhile misexpressing sspi in all cells in the posterior compartment (en-GAL4 UAS-sspi) would dramatically increase the number of cells producing ligand in addition to C1, resulting in the recruitment of excess oenocytes.

The homeodomain protein encoded by hth is essential for the nuclear localisation of EXD, the Hox cofactor. Interestingly the PNS phenotype reported in both exd and hth mutants matches that observed in abdA mutants, whereby the Ich5 array is transformed into a thorax-like dch3 array (Heuer and Kaufman, 1992; Kurant et al., 1998). In addition, I have shown that oenocytes are absent in both abdA and exd mutants. One explanation for the similarity of these results would be a lack of EGFR-mediated recruitment in abdominal segments in abdA, exd and hth mutants. If this is the case, abdA could fulfil its oenocyte identity function by regulating EGFR signalling in a cofactor-dependent manner. Therefore the work presented in this thesis has come round full circle, and as in my study of the LNE described in Chapter 3, I implicate cofactors in the regulation of Hox target genes.
There comes a time when...“Only daring speculation can lead us further, and not accumulation of facts.” ALBERT EINSTEIN
6.1 From Hox genes to morphogenesis

How genes control morphogenesis is one of the fundamental questions in developmental biology. Since Ed Lewis' seminal work on mutations in the BX-C, and the segment transformations these generated (Lewis, 1963; Lewis, 1978), the Hox genes have been the focus of many studies. Despite over 20 years of research in Drosophila, little is known about how even a single Hox gene regulates diverse morphological events within a single segment. The principle aim of my first study was to investigate the transcriptional mechanisms by which HOX proteins regulate their targets. To achieve this, I dissected the LNE model HOX target gene at the molecular level. In a second study, I took a novel approach and investigated how one Hox gene specifies one cell identity, the larval oenocyte.

6.2 The LNE is a target for multiple HOX proteins

The most important conclusion from my study of the LNE in Drosophila is that it is regulated by multiple Hox genes in a highly complex manner. This 370bp enhancer from the mouse Hoxb4 gene contains two closely spaced HOX sites, HS1 and HS2, both of which are essential for enhancer function. Each site has a different Hox specificity and both modulate a response to more than one Hox regulator (Figure 3.10). Together, HS1 and HS2 integrate the responses to three Hox activators (Dfd, Scr and Antp) and potentially two Hox repressors (Ubx and abdA). Unlike studies on minimal HOX-PBC sites of 20bp (Chan et al., 1997), altering two specificity conferring nucleotides in the HS2 HOX-PBC site, in the context of the full length LNE, did not result in a switch of HOX specificity. This suggested that sequences remote to HS1 and HS2 also help to define its HOX-responsiveness. In
agreement with this, I have shown that sequences upstream, downstream and in-between HS1 and HS2 are important for LNE function. The loss of LNE activity following the mutation of sequences located upstream and downstream of the HOX sites indicates a positive role for these highly conserved sequences. Meanwhile the ectopic LNE activation observed upon manipulating the sequence between the HOX sites indicates that this linker region is involved in restricting the Hox response to a subset of the Dfd/Scr/Antp territory. Thus in order to gain a true picture of HOX target gene regulation, full-length enhancers rather than minimal HOX-PBC sites should be analysed. Although they are an essential component of any target enhancer, the HOX-PBC sites appear to be just one of a number of regulatory modules, each of which are required for correct temporal and spatial expression.

The Hox cofactor exd is essential for proper LNE activation. Previous in vitro binding assays showed that EXD does not heterodimerise with DFD on HS2 oligonucleotides but somehow stimulates recruitment of the HOX protein to the DNA (Chan et al., 1997). However, I have observed a loss of in vivo LNE activity when an essential nucleotide for EXD but not HOX binding (Chan and Mann, 1996) is mutated in the HS2 site. This suggests that the lack of EXD binding to DNA in vitro might not reflect the in vivo situation. Perhaps the in vitro experiments lacked some tertiary factor that assists EXD recruitment to the DNA. One candidate for this might be HTH, which can interact with HOX/EXD complexes on DNA (Ryoo et al., 1999).

The LNE results presented here are consistent with all three models that have been proposed to explain HOX specificity in vivo: the binding site selection, activity regulation and composite models (see sections 1.9 and 1.10). To
discriminate between these possibilities, *in vitro* binding studies would be necessary in the future to compliment the *in vivo* mutational analysis presented in this thesis. Together with the transcriptional activation data presented here, binding studies would greatly increase our molecular understanding of how this Hox target gene is regulated.

6.3 Larval oenocytes - a new model system to study Hox specification at the single cell level

For many years it has been known that Hox genes specify segment identity in *Drosophila*. However, a single segment is composed of numerous different cell types, and in order to specify them, it is predicted that a large number of genes act downstream of the Hox specifier (Mastick et al., 1995; Akam, 1998; Liang and Biggin, 1998; Weatherbee et al., 1998; Mannervik, 1999). This makes the study of how Hox genes control segment identity extremely complex. The current hypothesis for the function of Hox genes is that they micromanage many individual cell fates and behaviours, and it is the integrated action of these regulations that defines segment identity (Akam, 1998b). I have therefore taken a novel approach to studying Hox regulation of morphogenesis, asking how a single Hox gene specifies a single cell identity. The larval oenocytes were chosen for this study because they represent a clear and well-defined identity that is on in some segments and off in others. In addition they are under the exclusive control of one Hox gene, *abdA*. Removing this gene results in the loss of oenocytes, whilst misexpressing it is sufficient to specify ectopic oenocytes in the thorax.
6.4 Oenocyte movement can be separated into AP and DV phases

Prior to the work presented in this thesis, there were very few studies of the larval oenocytes in *Drosophila*. Thus to facilitate my investigation of how *abdA* controls the identity of this cell type, I initially characterised their embryonic origin. Using a range of markers identified in an enhancer trap screen, I showed that oenocytes undergo two distinct phases of cell movement, in anterior and ventral directions respectively. As detailed in Chapter 4, ectodermal oenocyte and lateral chordotonal organ precursors derive from the posterior compartment and move anteriorly. These cells bulge out from the *en* stripe and thus may cross the parasegmental boundary. This was unexpected as the anterior border of the *en* stripe is considered a boundary of lineage restriction. As the moving cells form a bulge with a defined anterior edge, and do not mix with anterior cells, I would like to speculate that these cells lose the adhesive properties of the posterior compartment, whilst not gaining the adhesion properties of cells in the anterior compartment. Hence, anterior movement of oenocytes and lateral COPs might be mediated by repulsion from cells in the posterior compartment. Studying how oenocytes overcome the lineage restriction of the posterior compartment might provide an insight into the normal mechanisms of cell sorting and boundary formation.

In a second phase of movement, the delaminated oenocyte precursors migrate ventrally from their dorsal origin to finally lie in a ventrolateral position just beneath the epidermis. I have identified a gene, *svp*, required for the ventral aspect of migration (see section 4.2.3). In addition, when *wg* was misexpressed, normal ventral migration was disrupted (see section 4.2.6). Given the apparent lack of any gross ventral cell movements in abdominal segments during embryonic stage 12, I
think it likely that the ventral migration of oenocytes is an active process. This would occur in response to a specific signal and is likely to be driven by the formation of filopodia. Interestingly, the COPs and their progeny also migrate ventrally but at a slightly later stage, beginning after the oenocyte clusters have assumed their final position (data not shown). Oenocytes and COPs could prove to be a valuable model for active cell migration.

6.5 Do oenocytes have serial homologues in other segments?

_Hox_ genes often function to individualise serially homologous structures. The classic example of this is the differentiation of the _Drosophila_ haltere from the wing through the action of _Ubx_ (Weatherbee et al., 1998). In a recent study, Casares and Mann (2001) defined the developmental ground state for the ventral appendages and showed how this is modified by _hth_ and _Antp_ to generate antennal and leg structures, respectively. Other serially homologous structures, the genitalia and the analia, are likely to be formed when this ventral groundstate is modulated by _AbdB_ (Estrada and Sanchez-Herrero, 2001) and _caudal_ (Moreno and Morata, 1999), respectively. In the oenocyte system, clusters only form in segments A1 to A7. Are there serial homologues in other segments? In the thorax the answer appears to be no, as there is no EGFR recruitment around C1. For A8, however, there is a subset of dorsoposterior ectodermal cells that closely resemble oenocyte precursors. These A8 cells were identified because, like the oenocyte precursors in A1 to A7, they label with the early oenocyte marker _svp^dom1_. Presumably because they are under the control of _AbdB_, rather than _abdA_, these cells do not form oenocytes but instead become associated with the posterior spiracles. It would be interesting to investigate whether the dorsoposterior...
precursors in A1-A7 and those in A8 are equipotent and their cell fate simply depends upon the choice of Hox specifier. Given that misexpression of AbdB is sufficient to direct the development of spiracle structures on more anterior segments (Kuziora, 1993), would AbdB also transform oenocytes into these posterior spiracle-associated cells? In the reciprocal experiment, is an oenocyte cluster generated in A8 upon removing AbdB and misexpressing abdA using the heat-shock system?

In summary, oenocytes appear to have serial homologues in A8, associated with the posterior spiracles, but not in the thoracic segments. It is therefore possible that the Hox-independent ground state, which is partially thoracic (Struhl, 1983; Macias and Morata, 1996), is a segment completely lacking oenocytes. To test this idea further, embryos mutant for Scr/Antp/Ubx/abdA/AbdB, which lack all Hox expression in trunk segments, would need to be examined. Oenocytes and the posterior spiracle-associated cells might provide a simple two-fate system in which to address how initially equipotent cells achieve different fates through the action of two distinct Hox genes.

6.6 Multiple responses from one receptor: the role of prepatterns

The Drosophila EGF receptor is used in many different developmental contexts to initiate specific cell fate decisions (Perrimon and Perkins, 1997; Schweitzer and Shilo, 1997). In elucidating the mechanism of oenocyte induction, I have begun to address how this universal signalling pathway directs the development of two distinct cell types, the oenocyte and secondary COP. The moderate expression of sal in the dorsal ectoderm modifies the competence of these cells, promoting oenocyte rather than COP induction upon EGFR activation. Interestingly, sal is
also one of the transcriptional regulators that prepatterns sensory hairs on the
*Drosophila* thorax (Modolell and Campuzano, 1998; de Celis et al., 1999). A
genetic prepattern that predisposes a cell to adopt a defined fate, when it receives
the correct signal, is an attractive hypothesis to account for the specificity of
responses to a universal signal (Simon, 2000). In two recent studies, Lozenge, the
runt domain transcription factor, was shown to prepattern cells in the developing
eye, promoting specific transcriptional responses to the RAS signalling pathway
(Flores et al., 2000; Xu et al., 2000). These studies, together with related studies of
Halfon et al. (2000), provide evidence for the combinatorial regulation of specific
target genes. This occurs through the integrated action of transcription factor
prepattern genes and the downstream nuclear components of common signalling
cassettes. In conclusion, the idea of a prepattern, included in the prime-and-
respond model, provides a generic mechanism for the control of cell fate decisions,
whereby a universal signal(s) triggers a cryptic cell identity that is encoded by the
particular combination of prepattern factors.

6.7 Keeping an eye on oenocytes and COPs

In *Drosophila*, striking parallels can be drawn between the oenocyte/COP system
and photoreceptor recruitment in the eye. The development of the abdominal
chordotonal organs and oenocytes is linked in an induction process that is
comparable to that observed in the assembly of each ommatidial unit in the eye
imaginal disc. Similar to the formation of primary COPs, the first photoreceptor (R8)
of each ommatidial unit is selected from a cluster of equipotent cells expressing the
proneural gene *ato* (Jarman et al., 1994). Secondary COP, oenocyte and most
alternative ommatidial cell fates are determined by a phase of induction which is
initiated when the committed R8 cell or primary COP produces active SPI (Freeman, 1994; Tio et al., 1994; Okabe and Okano, 1997; zur Lage et al., 1997). In both systems, activation of the EGFR in nearby uncommitted cells results in their differentiation and recruitment as photoreceptors, secondary COPs or oenocytes. In the eye imaginal disc, this process is repeated when the recruited cells subsequently produce SPI (Freeman, 1996). This reiterative recruitment event is precisely controlled by the EGFR inhibitor argos, which is secreted by EGFR activated cells (Golembo et al., 1996). In my discussion to Chapter 5 (section 5.3.1), I suggested that oenocytes may also undergo more than one round of recruitment, however I would predict that this is not so precisely regulated by argos, given the variable number of oenocytes per cluster (4 to 9 cells). To account for the EGFR-mediated recruitment of both secondary COPs and oenocytes, I proposed that cell fate is determined by the repertoire of prepattern genes expressed in the cells receiving the signal. It appears as though precursors in the eye imaginal disc are similarly primed (see previous section). The analogy extends further as the oenocyte prepattern gene sal, and its sister gene spalt-related (sail), are also expressed in a subset of developing photoreceptors and accessory cells (Barrio et al., 1999). These two genes were recently shown to control photoreceptor fates (Mollereau et al., 2001), thus adding to the parallels described above between the eye and oenocyte/COP systems.

6.8 What defines the oenocyte prepattern?

In a recent review, Affolter and Mann (2001) suggested that morphogenetic events are the result of the integrated action of selector transcription factors with the downstream components of intercellular signalling pathways. In this model the
signalling pathways provide positional information within a field, culminating in a genetic prepattern. Meanwhile the selector genes interpret this "grid reference" and act either individually or in combination with the prepattern factors to generate specific structures in defined body locations. In this thesis I have described two essential components of the oenocyte ectodermal prepattern. The first, *engrailed*, narrows the field of competence down to the posterior compartment and lies downstream of the *wg* signalling pathway. The second, SAL is the dorsal competence factor for oenocyte formation. The intersection between SAL and EN defines the oenocyte prepattern grid reference. Although the upstream activator of *sal* is currently unknown, the DPP signalling pathway would be a good candidate, given its role in dorsal patterning. Therefore the oenocyte prepattern may have input from signalling components (*dpp* and *wg*) that provide DV and AP positional information, narrowing down the field of competence to a subset of dorsoposterior ectodermal cells. I would predict that there is at least one additional prepattern factor that restricts oenocyte induction to the ventral-most cells in this dorsoposterior domain. This is where the whorl always forms, even when inducing signal is provided throughout the DV axis. In this way the oenocyte prepattern is defined by an *abdA*-independent mechanism. This cryptic identity is present in all trunk segments and only requires some sSPI signal to reveal it.

6.9 Does *abdA* function in the C1 COP, in the responding ectoderm or in both?

In Chapter 5, I proposed a prime-and-respond model to explain the dual role of *sal* in the induction of oenocytes. According to this model, a subset of ectodermal cells expressing the *sal* prepattern factor are primed to become oenocytes, and these
cells respond to EGFR signalling by upregulating SAL. Where does the oenocyte Hox specifier, \textit{abdA}, act in this scheme? Expressed in both the C1 COP and the sickle-shaped oenocyte precursors (V. Brod and A. Gould, unpublished), \textit{abdA} could in principle function in either the signalling and/or the responding cell. As discussed in Chapter 5, one possible role for \textit{abdA} could be to control the degree (level and/or duration) of EGFR ligand production that is required in C1 for the recruitment of oenocytes. If this is the case, it is possible that the ectodermal expression of \textit{abdA} non-autonomously controls ligand production in the underlying C1 COP. However, this would require two-way signalling with an ectodermal signal acting on C1 to regulate EGFR ligand production, which would in turn result in the recruitment of oenocytes. The simpler, and more likely scenario, is that \textit{abdA} functions cell autonomously within the C1 COP to regulate EGFR signalling. If this hypothesis is correct, then we are left with the question of whether \textit{abdA} plays any role in the oenocyte precursors themselves. To address this issue would require developing tools for separating \textit{abdA} functions in C1 and the responding ectoderm. One further point is that in my \textit{en-GAL4 UAS-sspi} experiments, only relatively early markers such as anti-SAL and \textit{svp-lacZ} were used to demonstrate the induction of thoracic oenocytes. Further studies using terminal differentiation markers, for example those identified in the enhancer trap screen described in Chapter 4, would be necessary to assay whether these ectopic thoracic cells express the appropriate late markers and therefore develop into fully differentiated oenocytes.

If the thoracic oenocytes observed in \textit{en-GAL4 UAS-sspi} embryos prove to be fully differentiated in the complete absence of ABDA protein, then either there is no \textit{Hox} requirement in these cells or there is a partially redundant role. Consistent with the latter idea, although ectopic oenocytes can be produced where \textit{abdA} is not
normally expressed, they are only generated in Antp and Ubx territories and not more anteriorly. Indeed, other studies have shown that Hox genes are sometimes functionally interchangeable (see General Introduction), and ANTP/UBX/ABDA proteins are known to share the most highly conserved homeodomains (Passner et al., 1999). It is therefore conceivable that abdA has a specific non-autonomous role in C1, regulating EGFR ligand production, and a redundant autonomous role in the oenocyte precursors themselves.

6.10 Is rho the principal target of ABDA?

In the previous section, I speculated that abdA expression in the C1 primary COP might be responsible for the increased degree of EGFR signalling in abdominal segments, and hence the non-autonomous induction of oenocytes. Being an upstream component in EGFR signal production and, unlike spi, widely thought to determine the pattern of EGFR activation, rho is a good candidate for a Hox target gene. It is already known that the proneural gene ato is necessary for rho expression in C1 to C5 (Okabe and Okano, 1997; zur Lage et al., 1997). By analogy it seems likely that ato is also required in the thoracic primary COPs for rho expression. I would therefore like to speculate that ato initiates rho expression in all primary COPs, irrespective of whether they are in the thorax or abdomen. Later, rho expression would then be modulated in the abdominal segments by abdA, acting either directly or indirectly. Due to a requirement for EXD in oenocyte formation, I would therefore predict that ABDA activation of rho in C1 occurs in a cofactor dependent manner (Figure 6.1). ABDA could similarly increase the degree of EGFR signalling from C3, and thus be responsible for the recruitment of the two
Figure 6.1 A model for oenocyte induction by abdA and the EGFR pathway

This cartoon shows the signalling C1 COP (top) and the responding ectodermal oenocyte precursor (bottom), where solid and dashed arrows indicate proven and suggested regulatory events, respectively.

According to this model, ATO initiates rho expression in C1 and, at a slightly later stage, ABDA acts with its cofactor EXD (and HTH?) to maintain the high level of rho expression in this cell (top panel). Here I have suggested that ATO and ABDA regulate RHO through early and late enhancer elements, respectively, although this regulation could be direct or indirect. The continued high level expression of rho in C1 results in the production of active SPI ligand (SPI\textsuperscript{act}) from an inactive membrane-bound precursor (mSPI). SPI\textsuperscript{act} binds to the EGFR in overlying ectodermal cells (lower panel). Activation of the EGFR triggers the Ras/MAPK signal transduction cascade, which in turn switches on pnt. The EGFR inhibitor argos is one of the transcriptional targets for the PNT protein. This model predicts that SAL is also a PNT target in those ectodermal cells expressing a SAL prepattern (SAL\textsubscript{low}). The resultant upregulation of SAL (SAL\textsuperscript{high}) in response to EGFR signalling triggers the expression of the SAL target genes svp and vvl. The results presented here do not distinguish whether it is the SAL prepattern or SAL response that inhibits secondary COP induction. However, given the apparent requirement of a SAL prepattern for its own subsequent upregulation, I have indicated that SAL\textsubscript{low} inhibits secondary COP recruitment.
Cl COP

EXD

HTH

EXD

HTH

late early

rho

1st COP fate

mSPI

RHO

SPI_{act}

EGFR

Ras/MAPK Signalling Cassette

ARGOS

PNT

SAL_{low}

SAL_{high}

SVP, VVL

and other oenocyte differentiation genes

Oenocyte Precursor

2nd COP fate
secondary COPs required to form an Ich5. Again, direct or indirect abdA regulation of rho in C3 could be cofactor dependent which would explain the lack of secondary COP recruitment that has been observed in the abdominal segments of both abdA and hth mutants (Heuer and Kaufman, 1992; Kurant et al., 1998).

I have used the larval oenocytes in Drosophila to study how a single Hox gene, abdA, controls the identity of this cell type. Intriguingly, the results presented suggest, but do not prove, that abdA controls oenocyte specification in a non-autonomous manner. Although ato controls the initial expression of rho in C1, I suggest that ABDA maintains expression of this gene at the level required for oenocyte induction (see Figure 6.1). According to this model, rho would be the principal abdA target gene whose activation triggers a downstream cascade of oenocyte differentiation genes (Figure 6.1). This hypothesis could be tested by adding back RHO, or an active form of the EGFR ligand (sSPI), to abdA mutants. Although this still would not rule out a redundant role for Hox genes in the oenocyte precursors themselves, this type of experiment might show whether one target gene is sufficient to rescue the oenocyte deficit in abdA mutants. A positive outcome would then raise the exciting possibility that one Hox gene (abdA) regulates one principal target (rho) to non-autonomously specify one cell identity (the oenocytes).
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229


239


APPENDICES
Appendix I Expression levels of LNE-lacZ constructs in stage 13 embryos

Construct expression levels are given relative to wildtype LNE-mediated lacZ expression (lines C and L). The expression pattern for the wildtype LNE was identical in both HZ50pL (line C) and WZ50pL (line L) transformation vectors, however lacZ levels were lower in the latter. Therefore data for lines F and G is given relative to C, while lines M-T are relative to L, and comparisons of F or G lines to M-T lines cannot be made. The HS1^{mut}+HS2^{mut} construct (line D) is described in Gould et al. (1997).

(+++) indicates high expression, (+) refers to low expression and (-) denotes no expression unless otherwise stated in the footnotes.
<table>
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<th>Construct</th>
<th>Line</th>
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<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>A1</th>
<th>A2-7</th>
<th>LoR</th>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<td>++</td>
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\textsuperscript{1} Loss of Restriction resulting in a DV and/or AP intrasegmental expansion of expression.
\textsuperscript{2} (number of lines showing a consistent pattern)/(total number of lines established).
\textsuperscript{3} A subset of lines showed weak expression in these segments.
\textsuperscript{4} Posterior expansion of the T1 expression domain.
\textsuperscript{5} Dorsal expansion of the anterior T1 and T2 expression domains and expansion of \textit{lacZ} expression into posterior cells of T1 and T2.
\textsuperscript{6} Weak residual expression was observed in these segments in a subset of embryos.
Appendix II The only abdominal chordotonal organs to derive from the \textit{en} stripe are those in the Ich5 array

All embryos shown are at stage 16, carry \textit{en}-GAL4 and UAS-\textit{nls}lacZ and are immunolabelled with 22C10 (green) and anti-\textbeta gal (red). White arrowheads mark the dendrites of individual chordotonal organs. See Figure 5.3A for relative positions of chordotonal arrays.

(A) All components of the Ich5 lateral chordotonal array are \textbeta gal-positive and are observed anterior to the residual \textit{en} stripe (see also Figures 4.5E and 4.6F). Definite oenocyte nuclei are marked with asterisks while \textbeta-gal positive chordotonal components are labelled: ligament (l), neuron (n), scolopale (s) and cap (c) cells.

(B) A ventrolateral view of two adjacent segments showing the vchA and vchB doublet (labelled A and B respectively). Neither the neurons (labelled with 22C10) nor the chordotonal support cells label with anti-\textbeta gal. The vchA/B are positioned anterior to, and abutting the \textit{en} stripe.

(C) Two neighbouring hemisegments showing that the v'ch1 chordotonal (v') is also \textbeta gal-negative and located anterior to the \textit{en} stripe. Note that it is surrounded by large \textbeta-gal positive oenocyte nuclei (compare to Figures 4.5D and 4.6E).
spalt-dependent switching between two cell fates that are induced by the
Drosophila EGF receptor

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Accepted 11 December 2000; published on WWW 7 February 2001

SUMMARY

Signaling from the EGF receptor (EGFR) can trigger the differentiation of a wide variety of cell types in many animal species. We have explored the mechanisms that generate this diversity using the Drosophila peripheral nervous system. In this context, Spitz (SPI) ligand can induce two alternative cell fates from the dorsolateral ectoderm: chordotonal sensory organs and non-neural oenocytes. We show that the overall number of both cell types that are induced is controlled by the degree of EGFR signaling. In addition, the spalt (sal) gene is identified as a critical component of the oenocyte/chordotonal fate switch. Genetic and expression analyses indicate that the SAL zinc-finger protein promotes oenocyte formation and suppresses chordotonal organ induction by acting both downstream and in parallel to the EGFR. To explain these findings, we propose a prime-and-respond model. Here, sal functions prior to signaling as a necessary but not sufficient component of the oenocyte prepattern that also serves to raise the apparent threshold for induction by SPI. Subsequently, sal-dependent SAL upregulation is triggered as part of the oenocyte-specific EGFR response. Thus, a combination of SAL in the responding nucleus and increased SPI ligand production sets the binary cell-fate switch in favour of oenocytes. Together, these studies help to explain how one generic signaling pathway can trigger the differentiation of two distinct cell types.

Key words: Oenocytes, Chordotonal organs, Drosophila melanogaster, EGF receptor, spitz (spi), spalt (sal)

INTRODUCTION

One of the more surprising findings to come from the last twenty years of research is that only a relatively small number of highly conserved signaling molecules are used during animal development. This contrasts dramatically with the myriad of different cell types that are known to require induction by intercellular signals. Part of the explanation for this apparent discrepancy is that signaling pathways are multiply reused, each time producing a different outcome. Thus, one of the challenges for the future is to understand the context-dependent molecular mechanisms that link canonical signal transduction pathways to the induction of distinct cell fates.

Signaling via the epidermal growth factor receptor (EGFR) provides an impressive illustration of the wide range of cell types that can be induced by one highly conserved signaling pathway (reviewed by Perrimon and Ferons, 1997; Schweitzer and Shilo, 1997). For example, in the context of the developing Drosophila eye, the differentiation of at least seven distinct cell types is triggered by the reiterative use of the EGFR (Freeman, 1996). To account for at least part of this response diversity, it has been proposed that cell-type specificity is encoded in molecular differences that are generated prior to signaling (a prepattern; Dickson et al., 1992). Very recent work has identified the runt-domain transcription factor Lozenge as a component of such an EGFR prepattern (Flores et al., 2000; Xu et al., 2000). The emerging picture is that specific outputs from receptors such as the EGFR are generated in a combinatorial manner involving integration with other signaling inputs as well as transcription factor prepatterns (Halfon et al., 2000; Simon, 2000).

The EGFR also plays a critical role during the formation of a proprioceptive component of the Drosophila peripheral nervous system (PNS), the chordotonal organ. Indeed, the parallel between the formation of these sensory structures and photoreceptors extends way beyond the shared deployment of the EGFR. In both developmental contexts, the proneural gene atonal (ato) is expressed and required in the founding sensory cell (Jarman et al., 1993; Jarman et al., 1994). In the eye, the founder for each ommatidial cluster is the R8 photoreceptor whereas for the proprioceptors, the sensory mother cell is known as a primary chordotonal organ precursor (primary COP). ato-positive founder cells of both types produce SPITZ (SPI) ligand, a TGFα-like molecule that activates the Drosophila EGF receptor (EGFR) in neighboring cells (Freeman, 1994; Tio et al., 1994; Okabe and Okano, 1997; zur Lage et al., 1997). This induction results in the recruitment of additional sensory cells: the secondary chordotonal organ precursors (secondary COP), which contribute to proprioceptor arrays, and the majority of photoreceptor types in the eye ommatidium. In the case of chordotonal
development, primary COPs express the transmembrane product of the *rhomboid* (rho) gene, which is required for the correct presentation of active SPI to the EGFR (Bang and Kintner, 2000). Recent results suggest that *rhomboid* family members also play a similar role in the eye (Wasserman et al., 2000). One important component of the response of recruited cells to the EGFR signal is the production of the secreted EGFR inhibitor Argos (Golombo et al., 1996). Negative feedback by Argos spatially limits the activation of EGFR and thus provides a restriction on the number of cells recruited into each ommatidial or chordotonal cluster (Freeman, 1996; Okabe and Okano, 1997).

We have addressed the issue of how output specificity is achieved during EGFR signaling using a new model system, in which there is a choice between just two cell fates: the chordotonal organ and the oenocyte, a non-neural secretory cell. Genetic analysis indicates that, like secondary COPs, oenocytes require the functions of *ato, rho* and *spi*. We show that oenocyte precursors are induced as a single whorl of cells surrounding the most dorsal primary COP. In contrast, primary precursors that are more ventrally located recruit additional chordotonal precursors but not oenocytes. Manipulating EGFR signaling in a variety of ways can concomitantly increase or decrease the number of oenocytes and chordotonal organs but it is not sufficient to switch the qualitative nature of the EGFR response. In contrast, mutations in the *spalt* (*sal*) gene alter output specificity and lead to excess chordotonal recruitment at the expense of oenocyte formation. Epistasis tests and expression analysis reveal that *sal* acts both downstream and in a parallel pathway to EGFR signaling. To account for this, we present a prime-and-respond mechanism where the SAL protein is a critical component of an oenocyte nuclear prepattern and is also upregulated as part of the oenocyte-specific EGFR response.

**MATERIALS AND METHODS**

**Fly stocks**

The following loss-of-function mutations were used: *argos*<sup>47</sup> (Freeman et al., 1992b), *ato<sup>1</sup>* and *Df(3R)P13* (Jarman et al., 1993; Jarman et al., 1994), *spi<sup>+</sup>* (Nusslein-Volhard et al., 1984), *rho<sup>P35</sup>* (Freeman et al., 1992b) and a *rho<sup>7843</sup>* chromosome that also harbors a *roughoid/rhomboid-3* mutation (Jurgens et al., 1984; Wasserman et al., 2000). The apparent null alleles *sal<sup>445</sup>* and *sal<sup>18</sup>* (Jurgens, 1988; Kuhnlein et al., 1994) were used. Similar results were also obtained with *sal<sup>+</sup>* (Nusslein-Volhard et al., 1984) except that the lateral chordotonal migration/orientation phenotype was not observed with this allele.

For misexpression, *sal-GAL4* (Boube et al., 2000) and *en-GAL4* (A. Brand, Wellcome/CRC Institute, Cambridge, UK) were used in combination with UAS-*rho* (de Celis et al., 1997; Wasserman et al., 2000), UAS-*spi* (Schweitzer et al., 1995) and UAS-EGFR<sup>W120</sup> (O'Keefe et al., 1997). Three independent insertions of UAS-*sal* (Kuhnlein and Schuh, 1996) were used (RS58, RS90 and RS91), each giving a qualitatively similar phenotype. *BO-lacZ* is an oenocyte-specific reporter line containing an enhancer from the *sal* complex (Barrio et al., 1999) and *svp-lacZ* refers to the *svp<sup>bonl</sup>* enhancer trap (FlyBase, 1999). *rho-lacZ* refers to the *X81* line (Freeman et al., 1992b), an enhancer trap insertion about 100 nucleotides from the *rho<sup>lacZ</sup>* insert that is already known to be *C1* specific (Freeman et al., 1992a; zur Lage et al., 1997). *pnt-lacZ* refers to the *pnt<sup>1277</sup>* enhancer trap into exon 1 of the *pntP2* transcription unit (Scholz et al., 1993).

**Immunolabelling**

Embryo immunostaining was according to standard protocols using HRP or Alexa fluorescent conjugates (Molecular Probes). All fluorescent images were collected using confocal microscopy with a pinhole of 1. They are projections of several sections except Figs 1F, 2B, 2F and 3B where single sections are shown. Primary antibodies used were: rabbit anti-β-galactosidase (Cappell) at 1:6000, mouse anti-β-galactosidase (Promega) at 1:1000, 22Cl/anti-FUTSCH at 1:50 (Fujita et al., 1982; Hummel et al., 2000), anti-RHO (Sturtevant et al., 1996) at 1:600, anti-VVL (Anderson et al., 1995) at 1:1500, anti-SAL (Kuhnlein et al., 1994) at 1:30 or anti-SAL (Barrio et al., 1999) at 1:500.

**RESULTS**

The larval oenocytes of *Drosophila* are conspicuous secretory cells of ectodermal origin (Bodenstein, 1950; Hartenstein et al., 1992). They are arranged in clusters of, on average, 6 cells per abdominal hemisegment, occupying a characteristic lateral and subepidermal location. In contrast to the invariant peripheral nervous system, the number of cells in each larval oenocyte cluster can vary between 4 and 9. Using many different molecular markers, we have traced the development of larval oenocytes (hereafter called oenocytes) from the third larval instar back to the extended germ band stage of embryogenesis (P. E. and A. G., unpublished). In brief, we have found that developing oenocytes express four genes from very early stages, all of which encode DNA-binding proteins. These are *seven up* (*svp*), *pointed* (*pnt*), *spalt* (*sal*) and ventral veins lacking (*vvl*) which produce proteins of the nuclear receptor, ETS-domain, zinc-finger and POU-homeodomain class, respectively (Mlodzik et al., 1990; Scholz et al., 1993; Kuhnlein et al., 1994; Anderson et al., 1995).

**Oenocytes form around the most dorsal chordotonal precursor and require *ato***

In the late embryo, immunolabelling experiments were carried out with two independent oenocyte markers: *svp-lacZ*, an enhancer trap into the *svp* gene and *BO-lacZ*, a regulatory construct containing an oenocyte-specific enhancer from the *sal* complex (see Materials and Methods). Using these markers, in conjunction with the sensory neuronal marker anti-FUTSCH/22ClO (Fujita et al., 1982; Hummel et al., 2000), it can be seen that each cluster of oenocytes is closely associated with an array of five lateral chordotonal organs, termed *Lch5* (Figs 1A,B, 6A). In each abdominal hemisegment, there are eight chordotonal organs that are partitioned into arrays consisting of one dorsolateral (*V'ch1*), five lateral (*Lch5*) and two ventral (*VchAB*) organs (Brewster and Bodmer, 1995). The close apposition of mature oenocyte clusters and *Lch5* arrays in late embryos suggested that their formation might be linked in some way. In order to investigate this possibility, we examined the spatial relationship between the precursors of both cell types in early embryos.

Each chordotonal organ is formed by a single chordotonal organ precursor (COP) that divides asymmetrically to produce a sensory neuron, scolopale, ligament and cap cell (Fig. 6A, reviewed in Brewster and Bodmer, 1996). Previous studies using the *rho<sup>lacZ</sup>* reporter line (zur Lage et al., 1997) have determined that the progeny of the most dorsal COP (C1)
constitute the most anterior chordotonal organ of the lateral cluster (Lch5a, Fig. 6A). We have used a similar rho-lacZ insertion that is also specific for Lch5a and its precursor COP (Fig. 1C-E and materials and methods), together with anti-SAL to follow the development of C1 and oenocytes simultaneously. By stage 10, C1 has delaminated and does not express SAL, despite lying directly underneath a dorsal domain of SAL-positive ectoderm (termed the dorsal SAL domain, Figs 1D, 2B). By stage 11, C1 has already divided (zur Lage et al., 1997) and its progeny are surrounded by a whirl of sickle-shaped nuclei expressing higher levels of SAL than surrounding cells (Figs 1E,G, 2B). The whirl structure always appears in a dorsal and posterior segmental position, close to the ventral limit of the SAL domain, and corresponds to oenocyte precursors in the process of delamination (P. E. and A. G., unpublished). In addition to high levels of SAL, the oenocyte precursor whirl also expresses tsp-lacZ and vvl (Fig. 1F and data not shown). As we only observe one oenocyte precursor whirl per hemisegment and this surrounds C1, we conclude that more ventral COPs (Okabe and Okano, 1997; zur Lage et al., 1997) are not associated with the formation of oenocytes (also see Discussion).

To test the idea that the C1 cell might have an influence on oenocyte development, ato mutants were examined. Embryos carrying ato^1, a strong hypomorph, in trans with a deficiency, have hemisegments in which the Lch5 array is either completely missing or reduced to a single lateral chordotonal organ, termed Lch0 and Lch1 phenotypes respectively (Jarman et al., 1995). Where an Lch1 is produced, this single organ is known to derive from a residual C1 precursor (zur Lage et al., 1997). We observe that oenocyte formation is completely abolished in these segments lacking all lateral chordotonal organs (Fig. 2A). Interestingly, in the majority of cases where an Lch1 is formed, it is associated with an oenocyte cluster of normal or somewhat reduced cell number. Similar results were obtained with ato^0 homozygotes although the missing chordotonal/oenocyte phenotype is less penetrant (data not shown). Thus ato is required for oenocyte formation and there is a correlation between the presence of C1 and the ability to form an oenocyte cluster.

The above results do not rule out a scenario where ato is directly required in oenocyte precursors. However, given the correlation between the presence of C1 progeny and the formation of an oenocyte cluster in ato mutants, we think it more likely that ato functions in the C1 cell to facilitate oenocyte development in an indirect manner. This raises the possibility that an ato-dependent signal from C1 is involved in the induction of oenocyte precursors.

**Oenocyte induction requires rho, spi and EGFR activity**

C1 is one of a set of five primary COPs per hemisegment (C1-5, Fig. 6B) that delaminate from ato-positive proneural clusters. The continued expression of ato in these primary COPs switches on rho and thus activates SPI. In turn, this induces three secondary COPs via EGFR signaling, giving the full complement of eight COPs per hemisegment (Okabe and Okano, 1997; zur Lage et al., 1997). In rho mutants, where active SPI ligand is absent, the two lateral chordotonal organs that are descended from secondary COPs are missing, so producing an Lch3 phenotype (Bier et al., 1990). As C1 expresses rho and lies at the center of the whirl (Fig. 1E,F), oenocyte precursors are likely to be exposed to EGFR ligand. In addition, they express a reporter for pnt (Fig. 1G), a transcription factor target of the ras/MAP kinase pathway that is known to act downstream of the EGFR (O'Neill et al., 1994). For these reasons, we investigated the possibility that the EGFR pathway might be used to induce oenocytes.

Embryos homozygous for rho^1MV5 or rho^PA3 display an Lch3 phenotype and a complete lack of oenocytes at stage 16 (Fig. 2E and data not shown). This is consistent with data presented in a previous analysis of rho function (see Fig. 3 from Wappler et al., 1997). Embryos lacking the EGFR ligand, Spi, also present an Lch3 phenotype (Rutledge et al., 1992) and again lack all oenocytes (Fig. 2F). The absence of mature oenocytes in spi and rho mutants can be traced back to stage 11 where the oenocyte precursor whorls are missing (Fig. 2B-D). Importantly, although SAL upregulation in oenocyte precursor cells is a response to EGFR signaling, the dorsal SAL domain remains in rho and spi mutants and therefore is independent of SPI ligand (Fig. 2C,D).

In order to test the role of the EGFR itself, we used en-GAL4 (Brand and Perrimon, 1993) to misexpress a dominant-negative form of this receptor that lacks the cytoplasmic kinase domain (EGFR^DN, Freeman, 1996; O'Keefe et al., 1997). The en-GAL4 driver expresses in a domain containing all of the Lch5 and oenocyte precursors but not the founding COPs for V'ch1 or VehAB (P. E. and A. G., unpublished). Like rho and spi loss of function, misexpression of EGFR^DN at both 25°C and 29°C resulted in a complete loss of oenocytes (Fig. 2G). In contrast, the lateral chordotonal organ array was only reduced by one at 25°C with the rholspi Lch3 phenotype appearing only at the higher temperature (Fig. 2G). Thus, a reduction in EGFR activity can completely suppress oenocyte formation but only partially blocks secondary COP induction. This unexpected result suggests that the threshold for EGFR activation required to induce oenocytes may be higher than that for chordotonal precursors.

To test whether the EGFR is required directly in oenocyte precursors or more indirectly in C1, a sal-GAL4 driver was used (Boube et al., 2000). In contrast to en-GAL4, this particular driver line directs dorsal expression in a subset of oenocyte precursors but not in C1 (data not shown). Expressing the EGFR^DN using sal-GAL4 at 29°C had no effect on chordotonal organ number but did produce a significant reduction in the number of oenocytes per cluster (2.73±1.14 (mean±s.d.)), n=25) relative to controls from the same experiment (5.76±0.83 n=21). Thus, oenocyte precursors autonomously require EGFR function. This is consistent with the previous data indicating that they express a reporter for pnt, an EGFR and MAPK target gene that is essential for oenocyte production (Fig. 1G and data not shown).

These studies show that EGF pathway components are required for the formation of oenocytes, in addition to their previously characterized role in chordotonal organ recruitment. Together with the preceding results, we conclude that SPI ligand, secreted from C1, is required to activate the EGFR in the neighbouring ectoderm, in turn leading to oenocyte induction.

**Excess EGFR signaling increases the number of oenocytes and chordotonal organs**

Why does an oenocyte whorl form around C1 and not around more ventrally located primary COPs? Previous studies have
shown that rho and thus active SPI ligand is expressed in C2-C5 (Okabe and Okano, 1997; zur Lage et al., 1997) yet these cells only recruit chordotonal organs and not oenocytes. In light of the EGFR\textsuperscript{DN} results, a greater degree of signaling is likely to be required to trigger oenocyte induction than secondary COP recruitment. Consistent with this, C1 is known to express rho more strongly than other COPs (zur Lage et al., 1997) and this could provide the trigger for the induction of oenocytes rather than COPs. To test this possibility, we examined argos mutants where the degree of EGFR signaling should be increased in the vicinity of all primary COPs.

Removing argos function resulted in one extra lateral chordotonal organ (Fig. 3C) as previously described (Okabe et al., 1996). In addition, we observed that in argos mutants, a single oenocyte precursor whorl is present but it is expanded in diameter from one to two concentric rings of cells and thus gives rise to a large oenocyte cluster of 15-27 cells (Fig. 3A,B). Hence, removal of an EGFR inhibitor leads to an increase in oenocyte number but not to the induction of ectopic oenocyte whorls.

A second way of increasing EGFR activation is to misexpress rho using en-GAL4. As rho is rate limiting for spi activation, this should produce high levels of EGFR ligand in ectodermal cells throughout the dorsoventral axis. An Lch6 phenotype and an enlarged whorl producing a cluster of 17-27 oenocytes were observed (Fig. 3D-F) as in argos mutants. A more direct way of generating constitutively active EGFR ligand is to use a secreted form of the spi gene, sspi (Schweitzer et al., 1995). Driving sspi using cn-CAL4 results in EGFR activation in the responding ectoderm that is COP independent. As with rho misexpression, an enlarged oenocyte precursor whorl results, giving rise to a giant cluster of 21-39 oenocytes (Fig. 3C,H). In these embryos, the PNS is highly disorganized but it is possible to ascertain that excess lateral chordotonal organs are formed, frequently giving an Lch6-7 phenotype (Fig. 3I). Interestingly, in UAS-sspi but not argos embryos, sal is upregulated dorsal to the whorl of sickle-shaped nuclei. However, neither sal upregulation nor the production of oenocyte precursors is ever observed outside the dorsal SAL domain. Thus, while excess EGFR ligand can dramatically increase the number of oenocytes that are recruited around C1, it is not sufficient to induce ectopic oenocyte precursors around other primary COPs. We surmise that factor(s) other than SPI
Roles for sal and EGFR in oenocyte/COP induction

SAL ligand must limit the oenocyte response to the dorsal SAL domain.

sal promotes oenocyte induction and limits the number of lateral chordotonal organs

Given that manipulating EGFR activation does not switch the choice of recruited cell from COP to oenocyte, there could be a dorsoventral difference in the competence of the ectoderm to respond to EGFR ligand. As oenocytes can only be induced within SAL territory, even in the presence of excess EGFR signaling, it could be that sal itself is involved in the dorsal restriction process. To test this, we examined embryos carrying the null alleles sal^{16} and sal^{245} (Kuhnlein et al., 1994). Using both svp-lacZ and anti-VVL markers, almost no oenocytes were seen (Fig. 4A,B and data not shown) and with svp-lacZ we observed that this deficit is manifest as early as the whorl stage (data not shown). This provides a clear demonstration that oenocyte induction requires sal and also reveals that svp lies downstream of sal in the oenocyte response cascade.

In addition to the lack of oenocytes, sal mutants display a highly penetrant Lch6-7 phenotype (Fig. 4C,D). In contrast to argos mutants, extra chordotonal organs are restricted to the lateral cluster with the other abdominal chordotonal organs (V'ch1, VchAB) remaining wild type in number (data not shown). This is consistent with the finding that the primary precursors for these more ventral chordotonal organs are C4

Fig. 2. Oenocyte formation requires the functions of *ato*, *rho*, *spi* and the EGFR. In all panels SAL and FUTSCH/22C10 expression are shown in red and green, respectively. White arrowheads indicate the position of each lateral chordotonal organ in this and subsequent figures. (A) *ato^{1}/Df(3R) p13* embryo at stage 16. The anterior segment contains four oenocytes (asterisks) together with a single lateral chordotonal organ with the neuron (n) expressing FUTSCH/22C10 and the ligament (l) and scolopale (s) cells expressing SAL. The posterior segment contains no oenocytes and no lateral chordotonal organs, although other FUTSCH-expressing sensory neurons do remain. This represents the phenotype displayed in approximately 25% of hemisegments. The mixture of Lch0 and Lch1 seen at stage 16 in *ato^{1}/Df(3R) p13* correlates with the stage 11 phenotype where some segments contain oenocyte precursor whorls but others contain no recognizable whorl (data not shown). (B-D) At stage 11, oenocyte precursor whorls of large sickle-shaped nuclei expressing high levels of SAL are seen in wild type (B) but not in *rho^{SM43}* (C) or *spi* (D) homozygotes. However, the dorsal domain of moderate SAL expression is still present in these two mutants. Within this domain, some rings of nuclei expressing moderate but not high levels of SAL are observed but these lie in the wrong anteroposterior position to correspond to oenocyte precursors. Open arrowheads indicate whorls. (E,F) At stage 16, *rho^{SM43}* (E) and *spi* (F) embryos display a fully penetrant Lch3 phenotype and lack all oenocytes. (G) A stage 15 embryo carrying UAS-EGFR^{DN} and en-GAL4 at 25°C. At this temperature, a partial block in EGFR activity is observed, producing an Lch4 phenotype. In addition, no oenocytes are formed.

Fig. 3. Loss of *argos* or excess *rho* or *spi* lead to recruitment of supernumerary oenocytes and chordotonal organs. (A-C) In *argos* mutants at stage 11, the precursor whorl is enlarged and contains many extra cells (A) and by stage 16 oenocyte clusters containing 15-27 cells (20±4.2, n=10) are present (B). Loss of *argos* function also results in one extra lateral chordotonal organ, giving an Lch6 phenotype (C). (D-I) Overexpression of UAS-*rho* (D-F) or UAS-*spi* (G-I) using en-GAL4 produces an enlarged whorl at stage 11 (D,G) similar to those seen in *argos* mutants. By stage 16, giant clusters of 17-27 (23±2.7, n=9; E) or 21-39 (29±4.6, n=21; H) oenocytes are observed that are associated with disorganized Lch6-7 arrays (F,I). UAS-*spi* produces SAL upregulation throughout the dorsal sal domain but sickle-shaped nuclei are restricted to the normal whorl position (G). Asterisk indicates a sensory neuron of uncertain identity.

Fig. 4. Loss of *argos* or excess *rho* or *spi* lead to recruitment of supernumerary oenocytes and chordotonal organs. (A-C) In *argos* mutants at stage 11, the precursor whorl is enlarged and contains many extra cells (A) and by stage 16 oenocyte clusters containing 15-27 cells (20±4.2, n=10) are present (B). Loss of *argos* function also results in one extra lateral chordotonal organ, giving an Lch6 phenotype (C). (D-I) Overexpression of UAS-*rho* (D-F) or UAS-*spi* (G-I) using en-GAL4 produces an enlarged whorl at stage 11 (D,G) similar to those seen in *argos* mutants. By stage 16, giant clusters of 17-27 (23±2.7, n=9; E) or 21-39 (29±4.6, n=21; H) oenocytes are observed that are associated with disorganized Lch6-7 arrays (F,I). UAS-*spi* produces SAL upregulation throughout the dorsal sal domain but sickle-shaped nuclei are restricted to the normal whorl position (G). Asterisk indicates a sensory neuron of uncertain identity.
Fig. 4. *sal* acts downstream or in parallel to the EGFR to promote oenocyte and repress COP fates. The expression of VVL (red) marks oenocytes and FUTSCH/22C10 (green) labels chordotonal organs in all panels except E. (A,B) VVL expression indicates that, compared to wild type (A), mutants lack oenocytes (B). (C,D) Both *sal* (C) and (D) mutants display extra lateral chordotonal organs. Examples of an Lch6 and an Lch7 phenotype are shown. Asterisk indicates a sensory neuron of uncertain identity. (E) The expression of RHO in membrane plaques of the primary COPs (numbered) at late stage 10/early stage 11 appears normal in *sal* mutants. T indicates the position of the tracheal placode/pit, a site of strong RHO expression. At later stages, high levels of RHO persist in Cl but not other COPs (data not shown). (F) In *sal: rho* double mutants, oenocytes are completely missing and a fully penetrant Lch3 phenotype is observed. (G,H) *sal: argos* double mutants display a dramatic increase in lateral chordotonal organs, an example of a disorganized Lch11 is shown (G). In addition, these double mutants lack all oenocytes (H).

and C5 (Zur Lage et al., 1997), which both lie outside the dorsal SAL domain. In *sal* mutants, we also observed that some of the lateral chordotonal organ arrays are displaced dorsally and frequently point ventrally (data not shown).

The phenotypic analysis above demonstrates that *sal* plays a positive role in oenocyte induction and a negative role in chordotonal organ formation. However, further studies are required to determine whether *sal* functions in the induced cells themselves or alternatively in the primary precursor producing the SPI signal.

**sal acts downstream or in parallel to the EGFR**

To investigate the regulatory relationship between the EGFR pathway and the functions of *sal*, the expression of RHO was examined in a *sal* background. The pattern and level of membrane-plaque associated RHO in *sal* mutant COPs appears indistinguishable from wild type (Fig. 4E and data not shown). As *rho* expression is rate limiting for active SPI production, this observation suggests that EGFR ligand synthesized in primary COPs is independent of *sal*. This is consistent with the observation that SAL is not detected in delaminated COPs at stage 11 (Fig. 4D,E and data not shown).

Addressing the question of whether the extra lateral chordotonal organs observed in *sal* mutants derive from primary or secondary COPs, we generated embryos mutant for both *sal* and *rho*. These *sal: rho* double mutants lack all oenocytes and display an Lch3 phenotype that is identical to *rho* or *spi* single mutants (Fig. 4F). As the *rho* mutation abolishes secondary but not primary COP formation, the 1-2 extra lateral chordotonal organs formed in *sal* mutants must be derived from supernumerary secondary COPs. Furthermore, the *sal: rho* chordotonal phenotype indicates that *rho* loss of function is epistatic to *sal* loss of function. Together with the observation that RHO expression is normal in a *sal* background, we conclude that *sal* functions downstream or in a parallel pathway to the EGFR signal to repress chordotonal recruitment.

To explore further the relationship between *sal* and the EGF pathway, *sal*; *argos* double mutants were produced. These

Fig. 5. *sal* is sufficient for COP repression but not oenocyte induction. Expression of *svp-lacZ* is shown in red and FUTSCH/22C10 is in green. (A,B) Misexpression of *sal* using en-GAL4 with the UAS-*sal* (RS90) insertion at 25°C produces a modest reduction in oenocyte number (A) with a mean of 4.6 cells per cluster (±1.2, n=25) and a mixed Lch3/4 phenotype (B). An example of a 6-cell and 4-cell oenocyte cluster is shown in A and an Lch3 in B. (C,D) Combined misexpression of UAS-*sal* (RS85) and UAS-*sspi* using en-GAL4 at 25°C results in giant oenocyte clusters of 21-35 cells (28±4.7, n=14). A highly penetrant Lch3 phenotype is also seen (D).
have a dramatic chordotonal overproduction phenotype that is more severe than that seen in either single mutant. Frequently, there are 9 or more lateral chordotonal organs per hemisegment that, as in *sal* mutants, are often scattered in lateral and dorsal positions (Fig. 4G and data not shown). This implies that the EGFR pathway is hyperactivated within the dorsal territory surrounding C1, where *sal* would normally repress secondary COPs and promote oenocyes. Importantly, the *sal; argos* genotype also abolishes oenocyte formation (Fig. 4H). This demonstrates that, for oenocyte induction, *sal* loss of function is epistatic to *argos* loss of function. Hence, despite the high levels of EGFR signaling around Cl that lead to excess secondary COP production, no oenocytes are recruited. These results strongly suggest that *sal* is required in the responding cell, either downstream or in parallel to the EGFR, for the induction of the oenocyte fate.

**SAL raises the apparent threshold for induction by SPI**

To test further the idea that *sal* biases the choice of EGFR recruited fate from COP to oenocyte, *sal* was misexpressed in the ectoderm overlying more ventral COPs using *en-GAL4*. At 25°C, this results in a high frequency of Lch3-4 arrays (Fig. 5B) and at 29°C these become predominantly of the Lch3 type (data not shown). The Lch3 phenotype is identical to that seen with *rho* and *spi* and is therefore consistent with a loss of secondary COP recruitment. This is opposite to the phenotype of *sal* loss-of-function mutants, and implies that during normal development *sal* plays a role in repressing COP recruitment around C1. In addition to the Lch3 phenotype, *sal* misexpression is also associated with a moderate reduction in oenocyte number (Fig. 5A). Examination of stage 11 embryos indicates that the oenocyte deficit can be traced back to the precursor whorl stage (data not shown). To confirm that this effect is not insertion-site specific, three different UAS-*sal* lines of varying strengths were tested at 25°C and gave a range of mean oenocyte cluster sizes that were all less than wild type (data not shown). One to the phenotype of *sal* loss-of-function mutants, and implies that during normal development *sal* plays a role in repressing COP recruitment around C1. In addition to the Lch3 phenotype, *sal* misexpression is also associated with a moderate reduction in oenocyte number (Fig. 5A). Examination of stage 11 embryos indicates that the oenocyte deficit can be traced back to the precursor whorl stage (data not shown). To confirm that this effect is not insertion-site specific, three different UAS-*sal* lines of varying strengths were tested at 25°C and gave a range of mean oenocyte cluster sizes that were all less than wild type (data not shown). One

**Fig. 6. Summary and prime-and-respond model for oenocyte and chordotonal induction.** (A) Summary of the mean number of oenocytes (red) and lateral chordotonal organs (green) present in each abdominal hemisegment in the wild-type (WT) and mutant backgrounds indicated. In the wild-type panel the relative positions of the cap(c), scolopale (s), neuron (n) and ligament (l) cells that constitute each of the five organs (a-e) of the Lch5 are shown. Missing lateral chordotonal organs and oenocytes are indicated by unfilled outlines and the UAS results shown are with the *en-GAL4* driver. (B) The prime-and-respond model. The central panel indicates the position of the five ATO-expressing primary COPs (C1-C5) relative to the dorsal oenocyte prepattern of low SAL expression. C2 is shown half obscured as it is not clear if it lies within or just ventral to the SAL domain. Either way, C2 does not express *rho* strongly (zur Lage et al., 1997) and therefore is unlikely to induce oenocytes. C1-3 contribute to the Lch5 but C4 and C5 (dashed circles) do not. The left panel shows the induction of oenocytes (O) via strong and persistent SPI signaling (large blue arrows) from C1 to the EGFR in overlying ectodermal cells. The oenocyte prepattern of low SAL raises the apparent threshold for induction by SPI. Low SAL also serves to prime the responding cell so that *sal* can be subsequently upregulated as part of the response to EGFR activation. In turn, this stimulates the expression of the *sal* target gene, *svp* (not shown). The right panel shows the induction of secondary COPs (2°) by moderate SPI signaling (small blue arrows) from C5 to ectodermal cells that are SAL-negative. In this case, EGFR stimulation does not lead to the activation of *sal* or *svp*. Instead, sensory organ precursors that divide and differentiate into lateral chordotonal organs are produced.
explanation for the reduction in precursor number is that increasing the SAL concentration in responding cells reduces their sensitivity to the EGFR ligand produced by C1. The main conclusion from this experiment, however, is that misexpression of sal in the vicinity of ventrally located COPs is sufficient to suppress chordotonal recruitment but not to promote ectopic oenocyte induction.

To mimic the conditions around C1 in a wild-type embryo, we tested whether a combination of increased EGFR signaling and the presence of SAL might be sufficient to induce ectopic oenocyte formation in regions outside the dorsal SAL domain. We used the en-GAL4 driver to coexpress sspi and sal (Fig. 5C,D). The effect of this coexpression on the lateral chordotonal organs is to produce Lch6-7 forms seen with UAS-sspi and is identical to the phenotype obtained with UAS-sal alone. Hence, with respect to COP induction, overexpression of sal is epistatic to constitutive sspi. This is consistent with the findings in sal; rho and sal; argos double mutants and further supports the conclusion that sal acts downstream or in parallel to SPI ligand production during oenocyte induction and COP repression.

Combined misexpression of sspi and sal does not produce ectopic oenocyte induction in ventral regions. Instead, this results in giant oenocyte clusters (21-35 cells) that are induced dorsally, as seen with excess sspi alone (Fig. 5C and data not shown). Importantly, the reduction in oenocyte numbers seen with sal overexpression can be completely abrogated by simultaneously providing excess SSPI. This strongly suggests that raising the concentration of SAL in oenocyte precursors leads to a reduced sensitivity to induction by SSPI. Such an effect is consistent with the previous results of EGFRDN misexpression, where a partial block of receptor activation completely abolished SAL-positive oenocyte induction but not SAL-negative COP recruitment. Such preferential blocking of oenocyte induction by EGFRDN can not be explained by reduced SPI ligand production as C1 expresses RHO more strongly than the other primary COPs. Taken together, these experiments suggest, but do not prove, that the presence of SAL in the nucleus of the responding cell raises the threshold for induction by SPI.

**DISCUSSION**

Little is known about the molecular linkage between the canonical EGFR pathway and the induction of different cell fates. Here we have addressed this issue with a novel and relatively simple model system where receipt of SPI signal can be only interpreted in two qualitatively different ways (Fig. 6). We have dissected the mechanism underlying this binary fate switch and find that sal encodes a critical component that functions downstream or in parallel to the EGFR. We discuss the qualitative and quantitative contributions of sal and the EGF pathway and propose a prime-and-respond model that explains how one signaling pathway can trigger the differentiation of two alternative cell fates.

**Induction of oenocytes by the PNS**

We have shown that oenocytes are induced from the ectoderm by an inductive signal that is generated in the developing PNS. The production of active SPI by the C1 precursor cell, under the control of ato and rho, triggers EGFR activation and thus oenocyte induction in adjacent ectoderm. Oenocyte induction by the PNS appears to be a short-range event with only the cells immediately surrounding C1 switching on oenocyte markers. In argos mutants, however, the range of the response is increased from one to two concentric rings of cells. Hence, as in photoreceptor recruitment, SPI ligand is not intrinsically limited to immediate neighbors but the response is nevertheless kept short-range by argos-mediated feedback inhibition of the receptor.

In wild-type embryos and in all of the mutant backgrounds that we have examined, the number of cells in the whorl at any one time is less than the final number of mature oenocytes. For example, a wild-type whorl contains 3-4 cells with sickle-shaped nuclei but the final oenocyte cluster contains on average 6 cells. We do not yet understand the basis for this difference but note that it might be explained by cell division or by sequential delamination of oenocyte precursors.

**Control of cell number by the EGF pathway**

The specification of secondary COP and oenocyte fates requires the EGF pathway. In ato, rho, spi and EGFRDN backgrounds, where signaling is compromised, the induction of both cell types is blocked (Fig. 6A). Conversely, when the EGFR is hyperactivated, both cell types become more numerous. These results indicate that the number of recruited cells is controlled by the amount of EGF pathway signal. It is important to realize, however, that the level, duration and spatial extent of ligand production are all being altered in our experiments. More sophisticated methods would be needed to clearly distinguish which of these three signaling parameters is critical for controlling cell number.

Surprisingly, there is no parity between the numbers of excess oenocytes and lateral chordotonal organs that are produced by EGFR hyperactivation. Thus for a given increase in ligand, more oenocyte precursors than COPs are recruited (Fig. 6A). This implies the existence of an additional tier of control that restricts neural but not oenocyte induction. Such a selective inhibition process would ensure that the number of chordotonal organs is more tightly controlled than that of oenocytes, as is observed in wild-type embryos.

**Choice of fate induced by EGF signaling depends on a SAL switch**

As we have described, the expression pattern and mutant phenotype of sal can account for the restriction of oenocyte induction to a single whorl surrounding C1, the most dorsal primary COP. Previously it was suggested that C1 and C3 each induce one secondary COP (zur Lage et al., 1997). However, the results presented here argue that the presence of SAL is incompatible with chordotonal recruitment. Therefore, we favor the idea that C3 recruits both of the secondary COPs that contribute to the Lch5 array.

The sal gene plays opposite roles in oenocyte and chordotonal induction. It is both necessary and sufficient for repressing secondary COP induction and it is necessary but not sufficient for promoting oenocyte formation. The lack of sufficiency for oenocyte induction is revealed when sal is misexpressed using the en-GAL4 driver. Oenocytes are not ectopically induced in ventral regions, even in the presence of...
excess sp si (Figs 3, 5 and data not shown). It is likely that other factors are required, together with SAL, to promote the oenocyte induction process.

Using epistasis tests and gene expression analysis we have elucidated the regulatory relationship between sal and the EGF pathway in oenocyte and COP formation. These data allow us firmly to exclude the possibility that sal acts upstream of spi in the signaling cell. Importantly, our results indicate that sal functions in the responding ectoderm, either downstream of the EGFR or in a parallel pathway leading to oenocyte induction and secondary COP repression.

In fact, it is probable that sal plays a dual role that is downstream and also in parallel to the EGFR. In rho and spi mutants, the normal upregulation of SAL in the vicinity of C1 is abolished. Conversely, sp si misexpression produces ectopic SAL upregulation in dorsal locations. Both results indicate that sal lies downstream of the EGFR and that SAL protein levels are controlled by receptor activation. However, SAL is also expressed at moderate levels in presumptive oenocyte precursors prior to EGF pathway activation and this expression remains normal in rho and spi mutants. For these reasons, it is likely that at least part of the function of sal lies in a parallel pathway that, in conjunction with the EGFR signal, promotes oenocyte induction and inhibits COP recruitment.

### A prime-and-respond model

To integrate the dual roles of sal downstream and also in parallel to the EGFR, we propose a prime-and-respond model (Fig. 6B). In this model, sal functions in the parallel pathway as a competence switch. Thus, SAL prepatterns the dorsal ectoderm so that, on receipt of the EGFR signal, oenocytes rather than COPs are induced. As we have argued, one consequence of the SAL oenocyte prepattern is to increase the apparent induction threshold in responding cells. This makes the prediction that the signaling cell inducing oenocytes needs to express more ligand than those that recruit secondary COPs, and this is indeed the case. C1 is known to express high levels of rho for longer than any of the other primary COPs (zur Lage et al., 1997). Thus, the EGF pathway does contribute to the cell-type specificity of the induction event in the sense that more SPI ligand is required to induce oenocytes than to recruit chordotonal organs.

One of the early oenocyte-specific responses to the SAL prepattern is the subsequent upregulation of SAL itself. This, in turn, stimulates the expression of the sal target gene svp, one member of the repertoire of oenocyte early differentiation genes (P. E., V. B. and A. G., unpublished). A key feature of the prime-and-respond model is that moderate levels of sal expression serve to prime the responding cells to further upregulate SAL when they receive SPI ligand. In support of this priming mechanism, we have demonstrated that upregulation in response to constitutive sp si expression is restricted to those cells that have already expressed sal. Hence, SAL proteins provide a molecular link between the prepattern and the EGFR response.

In the prime-and-respond model, it is implicit that the early and late phases of sal expression produce distinct effects on the responding cell. As the levels of SAL are different in the two phases, it may be that there are at least two different concentration-dependent effects for this transcription factor. In agreement with this, we have shown that strong expression of the sal target gene, svp, correlates with the domain of sal upregulation and not with the lower-level prepattern. In another system, wing vein development, there is a very extreme example of a concentration difference, with low and high levels of SAL producing completely opposite transcriptional effects on the knirps target gene (de Celis and Barrio, 2000).

### PERSPECTIVES

As the EGF pathway and sal play essential roles in the development of many different tissues, the prime-and-respond mechanism described here may well be deployed in other contexts. For example, in the developing Drosophila tracheal system, it has been suggested that the initial phase of sal expression during primary branch formation is EGF independent but that later expression in the dorsal trunk is maintained by the EGF pathway (Chen et al., 1998). Furthermore, as has been already stated, there are striking parallels between the oenocyte/chordotonal system and the developing Drosophila eye. In this regard, we note that sal is expressed in the eye imaginal disc in a subset of developing photoreceptor and accessory cells (Barrio et al., 1999). It remains to be seen whether sal or its sister gene, spalt related, also switch the outcome of EGF signaling in this developmental context.

Orthologs of sal have been identified in many animals other than Drosophila. In humans, it has been shown that one sal ortholog is associated with Townes-Brocks syndrome, an autosomal dominant condition affecting numerous structures including the limb (Kohlhase et al., 1998). Moreover, recent studies of the developing chick limb have revealed that a sal ortholog can be regulated by sonic hedgehog, BMP, FGF and Wnt signals (Farrell and Munsterberg, 2000). This raises the possibility that vertebrate sal family genes may be involved in modulating the responses to a wide range of signals and not just EGF ligands.

We wish to thank R. Schuh, S. Benzer, E. Bier, A. Jarman, M. Freeman, J. de Celis, R. Barrio, F. Kafatos, W. Johnson, J.-P. Vincent, M. Calleja, G. Morata, R. White and the Bloomington and Umea stock centers for stocks and antibodies. We also thank C. Alexandre, B. Bello and S. Pagakis for advice, and J. Smith and J.-P. Vincent for critical reading of the manuscript. This work was supported by the Medical Research Council.

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interactions among tracheal genes support a co-operative model for the


Insect oenocytes: a model system for studying cell-fate specification by Hox genes

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(Accepted 6 April 2001)

ABSTRACT
During insect development, morphological differences between segments are controlled by the Hox gene family of transcription factors. Recent evidence also suggests that variation in the regulatory elements of these genes and their downstream targets underlies the evolution of several segment-specific morphological traits. This review introduces a new model system, the larval oenocyte, for studying the evolution of fate specification by Hox genes at single-cell resolution. Oenocytes are found in a wide range of insects, including species using both the short and the long germ modes of development. Recent progress in our understanding of the genetics and cell biology of oenocyte development in the fruitfly Drosophila melanogaster is discussed.

In the D. melanogaster embryo, the formation of this cell type is restricted to the first 7 abdominal segments and is under Hox gene control. Oenocytes delaminate from the dorsal ectoderm of A1-A7 in response to an induction that involves the epidermal growth factor receptor (EGFR) signalling pathway. Although the receptor itself is required in the presumptive oenocytes, its ligand Spitz (Spi) is secreted by a neighbouring chordotonal organ precursor (COP). Thus, in dorsal regions, local signalling from this component of the developing peripheral nervous system induces the formation of oenocytes. In contrast, in lateral regions of the ectoderm, Spi signal from a different COP induces the formation of secondary COPs in a homeogenetic manner. This dorsoventral difference in the fate induced by Spi ligand is controlled by a prepattern in the responding ectoderm that requires the Spalt (Sal) transcription factor. Sal protein is expressed in the dorsal but not lateral ectoderm and acts as a competence modifier to bias the response to Spi ligand in favour of the oenocyte fate. We discuss a recently proposed model that integrates the roles of Sal and the EGFR pathway in oenocyte/chordotonal organ induction. This model should provide a useful starting point for future comparative studies of these ectodermal derivatives in other insects.

Key words: Hox; homeotic; oenocytes; chordotonal organs; EGF receptor; spalt.

INTRODUCTION
The fruitfly Drosophila melanogaster, with its one hundred years of genetics and a completely sequenced genome, holds a central position in the study of insect development. However, several recent technical advances look extremely promising for applying the kind of sophisticated genetic tools, normally associated only with D. melanogaster, to other insects. These new approaches include forward genetic screens in other insects such as the red flour beetle (Tribolium castaneum), gene mapping using PCR based methods and cross-species transfer of genes via ‘universal’ transposable element vectors and retroviral infection. Furthermore, genome projects have already been started in several insect species other than D. melanogaster and it is expected that these will come on-line in the very near future. For all of these reasons, the comparative developmental biology of insects is rapidly becoming a very promising area for the detailed study of evolutionary mechanisms.

Genetic pathways that control differences between segments during development are likely to provide a rich substrate for segmental variation during evolution (Akam, 1998b). Perhaps the best characterised genes in these segment-specific pathways are the...
Although the Hox protein sequences themselves are remarkably conserved across a wide range of animal species, their expression patterns and transcriptional targets appear to be highly variable (see for example, Palopoli & Patel, 1998; Weatherbee et al. 1999). Much current research is aimed at testing whether this source of variation is responsible for morphological innovation within Insecta.

The overall number of target genes modulated by the Hox genes during development is probably very large (Graba et al. 1997; Akam, 1998c; Pradel & White, 1998). Even in D. melanogaster, identifying all of the genetic networks under the control of any one Hox gene is a daunting prospect. Yet, if we are to understand how Hox genes modulate morphogenesis during development and evolution, the identification of these downstream targets is essential. This complex target problem can be reduced to something more manageable by focusing on the specification and differentiation of a single cell type that is under Hox control. Such a single-cell approach has been adopted recently in D. melanogaster for the larval oenocyte. The starting point for these studies is that larval oenocyte formation is completely blocked in embryos lacking all of the Hox genes of the bithorax complex: Ultrabithorax, abdominal A and Abdominal B (P. Elstob & A. Gould, unpublished observations). Thus the generation of the oenocyte fate is entirely dependent on Hox function. This dramatic Hox-dependence contrasts with many other cell types whose morphologies are only subtly modified by Hox inputs.

At present, it is not certain which of the 3 bithorax-complex genes are required for oenocyte formation. Neither is it known which Hox target genes are involved in making a larval oenocyte. Before we can hope to reconstruct the genetic cascade leading from Hox gene to oenocyte, it is important to understand the developmental origin of this rather mysterious cell type. The purpose of this article is to review the literature on larval oenocytes and to summarise recent progress in our understanding of their formation in Drosophila.

THE OENOCYTE AS A MODEL CELL FOR STUDYING HOX PATTERNING

The larval oenocyte is well suited to evolutionary-developmental studies of Hox patterning as it is both segment-specific and easily identifiable by morphological criteria across a range of insect species. Two separate populations of oenocytes exist in D. melanogaster, larval and imaginal (Koch, 1945). This review focuses only on the larval oenocytes (hereafter termed oenocytes) which are derived from the embryonic ectoderm of abdominal segments A1-A7 (Hartenstein et al. 1992; Elstob et al. 2001). In the mature embryo, the oenocytes occupy a characteristic lateral and subepidermal position and are clustered into a single group of cells within each hemisegment. Unlike many other ectodermal derivatives, such as the peripheral nervous system, the precise number of oenocytes in a cluster is not absolutely fixed and can vary anywhere between 4 and 9, with a mean of 6. After larval hatching, oenocytes undergo extensive cell growth without division so that by the late third instar they have attained a diameter of ~ 80 μm (Bodenstein, 1950). Due to their conspicuously large size and unusual ultrastructure, the oenocytes of Drosophila and other insects have long attracted the interest of invertebrate physiologists. The presence of densely packed smooth and rough endoplasmic reticulum, together with other morphological features characteristic of mammalian steroidogenic cells and hepatocytes, indicates that oenocytes are cells with a specialised secretory function (Koller, 1928; Wigglesworth, 1933; Rinterknecht & Matz, 1983).

However, the repertoire of the substances that they secrete and their physiological function in the intact organism are far from clear. On the basis that oenocyte morphology varies with the molting cycle and that maximal secretory potential is reached just prior to ecysis, it was postulated that these cells secrete lipid and protein components of the insect cuticle (Wigglesworth, 1933, 1970; Baikova et al. 1993). An alternative, but not mutually exclusive, function in synthesising molting hormones such as ecdysteroids has also been suggested (Locke, 1969; Dorn & Romer, 1976). Indeed, there is evidence that the oenocytes of 2 beetle species, Tribolium castaneum and Tenebrio molitor, are capable of secreting derivatives of ecdysone in vitro (Romer, 1971; Romer et al. 1974). Despite all of this historical interest in oenocytes, surprisingly little was known about the formation of these interesting cells until recently. Two new studies, however, have revealed that the embryonic origin of oenocytes in D. melanogaster is closely linked with that of another cell type: a proprioceptive component of the peripheral nervous system called a chordotonal organ (Elstob et al. 2001; Rusten et al. 2001). Therefore, in order to understand oenocyte development, it is necessary to be familiar with the mechanisms of chordotonal organ formation.
PRIMARY AND SECONDARY CHORDOTONAL ORGAN FORMATION

In each abdominal hemisegment of the Drosophila embryo, there are 8 chordotonal organs that are partitioned into arrays consisting of 1 dorsolateral (V'ch1), 5 lateral (Lch5) and 2 ventral (VchAB) organs (Brewster & Bodmer, 1995) (Fig. 1). The Lch5 array lies in the same lateral position, and just internal to, the mature oenocyte cluster of ~6 cells. As the remainder of this review will describe, this close association can be traced back in development to the

Fig. 1. Fate map for oenocytes and chordotonal organs. Each panel represents a single abdominal hemisegment at early (left) and late (right) stages of embryogenesis. Anterior (A) is to the left and dorsal (D) up, as indicated. The left panel shows the 5 primary COPs (C1-C5, light grey) that express Atonal. Arrowheads indicate the site of Spitz/EGFR induction events. Within the Sal dorsal domain (light grey shading), 6 oenocyte precursors (dark grey, sickle shape) are induced around C1. More ventrally, outside the Sal dorsal domain, 2 secondary COPs (black, circular) near C3 and one secondary COP near C5 are induced. The derivatives of all 14 precursor cells are shown in the right panel. The 6-cell oenocyte cluster (dark grey, circular) derived from the oenocyte precursors is depicted above the 5 primary chordotonal organs (C1-C5, light grey) and the 3 secondary chordotonal organs (black). The precursors for the 2 secondary chordotonal organs of the Lch5 are induced near C3, while the founder for the VchA is induced near C5. In the real embryo, there is spatial overlap between the oenocytes, V'ch1 and Lch5 but for clarity they have been drawn well separated. Note that there are 2 remaining uncertainties in the mapping. First, C2 is shown half-in and half-out of the Sal dorsal domain as its precise location is not clear. Secondly, within the Lch5 array, the anteroposterior order of the organs derived from C2 and C3 is not known. Data for this figure were compiled from zur Lage et al. (1997) and Elstob et al. (2001); see text for more details.
EGF Receptor Pathway

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EGF Receptor Pathway

Fig. 2. The components of the EGFR Pathway in chordotonal organ/oenocyte induction. A primary COP (upper cell) signalling to a secondary COP or oenocyte (lower cell) is depicted. The expression of Atonal (Ato) protein in the nucleus of the primary COP results in the transcriptional activation of the rhomboid (rho) gene. Rho, a 7-pass transmembrane protein, is required for the conversion of inactive, membrane-bound Spitz (mSpi) to its active, secreted form (sSpi). In turn, sSpi binds to and activates the EGFR, resulting in the stimulation of the RAS/MAPK signal transduction cascade. This results in transcriptional activation of pointed in the nucleus of the responding cell. One of the transcriptional targets for the Pointed protein is argos. Argos protein is secreted by the responding cell and inhibits further activation of the EGFR, thus completing a negative feedback loop that limits the extent of signalling.

time when the precursors for both cell types delaminate from the dorsolateral ectoderm of the extended-germ band embryo.

Each chordotonal organ is formed by a single chordotonal organ precursor (COP) that divides asymmetrically to produce a sensory neuron, sclopale, ligament and cap cell (Fig. 3A; Brewster & Bodmer, 1996). The proneural gene that specifies COPs and therefore mature chordotonal organs is atonal (ato) which encodes a basic helix-loop-helix transcription factor (Jarman et al. 1993). Previous studies have elegantly demonstrated that, in the embryo, COPs are produced in a 2-step delamination process (Okabe & Okano, 1997; zur Lage et al. 1997). Initially, several primary COPs that are Ato-positive delaminate from the ectoderm. A subset of these then induce the delamination of Ato-negative secondary COPs in a recruitment process that requires the EGFR signalling pathway (Fig. 2). In each abdominal hemisegment, 5 primary COPs (C1-5) are produced, with each occupying a characteristic dorsoventral position: C1 is the most dorsal and C5 the most ventral (Fig. 1). Ato expression in these primary COPs is thought to switch on the transcription of the rhomboid (rho) gene, which encodes a transmembrane protein that is rate-limiting for the conversion of the EGFR ligand, Spitz (Spi), from an inactive to an active form (Freeman, 1994; Tio et al. 1994; Bang & Kintner, 2000). In turn, active Spi induces 3 secondary COPs via binding to and activation of the EGFR, giving the full complement of 8 COPs and thus 8 chordotonal organs per hemisegment (Fig. 1, 2). When EGFR signalling is abolished, such as in rho mutants, the 3 chordotonal organs that are descended from the secondary COPs are missing, producing a deficit of 2 units from the Lch5 array and a deletion of the VchA organ (Bier et al. 1990). Thus, 5 primary COPs induce just 3 secondary COPs, which raises the question of exactly which primary cells induce which secondary cells. Part of the answer to this anatomical puzzle came from observations suggesting that C5, the VchB precursor, induces the secondary COP that gives rise to VchA (zur Lage et al. 1997). However, as described below, the question of which primary COPs induce the 2 secondary COPs that contribute to the Lch5 was only resolved very recently (Elstob et al. 2001).

OENOCYTES ARE INDUCED BY THE DEVELOPING PNS

By using several molecular markers to track the development of oenocytes and chordotonal organs, it was found that oenocyte precursors delaminate from the ectoderm overlying the most dorsally located primary COP (C1) and that the presence of this particular sensory mother cell is required for oenocyte formation (Elstob et al. 2001) (Fig. 1). Around the time of delamination, oenocyte precursors can be identified as a whorl of sickle-shaped cells that
surround the dividing C1 cell. Just before the onset of oenocyte precursor formation, C1 begins to express high levels of Rho and thus presumably acts as a source of active Spi. In rho and spi mutants, where production of Spi signal by C1 is blocked, oenocyte formation is completely abolished. Thus Spi signalling from C1 induces oenocyte precursor formation in the neighbouring ectoderm. Receipt of Spi ligand activates the EGFR and its target gene pointed (O’Neill et al. 1994) in the precursors themselves, triggering differentiation along the oenocyte pathway. Consistent with this scenario, reducing the level of EGFR activation using a dominant-negative form of the receptor or removing pointed function has the effect of abolishing induction. Oenocyte induction is a short-range signalling event, with only the cells immediately surrounding C1 switching on markers specific for this cell type. In argos mutants, however, the range of the response is increased from 1 to 2 concentric rings of cells. Argos is a secreted inhibitor of the EGFR that is produced in response to EGFR activation (Freeman, 1996; Okabe & Okano, 1997). Hence, Spi ligand is not intrinsically limited to immediate neighbours but the response is nevertheless kept short-range by argos-mediated negative feedback to the receptor.

By combining the recent oenocyte/chordotonal data together with that from earlier studies, most of the uncertainties in chordotonal organ fate mapping have now been resolved. The revised fate map for the complete set of all 14 oenocytes and chordotonal organs in an abdominal hemisegment is shown in Figure 1. With our current knowledge of the oenocyte induction event around C1, the previous suggestion (zur Lage et al. 1997), that C1 and C3 each induce one of the secondary COPs that contribute to the Lch5, now looks extremely unlikely. Therefore the revised map indicates that C1 induces approximately 6 oenocytes whilst C3 induces both of the secondary COPs for the Lch5.

CONTROL OF CELL NUMBER BY THE EXTENT OF EGFR PATHWAY SIGNALLING

The formation of both secondary COP and oenocyte fates requires the EGFR pathway. In ato, rho, spi, and EGFRDN backgrounds, where signalling is compromised, the induction of both cell types is blocked (Fig. 3A). Conversely, when there is more EGFR signalling both cell types become more numerous. This has been demonstrated in multiple ways, using mutants that lack argos function or when Rho or constitutively secreted Spi are overexpressed using the GAL4/UAS system. Together, these results indicate that the number of recruited cells is controlled by the amount of EGFR pathway signal. Rather surprisingly, however, there is no parity between the numbers of additional oenocytes and lateral chordotonal organs that are produced by excess EGFR signalling. Thus, for a given degree of ligand overproduction, more oenocyte precursors than COPs are recruited (Fig. 3A). This implies the existence of an additional tier of control that restricts neural but not oenocyte induction. Such a selective inhibition process would ensure that the number of chordotonal organs is more tightly controlled than that of oenocytes, as is observed in wild-type embryos.

SPALT SWITCHES ECTODERMAL COMPETENCE FROM COP TO OENOYCYTE

Both oenocytes and chordotonal organs are induced by the EGFR, but how can the same signalling pathway produce two such different outcomes? More specifically, this question centres on understanding how Spi signal from C1 induces oenocytes, while that from C3 induces Secondary COPs. One possibility would be that the information for specifying the choice of cell fate is somehow contained in differences in the level or timing of active Spi production. This, however, seems unlikely as the factors induced in the vicinity of C1 and C3 are not qualitatively altered by over expressing Rho or active Spi, or by mimicking reduced Spi production using a dominant-negative EGFR. An alternative explanation for the C1/C3 difference is that the 2 populations of ectodermal cells responding to active Spi are differentially prepatterned prior to signalling. Strong evidence in favour of this scenario comes from a detailed analysis of the expression and function of the spalt(sal) gene which encodes a zinc-finger transcription factor that interacts with the EGFR signalling pathway (Kuhnlein et al. 1994; Chen et al. 1998; Elstob et al. 2001; Rusten et al. 2001).

Epistasis tests, using combinations of loss- or gain-of-function mutations in sal together with EGFR pathway genes were used to rule out a function for sal in the primary COP producing the Spi inductive signal (Elstob et al. 2001; Rusten et al. 2001). Instead, the results of these experiments are consistent with sal acting in the responding ectoderm. Here it appears to play a dual role: first acting prior to Spi signalling to modify ectodermal competence and secondly, functioning downstream of the EGFR, as part of the oenocyte-specific response (Elstob et al. 2001).
Fig. 3. Summary and prime-and-respond model for oenocyte and chordotonal induction. (A) Summary of the number of oenocytes (red) and lateral chordotonal organs (green) in wild-type (WT) and mutant backgrounds. Each panel represents a single abdominal hemisegment and Vchl and VchAB are not shown. In the wild-type panel the relative positions of the cap (c), scolopale (s), neuron (n) and ligament (l) cells that constitute each of the 5 organs (a-e) of the LchS are shown. Missing lateral chordotonal organs and oenocytes are indicated by

(B) primary COPs

<table>
<thead>
<tr>
<th>oenocyte induction</th>
<th>Dorsal</th>
<th>chordotonal induction</th>
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<tbody>
<tr>
<td>C1</td>
<td>C1</td>
<td>C3</td>
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<tr>
<td>SPI</td>
<td>C2</td>
<td>C4</td>
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<tr>
<td>SPI</td>
<td>C5</td>
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Legend: 
- ATO
- low/high RHO
- EGFR activation
- low/high SAL
Interestingly, these 2 roles for sal can be accounted for by 2 distinct phases of Sal protein expression. An early dorsal domain (the Sal dorsal domain), independent of rho and spi function, appears to define a zone of ectodermal competence to form oenocyte precursors. Later, at the time of induction, Sal levels are upregulated but only as a response to EGFR activation within the oenocyte precursors themselves.

The Sal dorsal domain is present in the extended germ-band embryo prior to oenocyte and secondary COP induction. It includes ectodermal territory in the vicinity of C1 but not C3, which lies too ventrally. Thus Sal protein is present in the right place and at the right time to have a role in modifying the competence of the ectoderm to respond to EGFR ligand from C1 but not C3. In support of this, oenocyte induction is blocked in sal null mutants (Elstob et al. 2001; Rusten et al. 2001). In contrast, in the same mutant background, 2 supernumerary lateral chordotonal organs are formed. As these ectopic organs can be suppressed by simultaneously removing rho function, they must be derived from supernumerary secondary and not primary COPs. This strongly suggests that, in the absence of Sal, C1 induces two secondary COPs at the expense of the entire oenocyte cluster. Hence, in the wild-type situation, the presence of Sal in the ectoderm overlying C1 plays 2 roles: the suppression of COP recruitment and the promotion of oenocyte induction. Sal is sufficient for the first of these roles, as when ectopically expressed in the ectoderm overlying C3 it can block the induction of the 2 secondary COPs that would normally form there (Elstob et al. 2001; Rusten et al. 2001). In this context, however, oenocytes fail to be induced near C3, arguing that Sal is not sufficient for oenocyte induction (Elstob et al. 2001). The most likely explanation for this lack-of-sufficiency is that Sal is only one of several essential components needed to define the ectodermal prepattern for oenocyte induction. Presumably these other competence factors are present within the wild-type dorsal Sal domain but, like Sal itself, are not expressed in more ventral regions.

**Two cell fates from one signal: a prime-and-respond model for the role of Sal.**

The dual roles for sal as a competence modifier, and also a part of the oenocyte-specific EGFR response, have been integrated in a prime-and-respond model (Elstob et al. 2001). This model illustrates how both the oenocyte and the chordotonal cell fates might be induced by one signal (Fig. 3B). First considering the early role of sal, where it functions prior to signalling as a competence switch: here, Sal prepatterns the dorsal ectoderm so that, on receipt of the Spi signal, oenocytes rather than COPs are induced. Experiments varying Sal concentration and levels of EGFR activity suggest that one consequence of the presence of Sal in the responding nucleus is to increase the apparent threshold for an inductive event (Elstob et al. 2001). This makes the prediction that a signalling cell inducing oenocytes may need to express more ligand than one that recruits secondary chordotonal organs. In fact, this does appear to be the case as in wild-type embryos C1 is known to express high levels of rho for longer than C3 (Zur Lage et al. 1997). Hence, the EGFR pathway does contribute to the cell-type specificity of the induction event in the sense that more Spi ligand is required to overcome the higher induction threshold for oenocyte precursors than for secondary COPs.

Turning now to the later role of sal that is downstream of EGFR activation: here the up-regulation of Sal protein is an early oenocyte-specific response to Spi signalling. In turn, this high level of Sal appears to stimulate expression of seven up (svp), which encodes a member of the steroid receptor superfamily (Mlodzik et al. 1990; Elstob et al. 2001). Presumably a large set of genes is turned on in response to EGFR activation in oenocyte precursors but most of these have not yet been characterised. In the future, it will be interesting to see how much overlap there is between this gene set and the repertoire of genes that is switched on during COP induction.
A key feature of the prime-and-respond model is that moderate levels of sal expression serve to prime the responding cells to further upregulate Sal when they receive Spi ligand. In support of this priming mechanism, it was demonstrated that sal upregulation in response to constitutively secreted Spi is restricted to those cells that lie within the Sal dorsal domain (Elstob et al. 2001). Hence, Sal proteins appear to provide a molecular link between the prepattern and the EGFR response. As the levels of Sal are different in these 2 phases, it may be that there are at least 2 different concentration-dependent effects for this transcription factor. There is a precedent for this in another context, macrochaete formation, where low levels of Sal are known to promote sensory organ precursor formation but high levels have an inhibitory effect on the differentiation of this cell type (de Celis et al. 1999). Even more strikingly, during wing vein development, low and high levels of Sal are known to produce completely opposite transcriptional effects on the knirps target gene (de Celis & Barrio, 2000).

**REFERENCES**


Roles for sal and EGFR in oenocyte/COP induction


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