A PHOSPHORYLATION SITE IN THE FTZ HOMEODOMAIN IS REQUIRED FOR SEGMENTATION IN *DROSOPHILA*

by

Jianli Dong

A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy
Graduate Department of Molecular and Medical Genetics
University of Toronto

© Copyright by Jianli Dong 1999

National Library of Canada

Acquisitions and Bibliographic Services

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale du Canada

Acquisitions et services bibliographiques

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-41015-3

Canada

A Phosphorylation site in the Ftz homeodomain is required for segmentation in *Drosophila*
Doctor of Philosophy, 1999
Jianli Dong
Department of Molecular and Medical Genetics
University of Toronto

Abstract

The *Drosophila* homeodomain-containing protein Fushi tarazu (Ftz) is expressed sequentially in the embryo, first in alternate segments, then in specific neuroblasts and neurons in the central nervous system and finally in parts of the gut. During these different developmental stages, the protein is heavily phosphorylated on different subsets of Ser and Thr residues. This stage-specific phosphorylation suggests possible roles for signal transduction pathways in directing tissue-specific Ftz activities. The objective has been the testing of the importance of a single phosphorylation site for Ftz activity in the embryo. This was done by mapping Ftz phosphorylation sites, mutating the relevant Ser or Thr residues to Ala and Asp, and then testing the ability of the mutated proteins to rescue *ftz* mutant embryos.

My analysis has focused on T263, which is in the N-terminus of the Ftz homeodomain. This site is phosphorylated *in vitro* by *Drosophila* embryo extracts and by purified protein kinase A. In the embryo, mutagenesis of this site to the non-phosphorylatable residue Ala results in segmental defects. Conversely, replacement of T263 by Asp, which is also non-phosphorylatable, but which successfully mimics phosphorylated residues in a number of proteins, rescues the segmental phenotype. This suggests phosphorylation of Ftz on residue T263 is required for function in the ectoderm. In the CNS however, both T263A and T263D mutant proteins function normally, indicating that the requirement for T263 phosphorylation is stage-specific. In terms of

molecular properties, mutation of T263 to Ala and Asp does not affect Ftz DNA binding activity *in vitro*, nor do the mutations affect transcriptional activity in transfected S2 cells. I conclude that T263 phosphorylation is most likely required for a homeodomain-mediated interaction with an embryonically expressed protein.

To Mom and Dad

Acknowledgments

After I finish writing this lengthy dissertation, I am fully aware that this work of science and arts would not be completed without supports and contributions from many others whom I live and work with.

I am most grateful to my husband for his consistent support. He always stands by my side whenever I need him. Without him, I even could not imagine how I could have gone through these six years full of joys and disappointments. I specially thank my family members and relatives in China. They never stop delivering their blessings and encouragement from overseas.

In particular, I wish to thank my Ph.D. advisor, Dr. Henry Krause. I learned a lot from him during my Ph.D. training. He is the most patient scholar I have ever known. He always has wise solutions to test tough hypotheses. I am grateful to my committee members: Drs. Andrew Spence, and Mike Tyers. They offered much valuable advice and many critical suggestions for my project. I appreciate Drs. Thomas Kornberg, Barbara Funnell, and Ulrich Tepass for taking time for my thesis defence.

I would like to thank many people in the *Drosophila* community for their help. Drs. Daniel Kalderon, Ian Duncan, John Kiger, James Manley, Jim Jaynes, Andrew Travers, Michael Akam, and Manfred Frasch generously offered me fly lines, DNA constructs and antibodies. I thank Dr. Steve Mason for *Drosophila* embryo extracts and Dr. Laurent Ruel for S2 cell phosphate-free medium.

I also appreciate help from members of the people in the laboratory. Particularly, I would like to thank Ling-Hong Hung and Robert Strome for phosphoamino acid analyses on Ftz T263, Andrzej Nasiadka for fly crosses and Andrew Simmonds for computer support.

Table of Contents

Thesis Abstract ii

Acknowledgments v

Table of Contents vi

List of Tables x

List of Figures xi

List of Appendices xii

List of Abbreviations xiii

Chapter 1: Introduction 1

1.1 *Drosophila* Embryogenesis and Segmentation 2

1.1.1 *Drosophila* Embryogenesis, a Brief Overview 2

1.1.2 Segment and Parasegment 7

1.1.3 Genetic Control of *Drosophila* Segmentation 8

1.2 Homeobox Genes and Homeodomain Proteins 14

1.2.1 Homeobox Genes in Animal Development 14

1.2.2 Classification of Homeobox Genes 16

1.2.3 Homeodomain Proteins are Transcription Factors 19

1.2.4 Regulation of Homeodomain proteins by Protein-protein Interactions 23

1.2.4.1 α2, Mcm1 and **a**1 23

1.2.4.2 HOM/Hox and Exd/Pbx 25

1.2.4.3 Exd and Hth 27

1.3 *fushi tarazu*, a *Drosophila* Homeobox Gene 28

1.3.1 *ftz* Expression and Function 35

1.3.2 Transcriptional Regulation of the *ftz* gene 37

1.3.2.1 *Cis*-acting Transcription Elements 37

1.3.2.2 Trans-acting Factors 42

1.3.3 Regulation of *ftz* mRNA and Protein Stability 46

1.3.3.1 *ftz* mRNA Instability Elements 46

1.3.3.2 Ftz PEST Regions 47

1.3.4 Regulation of Ftz Activity by Protein-protein Interaction 48
1.3.4.1 Ftz and Prd 49
1.3.4.2 Ftz and Ftz-F1 50
1.4 Regulation of Transcription Factors by Phosphorylation 51
1.4.1 NF-κB/IκB and Dorsal/Cactus 51
1.4.2 CREB and CBP 53
1.4.3 Pit-1 54
1.4.4 Phosphorylation of Ftz 56
1.5 Objectives and Overview of this Dissertation 57

Chapter 2: A Phosphorylation Site in the Ftz Homeodomain is Required for Segmentation 61
2.0 Abstract 62
2.1 Introduction 63
2.2 Results 65
2.2.1 Ftz Phosphorylation by Embryo Extracts 65
2.2.2 *In vivo* Analysis of T263 Mutants 75
2.2.3 Cuticle Patterns 76
2.2.4 *ftz* Autoregulation 79
2.2.5 *en* and *wg* Expression 84
2.2.6 DNA-binding Activity 84
2.2.7 Transcriptional Activity in Cultured S2 Cells 85
2.3 Discussion 90
2.3.1 Evidence for T263 Phosphorylation *in vitro* and *in vivo* 90
2.3.2 Stage Specificity of T263 Mutant Defects 90
2.3.3 Variability in the T263A Phenotype 91
2.3.4 Activities Affected by T263 92
2.3.5 Proteins that Interact with Ftz 93
2.3.6 Conservation of Thr263 94
2.3.7 Identity of the T263 Kinase 97

2.3.8 Homeodomain Proteins and Phosphorylation 97
2.4 Materials and Methods 98
2.4.1 Site-directed Mutagenesis and Construction of Ftz Expression Vectors 98
2.4.2 Expression and Partial Purification of Ftz Polypeptides 99
2.4.3 *In vitro* Kinase Assays 100
2.4.4 Phosphopeptide Mapping and Phosphoaminoacid Analysis 101
2.4.5 Construction of P-element Rescue Vectors 101
2.4.6 Cuticle Preparations, *In situ* Hybridization and Immunolocalization 102
2.4.7 Electrophoretic Mobility Shift Assays 102
2.4.8 Transient Transfection Assays 103

Chapter 3: Tissue-specific Requirement for a Phosphorylation Site in the Fushi tarazu Homeodomain 104
3.0 Abstract 105
3.1 Introduction 106
3.2 Results and Discussion 108
3.2.1 Ftz Thr263Ala Embryos Have Normal Eve Staining in RP2 Neurons 108
3.2.2 RP2 Neurons Are Unaffected in Ftz T263D-rescued Embryos 110
3.2.3 Tissue-specific Requirements for T263 Phosphorylation 110
3.2.4 What is the Cause of T263D Lethality? 112
3.2.5 Phosphorylation as a Means of Switching Tissure-specific Homeoprotein Activities 112
3.2.6 Molecular Functions of Ftz Affected by Thr263 Phosphorylation 113
3.3 Materials and Methods 113
3.3.1 Fly Strains 114
3.3.2 Embryo Collection, Fixation and Immunostaining 114

Chapter 4: Discussion and Future Directions 119
4.1 Summary 120

4.2 Discussion 121
4.2.1 Ftz T263A is Defective in Segmentation 121
4.2.2 Ftz T263 Phosphorylation is not Required in the CNS 122
4.2.3 Molecular Consequences of Phosphorylation of Ftz T263 123
4.2.4 Is PKA the T263 Kinase? 125
4.2.5 Are Residues at Position 7 Phosphorylated in Other Homeodomains? 126
4.2.6 Multiple Levels of *ftz* Regulation 127
4.3 Future Directions 129
4.3.1 Identification of Proteins that Interact with Ftz and the Effect of Ftz T263 Phosphorylation on the Interaction 129
4.3.2 Analysis of the Effects of Mutations Affecting Other Ftz Phosphorylation Sites 133
4.4 Conclusion 135

Appendix I: Effects of Ftz T263 Mutation on Protein-protein Interactions 136
Introduction of Proteins Tested 137
Results 139
Discussion 140
Materials and Methods 140

Appendix II: Sequence Alignment of Representative Homeodomain Classes 146

References 151

List of Tables

Table 1. Survival indices of Ftz T263 mutant offspring 73

List of Figures

Figure 1. *Drosophila* embryogenesis, stage 1 to 13 — 4
Figure 2. Parasegment and segment — 10
Figure 3. Schematic of the genetic hierarchy controlling antero-posterior (A-P) patterning — 12
Figure 4. Antenna-to-leg transformation resulting from a homeotic mutation — 17
Figure 5. Schematic of a homeodomain-DNA complex — 21
Figure 6. Ftz amino acid sequence and mapped phosphorylation sites — 29
Figure 7. *ftz* pair-rule expression in wild-type embryos — 31
Figure 8. *ftz* expression in later stages — 33
Figure 9. Schematic of the 5' *cis* elements that regulate *ftz* transcription — 39
Figure 10. Experimental Approach — 59
Figure 11. Labeling of Ftz by embryo extracts — 67
Figure 12. Phosphorylation of Ftz by PKA — 69
Figure 13. Mapping cAMP- and PKA-dependent phosphorylation sites — 71
Figure 14. Cuticle preparations of T263 mutant larvae — 77
Figure 15. *ftz* gene autoregulation in Ftz T263 mutants — 80
Figure 16. *en* and *wg* expression in Ftz T263 mutants — 82
Figure 17. Binding of Ftz T263 mutant homeodomains to DNA — 86
Figure 18. Transcriptional activity of Ftz T263 mutants in cultured cells — 88
Figure 19. Comparison of homeodomain N-terminal sequences — 95
Figure 20. Eve expression in wild-type and Thr263Ala mutant embryos — 116
Figure 21. Eve expression in wild-type and Thr263Asp mutant embryos — 117
Figure 22. The Ftz homeodomain/HMG-D interaction is diminished by T263D — 142
Figure 23. The Ftz T263D-DNA interaction is abolished by HMG-D — 144

List of Appendices

Appendix I: Effects of Ftz T263 Mutation on Protein-protein Interactions 136

Appendix II: Sequence Alignment of Representative Homeodomain Classes 146

List of Abbreviations

A1 first abdominal segment

aa amino acid

ABC avidin-biotin conjugate

abdA abdominal A

AbdB Abdominal B

Adf-1 alcohol dehydrogenase gene distal factor-1

AEL after egg laying

Antp Antennapedia

ANT-C Antennapedia Complex

AP alkaline phosphatase

A-P anterio-posterior

AS-C achaete-scute complex

asg **a**-specific genes

ATBF1 gene of human α-fetoprotein enhancer-binding protein

ATP adenosine triphosphate

bcd bicoid

BCIP 5-bromo-4-chloro-3-indolyl-phosphate

bp base pair

BSA bovine serum albumin

bx bithorax

BX-C bithorax Complex

bZip basic domain-leucine zipper

cad caudal

Cam PK calmodulin-dependent protein kinase

CBP CREB-binding protein

CKII casein kinase II

CNS central nervous system

CRE cAMP-response element

CREB cAMP-response element binding protein

Cy3 indocarbocyanine

DAB diamino-benzine

DABCO 1,4-diazabicyclo [2,2,2] octane

Dax divergent *Antennapedia* class homeobox gene

Df deficiency

Dfd Deformed

DIG digoxigenin

DMSO dimethyl-sulfoxide

DNA deoxyribonucleic acid

DNase deoxyribonuclease

dNTPs deoxynucleoside triphosphates

DTT dithiothreitol

D-V dorso-ventral

ECL enhanced chemiluminescence

EDTA ethylenediaminetetraacetic acid

EMSA electrophoretic mobility shift assay

en engrailed

eve even-skipped

exd extradenticle

FIE3 3' *ftz* instability element

FITC fluorescein isothiocyanate

ftz fushi tarazu

Ftz-F1 Ftz factor 1

GOF gain-of-function

GSK-3 glycogen synthase kinase-3

h hairy

h Brn human brain gene

h En-2 human *en* homologue gene

h Evx2 human *eve* homologue gene

HAT histone acetyltransferase

hb hunchback

HMG-D a *Drosophila* high mobility group protein

HOM-C Antennapedia Complex and bithorax Complex

HOM/Hox homeobox genes belong to the homeotic gene cluster

HRP horse radish peroxidase

hs heat shock

hsg haploid-specific genes

hth homothorax

IκB inhibitor of NF-κB

IKK-α IκB kinase α

IKK-β IκB kinase β

IL-1 interleukin-1

IPTG isopropyl-β-D-thiogalactoside

kni knirps

Kr Krüppel

LB Luria Broth

lab labial

LIM lin-11, islet-1, and mec-3

LOF loss-of-function

MADS MCM1, AG, DEFA and SRF.

MAPK mitogen activated protein (microtubule associated protein) kinase

mRNA messenger ribonucleic acid

nos nanos

NBT nitro-blue tetrazolium

NF-κB nuclear factor kappa B

NLS nuclear localization signal

NMR nuclear magnetic resonance

NP-40 Nonidet P-40

odd odd-skipped

opa odd-paired

ORF open reading frame

otd orthodenticle

PAGE polyacrylamide gel electrophoresis

PAX paired box

pb proboscipedia

PBS phosphate-buffered saline

PBT PBS plus Tween 20

PCAT p300/CBP associated factor

PCR polymerase chain reaction

PEST protein region rich in Pro, Glu, Ser, and Thr

Pit-1 pituitary factor 1

PKA cAMP-dependent protein kinase

PKC protein kinase C

POU Pit-1, Oct-1, Oct-2, and unc-86

PP-1 protein phosphatase-1

PP-2A protein phosphatase-2A

prd paired

r Isl-1 rat islet-1 gene

RHD Rel homology domain

RNA ribonucleic acid

RNase ribonuclease

SAR scaffold attachment region

Scr Sex combs reduced

SDS sodium dodecyl sulfate

S6K S6 protein kinase

slp sloppy-paired

SRY sex-determining region of the Y chromosome

T1 first thoracic segment

TAFs TBP-associated factors

TBP TATA-binding protein

TCL-3 T-cell leukemia gene 3

TFIIB transcription factor IIB

TFIIE transcription factor IIE

TNF-α tumor necrosis factor α

Tris-HCl tris-hydroxmethyl aminomethane hydrochloride

ts temperature sensitive

ttk tramtrack

Ual Ultra-abdominal-like

Ubx Ultrabithorax

USE upstream element

UTR untranslated region

UV ultraviolet

wg wingless

Chapter 1: Introduction

Homeodomain proteins are important in animal development. Where investigated, all so far are phosphoproteins. Phosphorylation provides a potentially economic, quick and reversible mechanism to modulate the activities of these proteins. In my dissertation, I use Fushi tarazu as a model homeodomain protein to explore the importance of phosphorylation in homeodomain protein function.

Chapter 1 begins with a brief overview of *Drosophila* embryogenesis followed by a general introduction about homeodomain proteins. This includes their function, structure and their regulation by protein-protein interactions. The *fushi tarazu* gene is then introduced and discussed in terms of its expression, function and regulation. I end with well-studied examples of phosphorylated transcription factors and their modulation by phosphorylation. Finally, the hypothesis and the objectives of the dissertation are given.

1.1 *Drosophila* Embryogenesis and Segmentation

1.1.1 *Drosophila* Embryogenesis, a Brief Overview

At 25°C, a *Drosophila* embryo takes about 22 hr to develop from a fertilized egg to a larva (Roberts 1986; Lawrence, 1992; Brody, 1996). Figure 1 lists the distinct morphological features that allow staging of embryogenesis (Lawrence, 1992).

Early *Drosophila* embryogenesis is unusual in that the nucleus undergoes synchronous divisions that are not accompanied by cytokinesis. Stage 1 is the freshly laid fertilized egg characterized by a superficially homogenous cytoplasm. The embryo is already polarized in both the anterio-posterior (A-P) and dorso-ventral (D-V) axes (St. Johnston and Nüsslein-Volhard, 1992; Lawrence, 1992; Brody, 1996). Nuclear cleavage starts at stage 2, and after nine divisions, most of the syncytial nuclei migrate outward to the peripheral cytoplasm. Those that reach the

posterior pole plasm divide twice more on their own schedule and form the germ line precursor, the pole cells, during stage 3. The zygotic nuclei continue to divide rapidly and synchronously without cytokinesis for a total of 13 cycles to form the syncytial blastoderm at stage 4. About 5000 nuclei are produced by these 13 cycles of nuclear division within a common cytoplasm. The embryo is called a syncytial blastoderm. The plasma membrane then folds inward between the blastoderm nuclei, causing the formation of mononucleate cells. The general activation of the zygotic genome is believed to occur at the tenth nuclear cycle, about 90 min AEL (after egg laying) at 25°C (Weir and Kornberg, 1985; Edgar and Schubiger, 1986).

During stage 5, cell membranes grow down between adjacent nuclei to isolate each nucleus. This results in the formation of a monolayered cellular blastoderm. The advancing front of the membrane is visible under the microscope and its progress is often used to further classify stage 5 embryos (see figure 1). Gastrulation starts immediately after the completion of cellularization. It is perhaps the most dynamic period of embryogenesis. The morphogenetic movements of gastrulation eventually segregate the embryo into three germ layers: ectoderm, mesoderm, and endoderm. The prospective mesoderm cells lie along the ventral midline of the cellular blastoderm. They fold in to form the ventral furrow at stage 6. The furrow later becomes the ventral tube and flattens beneath the ventral ectoderm. The ventral portion of the embryo by now consists of layered cells and forms the germ-band that covers the entire region from the cephalic furrow to the posterior pole. During stage 7, the future endoderm, comprising the primordia of the anterior and posterior midgut, starts to invaginate into the interior of the embryo. Body segments begin to appear, dividing the ectoderm and the mesoderm. Movements of the cells within the germ band cause it first to extend around the posterior end of the embryo until it

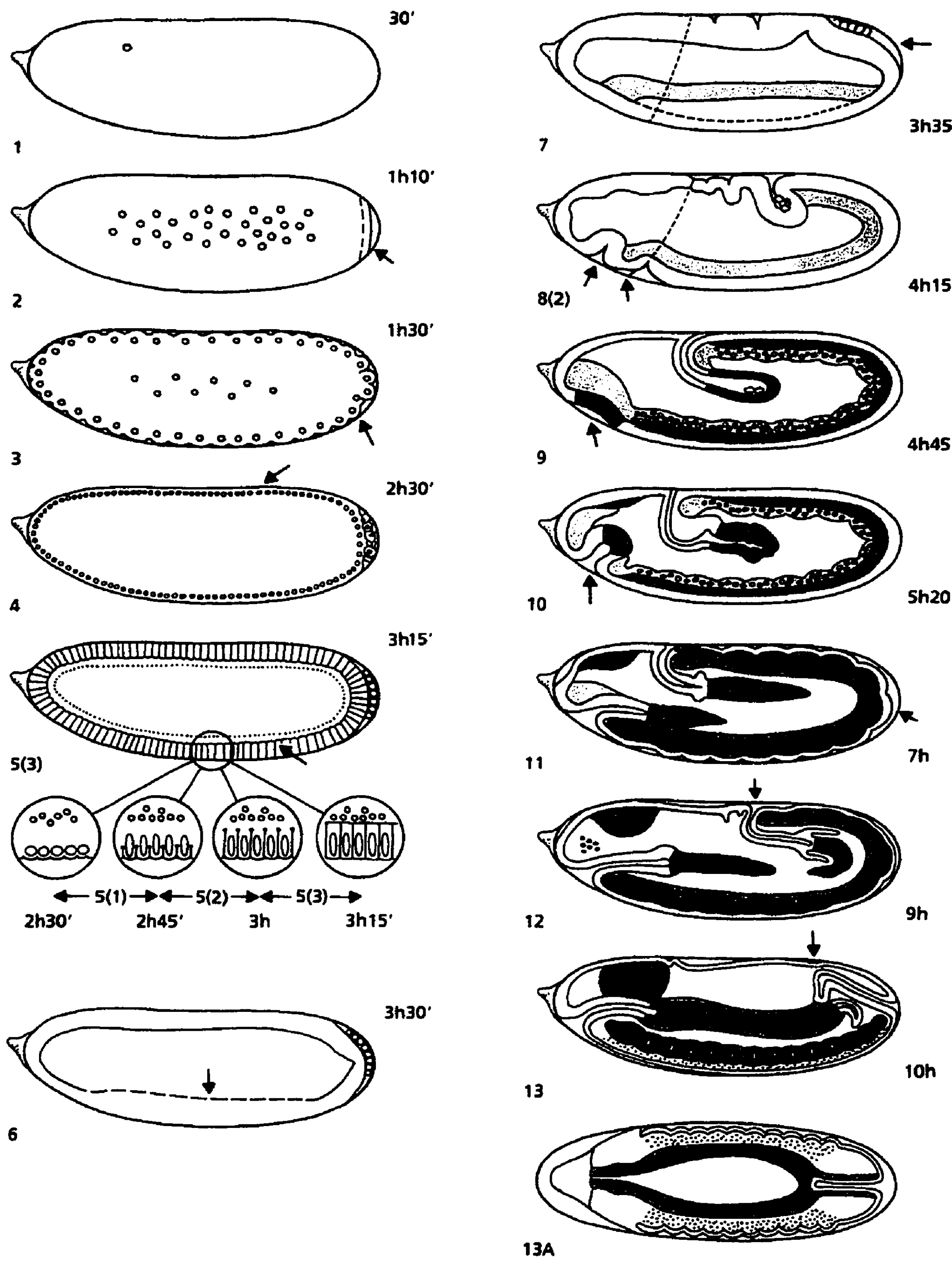
Figure 1. *Drosophila* embryogenesis, stage 1 to 13. Shown are schematic diagrams of wild-type *Drosophila* embryos, up to stage 13.

As in all figures in this thesis, anterior is to the left, and dorsal up. Numbers on the left-ventral side of the embryos indicate the developmental stages, those on the right-dorsal side mark the time points in development. Arrows point to those morphological features that are diagnostic of each particular stage and are explained below.

The arrow at stage 2 indicates "polar plasm", a cap of clear egg cytoplasm at the posterior pole of the embryo. The pole cells start to form at stage 3, as indicated, and the budded-off pole cells can be seen at the posterior pole of stage 4 embryos. The arrow in stage 4 indicates syncytial nuclei that have moved to the surface of the embryo forming the syncytial blastoderm. The advancing front of cell membranes that move down between adjacent nuclei are diagrammed in more detail in the stage 5 embryo. A furrow (ventral furrow) forms along the ventral midline of the embryo when gastrulation starts (stage 6), bringing the mesoderm primordium into the interior of the embryo. At stage 7, the posterior midgut primordium starts to invaginate at the posterior pole and form a plate. The plate is pulled dorsally and anteriorly resulting from the contraction of the dorsal side of the embryo. The germ-band is formed at this stage. The position of the cephalic furrow is shown as a dashed line. It forms on the lateral sides along the dorsal-ventral axis. The anterior midgut opening and cephalic furrow are indicated at stage 8 (arrows). The anterior midgut anlage is also indicated in stage 9 (arrow). Germ-band extension on the ventral side of the embryo pushes the posterior midgut invagination further anteriorly along the dorsal side. At stage 10, the anterior midgut starts to move posteriorly. The retraction of the embryo from the vitelline membrane at the posterior tip is indicated by the arrow at stage 11. The ectodermal segmentation is obvious as regularly spaced indentations. The posterior midgut

opening and the amnioserosa, on the dorsal surface of the embryo are pointed out in stage 12 and 13 embryos, respectively. The amnioserosa is an extraembryonic membrane that covers the dorsal side of the embryo. The germ-band retracts to the ventral side of the embryo at stage 13 (Roberts, 1986; Brody, 1996).

Different germ layers are shown in different colors, white for epidermis, pink for visceral mesoderm, brown for central nervous system, red for somatic mesoderm, and gray for endoderm. The figure is reprinted from Lawrence (1992). Figure ©1992 Blackwell Scientific publications. Reproduced here by permission.



30'
1
1h10'
2
1h30'
3
2h30'
4
3h15'
5(3)
5(1)
5(2)
5(3)
2h30'
2h45'
3h
3h15'
3h30'
6
7
3h35'
8(2)
4h15'
9
4h45'
10
5h20'
11
7h
12
9h
13
10h
13A

reaches about 2/3 of the way along the dorsal side, and then to retract until it is once again confined to the ventral surface between stages 8 and 12.

Later in gastrulation, the central nervous system (CNS), otherwise known as the ventral cord, develops from the neurogenic region, a single cell layer of the ventral ectoderm along the midline. The neuroectodermal cells enlarge and move into the interior of the embryo, where they differentiate into a stereotypical pattern of about 50 neuronal precursor cells (neuroblasts, NB) per segment. Each neuroblast is a stem cell that produces smaller progeny cells. The CNS of the embryo persists, with modifications, throughout the larval and pupa stages into adulthood (Doe and Scott, 1988).

The embryo gut is formed through assembly of foregut, midgut, and hindgut. The midgut is derived from endoderm. The foregut and the hindgut develop from the ectoderm surrounding the anterior and posterior midgut rudiments. Some parts of the hindgut become the Malpighian tubules. These are tissues that persist throughout metamorphosis into the adult when they serve a role analogous to kidney. The first instar larva hatches from the egg case at 22 hr after egg laying (AEL) at 25°C (Roberts, 1986; Brody, 1996).

1.1.2 Segment and Parasegment

Like most animals, the fruitfly *Drosophila melanogaster* has a body that consists of repeating and morphologically identical units (ie, metameres or segments) along the anterio-posterior (A-P) axis. This is evident in the form of epidermal grooves in the embryo, denticle belts in the larva, and segments (pigmented with stripes in the abdomen, and with legs and wings in the thorax) in adults. Segmentation in the adult head is not easily distinguished.

Segmentation is readily assayed by observation of the larval cuticle. Cuticle is secreted by epidermal cells at the end of embryogenesis. The most distinct feature of the larval cuticle is a series of denticle belts on the ventral surface. In each segment, the most anterior region is covered with denticles to varying extents, manifesting the diversity and arrangement of the underlying epidermal cells (Ashburner, 1989). The thoracic segments have short and stubby denticles arranged in 2-3 parallel rows, whereas the abdominal segments have 5-6 rows of trapezoidal denticles with varying size, shape and numbers. The larval segments, as reflected by the cuticle, coincide with the segmental boundaries of the adult fly.

The initial division of the embryonic epidermis is not in segments but rather in "parasegments" (Martinez-Arias and Lawrence, 1985; Lawrence, 1988). A parasegment consists of the posterior part of one segment and the anterior part of an adjacent segment (Figure 2). Morphologically, grooves appear at stage 11 in the extended germ band, and these coincide with the parasegmental boundaries (see Figure 1; Martinez-Arias and Lawrence, 1985). Well before this the parasegmental divisions are forecasted by segmentation gene expression patterns (Martinez-Arias and Lawrence, 1985; Lawrence, 1988, 1992).

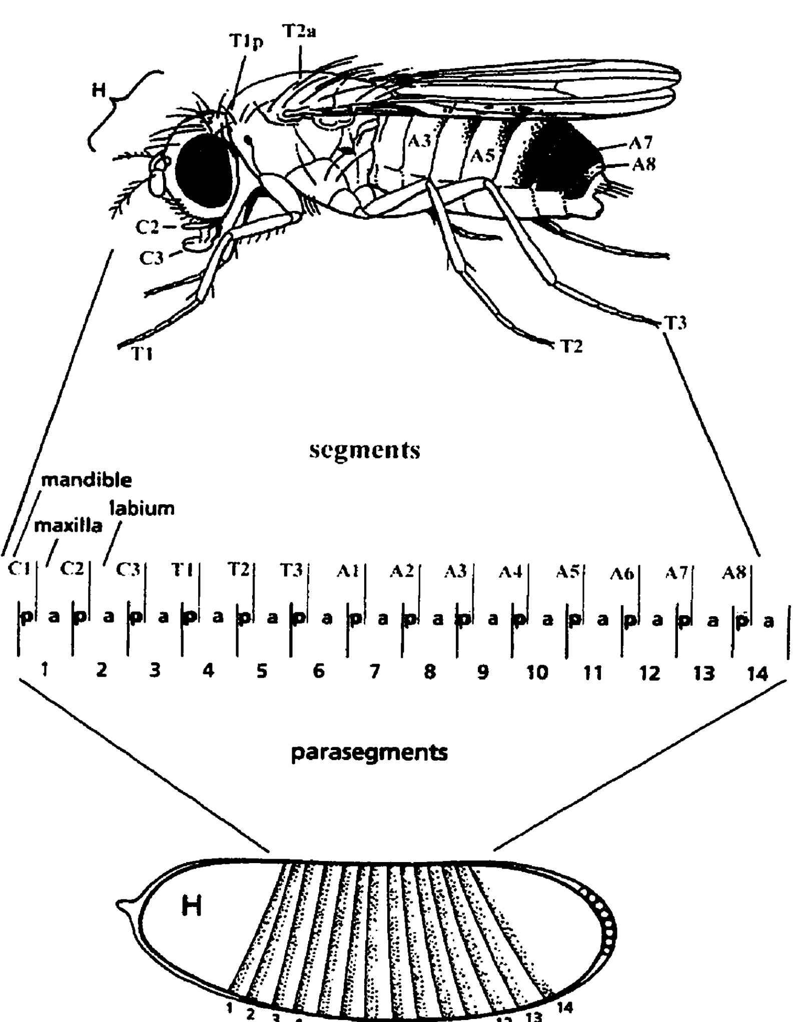
1.1.3 Genetic Control of *Drosophila* Segmentation

Pattern formation along the anterio-posterior (A-P) axis results in formation of the segmented trunk and the non-segmented termini. It occurs independently of the processes establishing dorso-ventral polarity (reviewed in St. Johnston and Nüsslein-Volhard, 1992). The *fushi tarazu* (*ftz*) gene, which is the focus of this thesis, functions in the segmentation of the A-P axis.

Segmentation is established in two phases, occurring before and after cellularization. During the first phase there is a cascade of interactions between genes and transcription factors. The second phase occurs after cellularization and depends on communication between cells (reviewed in Ingham, 1988). Combined genetic and molecular studies reveal that segmentation is controlled by a hierarchy of successively acting genes (Nüsslein-Volhard and Wieschaus, 1980; Ingham 1988; Lawrence, 1992; Kornberg and Tabata, 1993). These genes include the maternally active coordinate genes, and the zygotically active segmentation and homeotic genes. Based on mutant phenotypes and on the time of gene activation, the segmentation genes are grouped into gap, pair-rule, and segment polarity genes.

Each of the genes involved in segmentation shows a unique spatial expression pattern. Each is controlled by previously acting genes as well as interactions with other members of the same class. Transcripts of the anterior and posterior coordinate genes are deposited into the embryo by the mother, and are necessary for establishing embryonic polarity (reviewed in St. Johnston and Nüsslein-Volhard, 1992). The gap genes respond to these maternally derived signals and are expressed in several adjacent segmental primordia. These subdivide the embryo into broad sections. The next genes in the hierarchy, the pair-rule genes, are expressed in alternate segmental primordia. They are responsible for setting up segmental boundaries, and direct the expression of the segment polarity and homeotic genes. The segment polarity genes are expressed in a portion of every segment. They establish and maintain polarity within each segment. The homeotic genes, including the loci of the *Antennapedia* (*Antp*) and *bithorax* (*bx*) complexes, dictate the specific morphological characteristics of each segment (Lewis, 1978; Nüsslein-Volhard and Wieschaus 1980; Wakimoto and Kaufman, 1981; Lawrence and Morata,

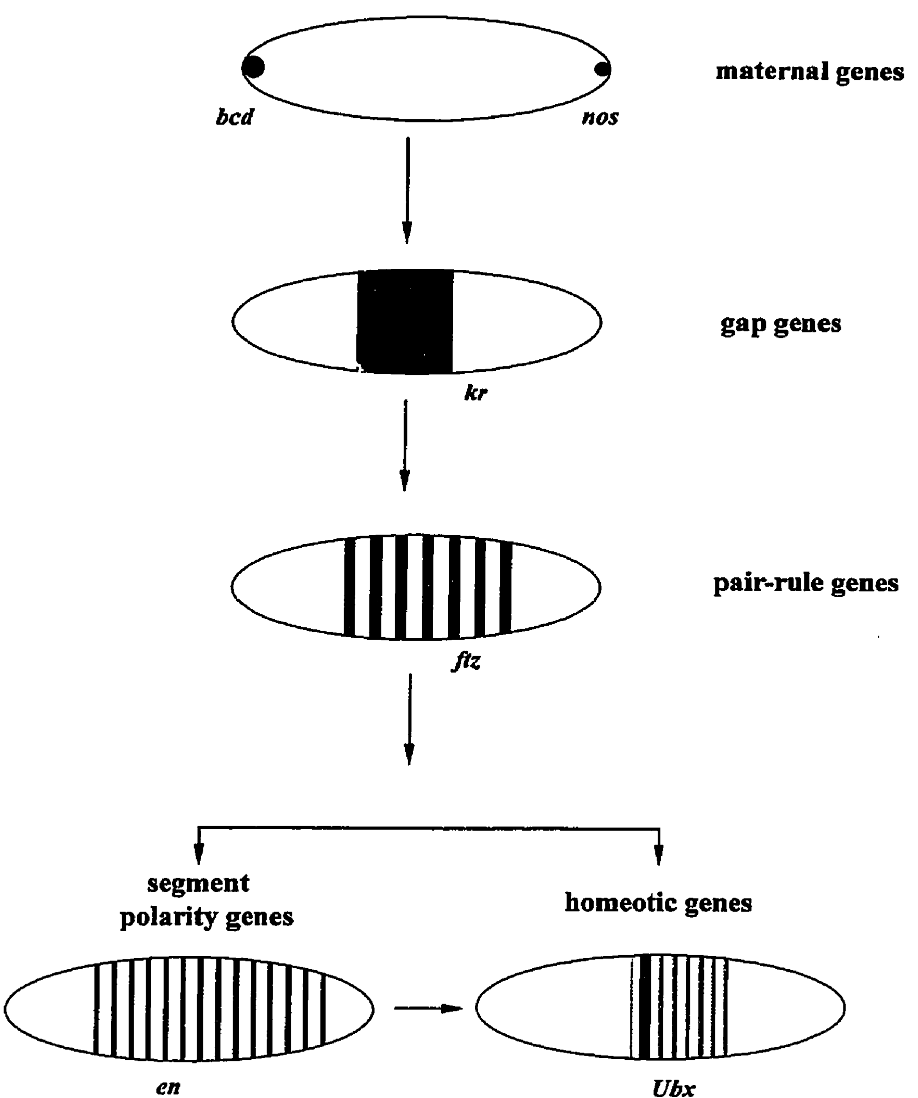
Figure 2. Parasegment and segment. Parasegments are out of register with the future segments. There are 14 parasegments, plus head (H) and tail regions in the embryo. The 14 parasegments later give rise to three head segments (C1-C3), three thoracic segments (T1-T3), and eight abdominal segments (A1-A8). The figure is reprinted from Lawrence (1992). Figure ©1992 Blackwell Scientific publications. Reproduced here by permission.



T1p
T2a
H
A3
A5
A7
A8
C2
C3
T1
T2
T3
segments
mandible
maxilla
labium
C1
C2
C3
T1
T2
T3
A1
A2
A3
A4
A5
A6
A7
A8
p a p a p a p a p a p a p a p a p a p a p a p a p a p a
1 2 3 4 5 6 7 8 9 10 11 12 13 14
parasegments
H
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 3. Schematic of the genetic hierarchy controlling antero-posterior (A-P) patterning. Maternal, gap, pair-rule, segment polarity and homeotic genes are presented with typical examples of mRNA expression patterns, shown for each class (adapted from Ingham, 1988).

bcd (*bicoid*) and *nos* (*nanos*) are maternal coordinate genes. Their mRNAs are deposited into the anterior and posterior ends of the embryo, respectively, by the mother. *Kr* (*Krüppel*) is a gap gene, expressed in a broad stripe. *ftz* (*fushi tarazu*) is a pair rule gene that is expressed in every other segment. *en* (*engrailed*) is a segment polarity gene and is expressed in the posterior part of each segment. *Ubx* (*Ultrabithorax*) is a homeotic gene. It is expressed most highly in part of the third thoracic segment and the first abdominal segment.


maternal genes
bcd
nos
gap genes
kr
pair-rule genes
ftz
segment
polarity genes
homeotic genes
en
Ubx

1983; Ingham, 1988; McGinnis and Krumlauf, 1992). The hierarchical regulatory relationship of these genes is depicted in Figure 3.

1.2 Homeobox Genes and Homeodomain Proteins

Homeobox genes are master control genes of axial patterning, organogenesis and cellular differentiation (Gehring, 1987; McGinnis and Krumlauf, 1992; Manak and Scott, 1994). Homeobox genes are defined by a common sequence element of 180 bp, the homeobox (McGinnis et al., 1984a, 1984b; Scott and Weiner, 1984; Shepherd et al., 1984). The homeobox encodes a 60 amino acid domain referred to as the homeodomain. The homeodomain serves as the DNA-recognition motif that allows these proteins to bind DNA sequence-specifically. Once bound to DNA, homeobox proteins function as transcription factors that regulate target genes in a precise spatial and temporal pattern (reviewed in Gehring, 1987; Levine and Hoey, 1988; McGinnis and Krumlauf, 1992; McGinnis, 1994; Manak and Scott, 1994).

1.2.1 Homeobox Genes in Animal Development

Abnormal expression or mutation of homeobox genes can result in remarkable phenotypes (reviewed in McGinnis and Krumlauf, 1992; Castronovo et al., 1994; Boncinelli, 1997). In *Drosophila*, for example, the homeobox genes *Antp* and *eyeless* are normally expressed and required in the leg and eye discs respectively. Misexpression of *Antp* in the antenna discs transforms the antennae into legs, and misexpression of *eyeless* transforms antennae into eyes (Schneuwly et al., 1987; Halder et al., 1995). These same transformations can be induced by ubiquitous expression of the vertebrate orthologs of the two genes, *Hoxb-2.2* and *Pax-6* (Malicki et al., 1990; Halder et al., 1995), exemplifying the importance of these genes in pattern formation

and the evolutionary conservation of these genes. Figure 4 shows the antenna-to-leg transformation characteristic of an *Antp* gain-of-function phenotype.

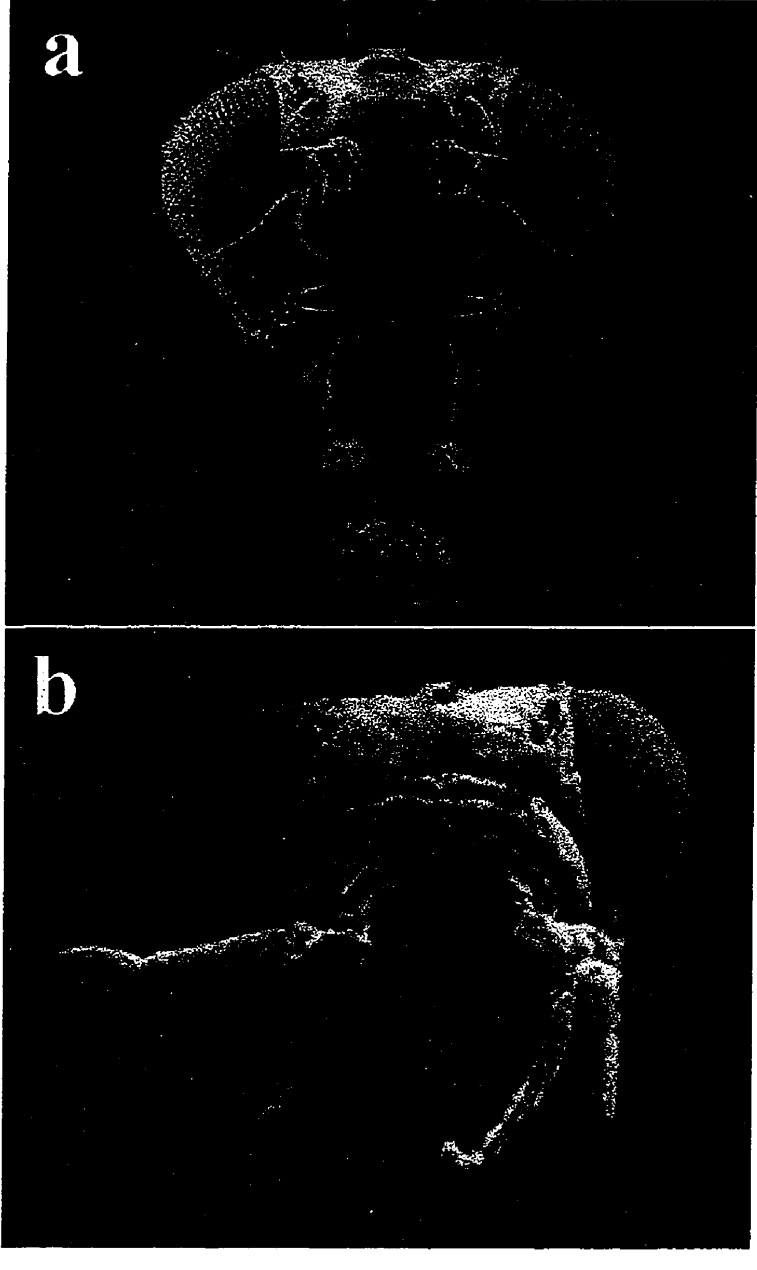
In addition to their role in embryo development, homeobox genes also play key roles in the fundamental processes of somatic cell growth and differentiation. It is not surprising, therefore, that several homeobox genes can be converted into oncogenes. A subset of homeobox genes form a family of nuclear proto-oncogenes (reviewed in Castronovo et al., 1994; Boncinelli, 1997). T-cell leukemia gene 3 (TCL-3), formerly Hox11, represents the first example of a homeobox gene functioning as an oncogene (Dube et al., 1991; Hatano et al., 1991). TCL-3 was isolated by means of its association with a chromosomal breakpoint that causes a human T-cell leukemia (Dube et al., 1991; Hatano et al., 1991). Transgenic mouse studies have since shown that TCL-3 is required for spleen formation during normal development (Roberts et al., 1994), and that its ectopic expression in the thymus causes T cell lymphoma (Hatano et al., 1992). Similarly, fusion between the homeodomain protein Pbx and the E2A protein, caused by another chromosomal translocation, induces B-cell lymphomas (Kamps et al., 1991; Dedera et al., 1993). Expression of the fusion genes in cultured cells also results in malignant transformation as assayed by tumor formation in nude mice (Kamps et al., 1991). Expression of the same fusion genes in transgenic mice induces leukemias and lymphomas (Dedera et al., 1993). *Cdx2*, the mouse homologue of the *Drosophila* homeobox gene *caudal*, behaves as a tumor suppressor gene; *Cdx2* homozygous null colon cells develop multiple intestinal adenomatous polyps (Chawengsaksophak, et al., 1997). The expression of several other homeobox genes has been shown to be associated with different kinds of cancers: examples include hLH-2, HOXB7, HOXB6, HOXB8, HOXC8 and HOXC9 (Wu et al., 1996; Carè, et al., 1996; Vider et al., 1997). However, in these instances causal relationships have yet to be established.

1.2.2 Classification of Homeobox Genes

The number of known homeobox genes has been growing steadily since their discovery more than a decade ago. Homeobox genes have been classified into two superclasses, the Complex Superclass, comprising the homeobox genes clustered in the homeotic complexes, and the Dispersed Superclass, whose genes are dispersed throughout the genome (Scott, 1992, 1993; Sharkey et al., 1997).

The *Drosophila* homeotic complex is referred to as the HOM complex (HOM-C) (Akam, 1989). In *Drosophila melanogaster*, the HOM-C has been split from a single ancestral complex into two separate complexes, the Antennapedia complex (ANT-C) and the bithorax complex (BX-C). The eight homeotic genes within the HOM-C (*labial, lab*; *proboscipedia, pb*; *Deformed, Dfd*; *Sex combs reduced, Scr*; *Antp*; *Ultrabithorax, Ubx*; *abdominal A, abdA*; and *Abdominal B, AbdB*) are called HOM genes. Four HOM-C homologous clusters exist in mammals and are thought to have arisen by two duplication events. The genes in these clusters are referred to as Hox genes (Scott, 1992, 1993; Sharkey et al., 1997). Although there are many homeobox genes, only those that are in these particular clusters are called homeotic genes. When this rule was applied, many homeobox genes were given new names, (e.g. Hox11 to TCL-3).
Homeobox genes can be further divided into at least 20 classes based on their primary sequence identity (Scott et al., 1989; Scott, 1992, 1993; Gehring et al., 1994a; Manak and Scott, 1994; Sharkey et al., 1997). Within the Complex Superclass there are 14 paralog classes (paralogs 1-13 and Evx). Paralogous genes share similar homeobox sequences and occupy the same relative positions within each Hox cluster (Scott, 1992, 1993; Gehring et al., 1994a; Manak and Scott, 1994; Sharkey et al., 1997). Interestingly, the order of these genes along the chromosome is

Figure 4. Antenna-to-leg transformation resulting from a homeotic mutation. The expression of the *Drosophila* homeotic gene *Antp* in antennal disks transforms antennae (a) to thoracic legs (b). *Antp* is normally expressed in T2 thoracic leg disks. Ubiquitous expression of *Hoxb-2.2*, the mouse ortholog of *Antp*, in antennal disks causes a similar transformation (Malicki et al., 1990). Images from: The Interactive Fly, http://sdb.bio.purdue.edu/fly/aimain/images.htm (Image allowed for all non-profit and educational purposes with attribution to Dr. Rudi Turner in the Department of Biology, Indiana University Bloomington, IN).


a
b

colinear with the order of their expression domains along the anterior-posterior body axis. Genes located more 3' in the cluster are expressed more anteriorly in the embryo, and genes located more 5' in the cluster are expressed more posteriorly in the embryo (Duboule and Morata, 1994). Such colinearity suggests a connection between the organization of the HOM/Hox complexes and the molecular mechanism of homeotic gene regulation.

The Dispersed Superclass of homeobox genes can be subdivided into more than 16 classes, each with its own distinguishing conserved sequences (Scott et al., 1989; Gehring et al., 1994a; Manak and Scott, 1994). The POU (Pit-1, Oct-1, Oct-2, and unc-86) (Herr et al., 1988), the LIM (lin-11, islet-1, and mec-3) (Karlsson et al., 1990), and the PAX (paired box) (Bopp et al., 1986) genes are examples of the Dispersed Superclass of homeobox genes (Gehring et al., 1994a; Manak and Scott, 1994). Each shares similar homeodomain sequences and contains separate domains outside the homeodomain, referred to as the POU-specific, the LIM, and the paired domains respectively (see Appendix II for the aligned sequences of a few representative homeodomain classes).

1.2.3 Homeodomain Proteins are Transcription Factors

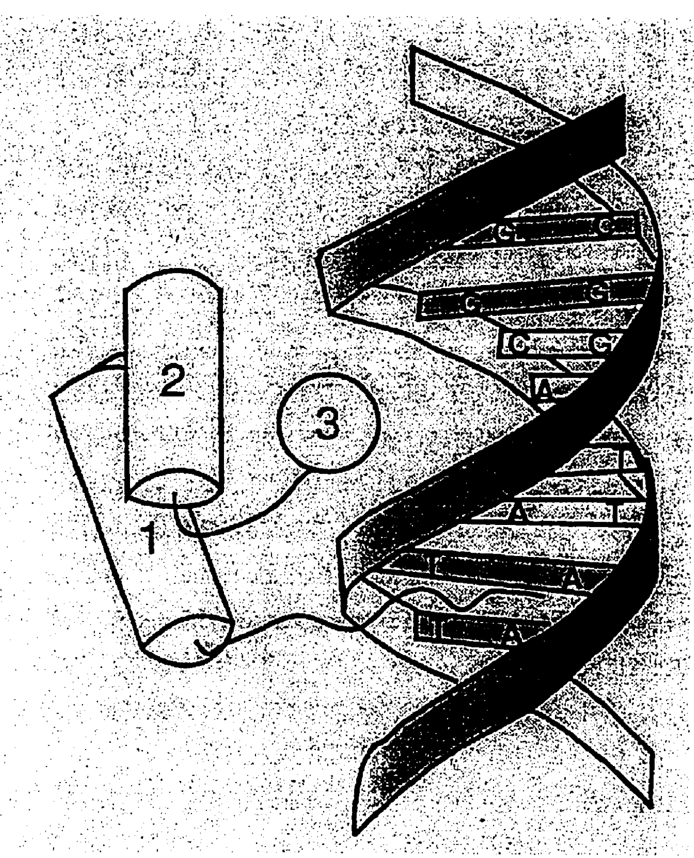
Homeodomain proteins are sequence-specific transcription factors (reviewed in Levine and Hoey, 1988). Like other transcription factors, homeodomain proteins contain modular domain structures (Frankel and Kim, 1991; Buratowski, 1994). For example, Fushi tarazu (Ftz) contains DNA binding domain, transcriptional activation domains, nuclear localization signals, and PEST sequences (detailed in section 1.3).

The homeodomain is both a DNA binding and protein binding motif. Its DNA binding ability has been analyzed *in vitro* by electrophoretic mobility shift assays (EMSA), DNase I

footprinting, immunoprecipitation assays, random oligonucleotide library screening, and cultured cell transcription assays (Desplan et al., 1985, 1988; Hoey and Levine, 1988; Hanes and Brent, 1989; Krasnow et al., 1989; Laughon, 1991; Kalionis and O'Farrell, 1993; reviewed in Gehring et al., 1994a, 1994b). These studies reveal that the homeodomain has intrinsic DNA binding preferences. Most of the DNA sequences that interact optimally with the homeodomain contain a 5'ATTA3' motif (reviewed in Hayashi and Scott, 1990; Gehring et al., 1994a, 1994b).

The three-dimensional structures of several homeodomains, both free and DNA-bound, have been solved. These include the Antp (Qian et al., 1989; Billeter et al., 1990, 1993), En (Kissinger et al., 1990), MATα2 (Wolberger et al., 1991), Ftz (Qian et al., 1994), and Oct-1 (Klemm et al., 1994) proteins. Despite only limited sequence conservation, for instance, the α2 and *en* homeodomains have only 27% sequence identity (Kissinger et al., 1990; Wolberger et al., 1991), all five of these homeodomains fold into a similar structure with three α helices, and all capable of binding DNA as monomers (reviewed in Gehring et al., 1994a, 1994b). Figure 5 is a schematic of a homeodomain-DNA complex. The flexible N-terminal arm (residues 1-9) precedes the three α helices and contacts the DNA minor groove via the highly conserved Arg-3 and Arg-5 residues (Billeter et al., 1993; Gehring et al., 1994a, 1994b). Helices I and II (residues 10-21 and 28-38) are separated by a loop and run parallel to the DNA. Helix III (residues 42-52) is connected to helix II by a tight turn to form a helix-turn-helix motif. The third helix is called the recognition helix since it fits into the DNA major groove and makes numerous base-specific contacts with the DNA (Kissinger et al., 1990; Wolberger et al., 1991; Billeter et al., 1993; Gehring et al., 1994a, 1994b). The residue at position 9 of the third helix (residue 50 of the homeodomain) has been shown to be the main determinant of homeodomain DNA binding specificity (Treisman et al., 1989; Schier and Gehring, 1992). The majority of homeodomain

Figure 5. Schematic of a homeodomain-DNA complex. The DNA backbone is represented by ribbons and the DNA base pairs by horizontal bars with the single-letter symbols of the bases. The homeodomain backbone is drawn as a solid line. It is folded into a helix-turn-helix structure. These helices are indicated by cylinders. The third helix, seen end-on, is the recognition helix and sits in the DNA major groove. The N-terminal arm of the homeodomain wraps around and contacts the DNA minor groove. This model is based on the structural analysis of homeodomain-DNA complexes (Gehring et al., 1994a, b). This figure is reprinted from Draganescu et al. (1995). Figure ©1995 Academic Press. Reproduced here by permission.



2
3
1

proteins possess one of three residues at this position; Gln, Lys or Ser. Homeodomains sharing the same residue at this position bind to closely related sequences.

Protein-protein interactions made by the homeodomains are exemplified in the next section (1.2.4). The gene transcription function of homeodomain proteins is achieved partially by directly recruiting general transcription factors to target promoter sites (reviewed in Mitchell and Tjian, 1989; Ptashne and Gann, 1997). Homeodomain proteins have been shown to interact with the general transcription apparatus. For example, Even-skipped (Eve) interacts with TATA-binding protein (TBP) (Um et al., 1995; Zhang et al., 1996), Ftz with transcription factor IIB (TFIIB) (Colgan et al., 1993, 1995), Hunchback (HB) and Bicoid (Bcd) with TBP-associating factor (TAF_{II}s) (Sauer et al., 1995), and Antp and Abd-B with TFIIEβ (Zhu and Kuziora, 1996).

1.2.4 Regulation of Homeodomain Proteins by Protein-protein Interactions

Differences in the primary sequences of homeodomains result in subtle differences in their *in vitro* DNA binding preferences. These differences probably have real consequences *in vivo* but are unlikely to account for all of their functional differences *in vivo* (reviewed in Hayashi and Scott, 1990; Mann, 1995). Additional mechanisms, especially protein-protein interactions, have been shown to play important roles in the regulation of homeodomain protein function (reviewed in Mann and Chan, 1996). Protein-protein interactions affect homeodomain protein DNA binding specificity and affinity, as well as sub-cellular localization (see below). These interactions involve contacts both within and outside of the homeodomain.

1.2.4.1 α2, Mcm1 and a1

The interactions of the homeodomain protein α2 with **a**1 and Mcm1 are the best examples of protein-protein interactions that direct a homeodomain protein to its proper gene target. The budding yeast *Saccharomyces cerevisiae* exhibits three cell (mating) types: haploid **a** and α cells and diploid **a**/α cells. The haploid **a** and α cells constitutively express **a**-specific genes (**a**sg's) and α-specific genes (αsg's), respectively, and both express haploid-specific genes (hsg's). The mating of haploid cells results in the formation of the **a**/α diploid. In the diploid, the **a**sg's and αsg's, as well as the hsg's, are repressed. α2 is involved in the regulatory system that specifies these cell mating types. α2 represses the transcription of two distinct sets of cell-type-specific genes (reviewed in Herskowitz, 1989). In haploid α cells, α2 combines with the general transcriptional regulator Mcm1, a MADS box protein (reviewed in Shore and Sharrocks, 1995), to bind the upstream sequences of **a**sg's and repress their transcription. In diploid **a**/α cells, α2 associates with **a**1, another homeodomain protein, to recognize a different set of operator sequences and to repress transcription of hsg's (Hall and Johnson, 1987; Keleher et al., 1988).

α2 and Mcm1 bind as a heterotetramer to partially symmetric 31 bp sites located in the promoters of **a**sg's. Mcm1 binds as a dimer to the center of each recognition site, flanked on each side by α2 monomers (Keleher et al., 1989, Vershon and Johnson, 1993). One role of Mcm1 is to increase the sequence-specificity of α2 by requiring a specific spacing and orientation of the α2 and Mcm1 binding sites (Smith and Johnson, 1992). The second role is to increase the binding affinity of α2. In the presence of Mcm1, α2 DNA binding affinity increases at least 100-fold for **a**sg sites, indicating that there are strong cooperative interactions between α2, Mcm1 and the promoter DNA (Keleher et al., 1989). Thus specific protein-protein and protein-DNA interactions both contribute to the functional specificity of α2. The protein-protein interaction

requires the MADS box of Mcm1 and a 20 aa flexible N-terminal extension of the α2 homeodomain (Keleher et al., 1989, Vershon and Johnson, 1993). This 20 aa homeodomain extension is both required and sufficient for cooperative binding with Mcm1 since grafting to the amino terminus of the En homeodomain results in cooperative binding of the fusion protein to Mcm1 (Vershon and Johnson, 1993). This 20 aa extension is not involved in DNA binding by α2 or for the interaction between α2 and **a**1.

α2 interacts with a1 in **a**/α cells (Dranginis, 1990). This interaction furnishes α2 with a new binding specificity so that it can bind to different DNA sequences, the hsg operators, and then repress the associated genes (Goutte and Johnson, 1988). The three-dimensional crystal structure of the α2/**a**1 homeodomain heterodimer bound to DNA has been determined (Li et al., 1995). The α2 and **a**1 homeodomains bind in a head-to-tail orientation. The cooperative binding of α2 and **a**1 on DNA causes a 60° bend in the DNA, allowing an interaction between a 16 aa carboxyl-terminal extension of the α2 homeodomain and helices I and II of the **a**1 homeodomain (Li et al., 1995). Upon binding to **a**1, the 16 aa α2 homeodomain extension becomes ordered and part of it forms a short amphipathic helix structure (Mak and Johnson, 1993; Li et al., 1995).

For interactions with both Mcm1 and **a**1, α2 uses peptide regions immediately adjacent to its homeodomain to interact with partner proteins. It is conceivable that proteins with similar homeodomain sequences, but divergent homeodomain extensions, could interact with different cofactors and regulate different target genes.

1.2.4.2 HOM/Hox and Exd/Pbx

Mutations in the *Drosophila* homeobox gene *extradenticle* (*exd*) result in homeotic transformations in thoracic and abdominal segments without affecting the expression of

homeotic genes (Peifer and Wieschaus, 1990), suggesting that *exd* acts genetically as a cofactor for some HOM genes. It was shown later that *exd* and its mammalian homolog *Pbx* (Rauskolb et al., 1993) encode homeodomain-containing cofactors for homeotic proteins (reviewed in Mann and Chan, 1996). Exd and Pbx bound cooperatively to DNA with a wide spectrum of homeotic proteins (Chan et al., 1994; Van Dijk and Murre, 1994; Chang et al., 1995; Lu and Kamps, 1996, reviewed in Mann and Chan, 1996).

Homeotic proteins share similar homeodomain sequences and recognize similar DNA sequences *in vitro* (Mann, 1995). Exd/Pbx binds cooperatively and selectively with different homeotic proteins, enabling them to recognize distinct binding sites (Chan et al., 1994; Van Dijk and Murre, 1994; Chang et al., 1995; Johnson et al., 1995; Phelan et al., 1995; Pöpperl et al., 1995; Lu and Kamps, 1996). The interaction between Exd/Pbx and the homeotic proteins requires the homeodomain of Exd/Pbx, and the homeodomain plus residues N-terminal or C-terminal to the homeodomains of HOM/Hox proteins (Chan et al., 1994; Pöpperl et al., 1995; Chang et al., 1995; Johnson et al., 1995; Phelan et al., 1995; Chan et al., 1996; Lu and Kamps, 1996).

Pöpperl et al. (1995) and Chan et al. (1997) tested the *in vivo* relevance of the Exd and Lab/Hoxb-1 interaction. A 20 bp oligonucleotide was identified in the 5' autoregulatory region of the mouse *Hoxb-1* gene, an ortholog of *Drosophila lab* (Pöpperl et al., 1995). Three tandem copies of this oligonucleotide, 3Xrpt3, can drive *lacZ* reporter gene expression in patterns that resemble the wild type *Hoxb-1* expression pattern in mice and the *lab* expression pattern in *Drosophila* embryos (Pöpperl et al., 1995; Chan et al., 1996). Hoxb-1 and Lab are unable to bind to this sequence on their own. However, they can cooperate with Exd to bind this site while other HOM/Hox proteins, such as Ubx or Hoxb-4, cannot (Pöpperl et al., 1995; Chan and Mann, 1996;

Chan et al., 1996). Labial can only turn on the expression of 3Xrpt3-*lac*Z in the embryo in the presence of nuclear Exd (Chan et al., 1996; Chan et al., 1997).

Another study (Chan et al., 1996) showed that Lab is unable to bind the 20 bp Hox-Exd binding oligo because of an inhibitory effect exerted by an IYPWMK-containing hexapeptide motif located amino-terminal to the homeodomain. This hexapeptide motif is found in most homeotic proteins (Gehring et al., 1994a). In Lab the hexapeptide and the homeodomain are separated by 110 aa. The interaction between Lab and Exd induces a conformational change in Lab that relieves the inhibitory effect of the hexapeptide, promoting a high affinity interaction between Lab and DNA (Chan et al., 1996).

1.2.4.3 Exd and Hth

Exd is not simply a ubiquitously available cofactor for homeotic proteins. The distribution of Exd is developmentally regulated. In regions where it is not functional, Exd is present only in the cell cytoplasm, whereas it accumulates in the nuclei of cells requiring *exd* function. For instance, Exd is initially uniformly distributed but is excluded from nuclei in blastoderm embryos until gastrulation (Mann and Abu-Shaar, 1996; Aspland and White, 1997; Azpiazu and Morata, 1998). Exd nucleocytoplasmic localization is regulated through its direct interaction with another homeodomain protein Homothorax (Hth) (Rieckhof et al., 1997; Pai et al., 1998; Casares and Mann, 1998).

Hth has extensive amino acid identity to the murine proto-oncogene Meis1. Genetic experiments show that, in the absence of *hth*, *exd* protein is nonfunctional and is present in the cytoplasm (Pai et al., 1998). Exd directly interacts with Meis1 and Hth (Rieckhof, et al., 1997). Exd is nuclear where *hth* is expressed and Meis1 or Hth is sufficient to induce the cytoplasmic to

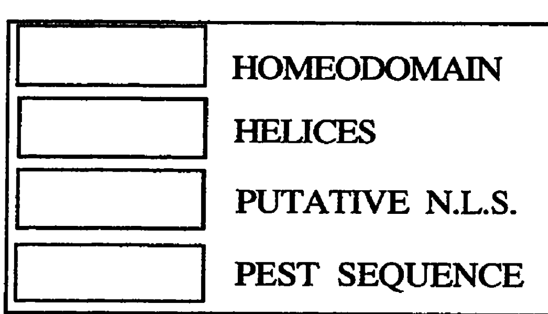
nuclear translocation of Exd in cultured cells and in *Drosophila* embryos (Rieckhof, et al., 1997; Pai et al., 1998). This is a novel mechanism for the regulation of homeodomain protein activity.

1.3 *fushi tarazu*, a *Drosophila* Homeobox Gene

fushi tarazu (meaning missing segments in Japanese), belongs to the pair-rule class of segmentation genes. The *ftz* gene maps to the right arm of the third chromosome within the *Antp* Complex (Lindsley and Zimm, 1992). It has one small intron and encodes a 413 amino acid protein (Kuroiwa et al., 1984; Weiner et al., 1984; Laughon and Scott, 1984). Figure 6 shows the amino acid sequence of the *ftz* protein (Ftz). Ftz has at least two nuclear localization consensus signals (NLS; Krause et al., 1988; LaCasse and Lefebvre, 1995), a PEST region (Kellerman et al., 1990), amino and carboxy-terminal transactivation domains (Fitzpatrick and Ingles, 1989; Fitzpatrick et al., 1992), and a DNA binding homeodomain (Kuroiwa et al., 1984; Weiner et al., 1984; Laughon and Scott, 1984; Qian et al., 1994). The *ftz* homeodomain is most closely related to that of Antp (50/60 amino acids are the same between the two homeodomains). Ftz binds to specific DNA sequences *in vitro* (Desplan et al., 1988; Müller et al., 1988; Percival-Smith et al., 1990; Pick et al., 1990; Florence et al., 1991), with the optimal Ftz binding sites being C/GCATTA and CAATTA (Percival-Smith et al., 1990; Pick et al., 1990; Florence et al., 1991). Ftz is localized to the nucleus (Carroll and Scott, 1985; Krause et al., 1988) and activates transcription in a sequence-specific fashion in *in vitro* transcription assays (Ohkuma et al., 1990), in *Drosophila* cultured cells (Jaynes and O'Farrell, 1988; Winslow et al., 1989; Han et al., 1989; Fitzpatrick et al., 1992; Ananthan et al., 1993), in yeast (Fitzpatrick and Ingles, 1989), and in developing *Drosophila* embryos (Ish-Horowicz et al., 1989; Fitzpatrick et al., 1992; Schier and Gehring, 1992), consistent with its role as a sequence-specific transcription factor.

Figure 6. Ftz amino acid sequence and mapped phosphorylation sites. The Ftz amino acid sequence (Laughon and Scott, 1984) is shown in single-letter symbols. The putative nuclear localization signals, PEST sequence, and the homeodomain are indicated. Mapped Ftz phosphorylation sites and the corresponding kinases are shown in colors, CK1 in blue, GSK-3β in green, MAPK in red, PKC in purple, PKA in black, and S6 kinase in orange. "Baculovirus" refers to those sites phosphorylated in Ftz expressed in the Sf9 insect cells from a baculovirus promoter. Other labeled sites are phosphorylated *in vitro* by the indicated kinases, using bacterial expressed Ftz as substrate. Figure courtesy of R. Strome.

M A T T N S Q S H Y S Y A D N M N M Y N M Y
H P H S L P P T Y Y D N S G S N A Y Y Q N T
S N Y H S Y Q G Y Y P Q G S Y S E S C Y Y Y
N N Q E Q V T T Q T V P P V Q P T T P P P K
A T K R K A E D D A A S I I A A V E E R P S
T L R A L L T N P V K K L K Y T P D Y F Y T
T V E Q V K K A P A V T T K V T A S P A P S
Y D Q E Y V T V P T P S A S E D V D Y L D V
Y S P Q S Q T Q K L K N G D F A T P P P T T
P T S L P P L E G I S T P P Q S P G E K S S
S A V S Q E I N H R I V T A P N G A G D F N
W S H I E E T L A S D C K D S K R T R Q T Y
T R Y Q T L E L E K E F H F N R Y I T R R R
R I D I A N A L S L S E R Q I K I W F Q N R
R M K S K K D R T L D S S P E H C G A Y T
A M L P P L E A T S T A T T G A P S V P V P
M Y H H H Q T T A A Y P A Y S H S H S H G Y
G L L N D Y P Q Q Q T H Q Q Y D A Y P Q Q Y
Q Q Q C S Y Q Q H P Q D L Y H L S



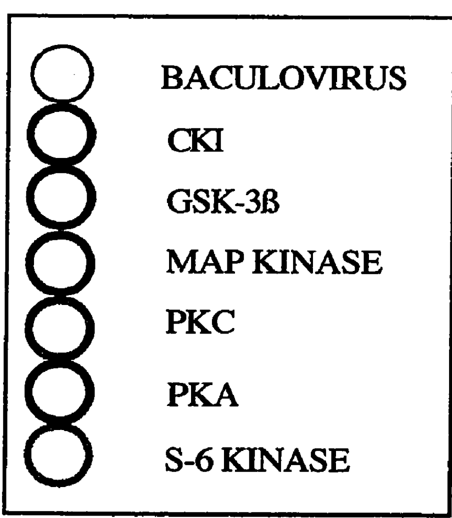


Figure 7. ***ftz*** **pair-rule expression in wild-type embryos.** *ftz* transcripts were visualized by *in situ* hybridization. *ftz* transcripts are first detected throughout the embryo at low levels and then accumulate at higher levels in the trunk (a). Gaps then appear in this broad band (b), eventually generating seven stripes (c, d). Later the stripes narrow (e, f) and then gradually fade away, with stripe 7 remaining the longest (g, h). See text 1.3.1 for further description of the dynamic changes in *ftz* stripes. Figure courtesy of A. Nasiadka.

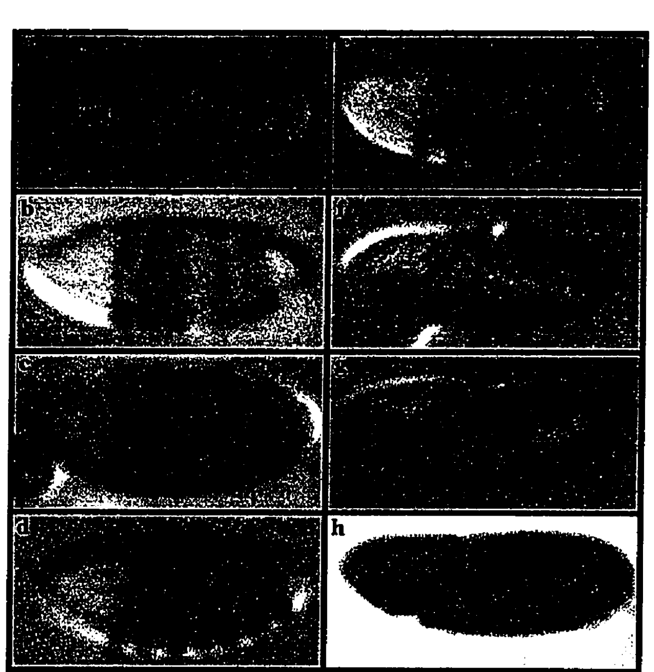
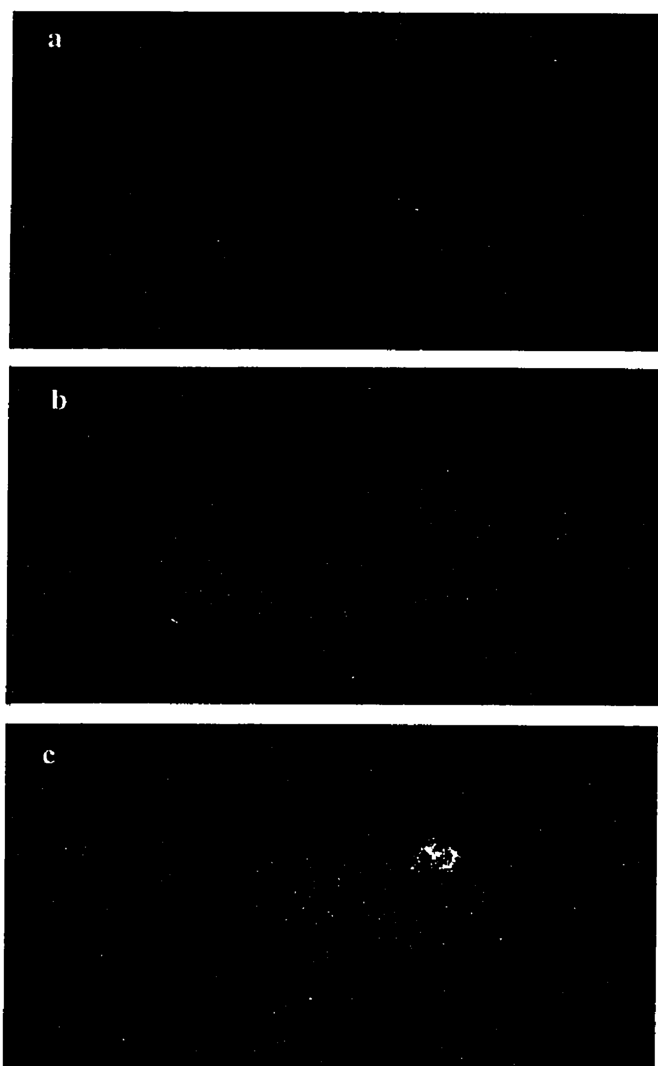


Figure 8. *ftz* expression at later stages. Whole-mount preparations of fixed embryos were stained with anti-*ftz* antibodies (Krause et al., 1988). Ftz immunofluorescence is detected in three different tissues: (a) blastoderm, ~3 ¼ hr AEL; (b) CNS, ~9.5 hr AEL; and (c) hindgut, ~14 hr AEL (all at 25°C).


a
b
c

1.3.1 *ftz* Expression and Function

Whole-mount *in situ* hybridization (Hafen et al., 1984; Weir and Kornberg, 1985; Yu and Pick, 1995) and antibody staining (Carroll and Scott, 1985; Krause et al., 1988) show that *ftz* mRNA and protein have three phases of expression (Figure 7, 8). Ftz is thus believed to control three temporally distinct aspects of embryogenesis: segmentation, neurogenesis and gut development.

ftz is first detected shortly after the cleavage nuclei have reached the egg surface. At this stage *ftz* transcripts are present at low levels and associated with nuclei throughout the embryo (Yu and Pick, 1995). Subsequently, during the syncytial blastoderm stages (nuclear cycles 11-13), expression of *ftz* RNA is restricted to a broad band between 15%-65% egg length (0% egg length is at the posterior pole). A zebra-like striped pattern evolves by the end of syncytial blastoderm (2.5 hr AEL, nuclear cycle 13) as gaps divide the broad bands into seven stripes, each 4 nuclei wide (Hafen et al., 1984; Carroll and Scott, 1985). Each stripe initially has a bell-shaped variation in intensity along its width. As gastrulation and germ band extension proceed, each *ftz* stripe sharpens at its anterior edge and begins to fade at its posterior edge. By stage 7, only the most anterior cells of each stripe remain (except stripe 7, which remains 3-4 cells wide). Finally at stage 8 (4.5-5.5 hr AEL), *ftz* transcripts are extinguished with stripe 7 being the last to fade. Figure 7 summarizes the evolution of *ftz* stripes.

Mutations in the *ftz* locus are embryonic lethal: homozygous *ftz*$^-$ embryos die as larvae that possess half the normal number of segments (Wakimoto and Kaufman, 1981; Wakimoto et al. 1984). The deleted regions are derived from cells normally expressing *ftz* (Hafen et al., 1984). These regions are the primordia of the even-numbered parasegments (Martinez-Arias and Lawrence, 1985; Lawrence, 1988; for parasegmental/segmental register see Figure 2). Thus, the

central function of *ftz* stripes in the blastoderm is to define every other parasegmental unit (Lawrence et al., 1987).

Shortly after its expression in blastoderm stripes has decayed (~6 hr AEL), Ftz reappears in a subset of neuronal precursors in the developing central nervous system (CNS) (Figure 8b). This expression is no longer pair-rule in nature, but rather occurs in a repeated pattern in all parasegments (Carroll and Scott, 1985; Hiromi et al., 1985; Krause et al., 1988; Doe et al., 1988a). Sixty of the 500 neurons in each segment express Ftz (Doe et al., 1988a). *ftz* expression in the CNS fades away by 12 hr AEL.

As Ftz is fading in the CNS, it reappears two hours later in a ring of expression in the developing hindgut, where the imaginal region of the hindgut is located (Figure 8C). It is also detectable at this stage (12-14 hr AEL) in the posterior-dorsal surface of the embryo and in the foregut (Krause et al., 1988). Since the imaginal tissues are the primordium of adult structures, Ftz may be involved in setting up the formation of the adult gut. Ftz is not known to be expressed in larvae or adult flies.

ftz protein (Ftz) appears to be involved in several regulatory activities in the blastoderm embryo. The enhancement and maintenance of the *ftz* striped pattern requires autoregulation through direct binding of Ftz to its own enhancer (Hiromi et al., 1985; Hiromi and Gehring, 1987; Ish-Horowicz et al., 1989; Schier and Gehring, 1992). Ftz appears to act as a negative regulator of *even skipped* (*eve*), since *eve*-dependent parasegments are narrower in gain-of-function *ftz* mutant embryos (Kellerman et al., 1990). Ftz is also involved in the activation of the segment polarity gene *engrailed* (*en*) (Howard and Ingham, 1986; DiNardo and O'Farrell, 1987; Ingham et al., 1988; Ish-Horowicz et al., 1989; Florence et al., 1997), repression of the segment polarity gene *wingless* (*wg*) (Ingham et al., 1988; Copeland et al., 1996), and activation of several

homeotic genes, including *abd-A* (Kellerman et al., 1990; Macias et al., 1994), *Ubx* (Duncan, 1986; Winslow et al., 1989; Müller and Bienz, 1992), *Antp* (Winslow et al., 1989; Riley et al., 1991), and *Scr* (Ingham and Martinez-Arias, 1986). *ftz* function in the CNS is not well understood. One study shows that Ftz is involved in the specification of RP2 neurons through activation of *eve* (Doe et al., 1988a). This regulatory relationship between the two genes is different from that in the blastoderm where Ftz may be acting as a repressor of *eve* (Lawrence et al., 1987; Kellerman et al., 1990). This switch in activities suggests that other Ftz target genes are also likely to vary in a stage-specific fashion. The function of *ftz* in the non-segmented gut has not yet been investigated.

1.3.2 Transcriptional Regulation of the *ftz* Gene

The spatial regulation of *ftz* expression is predominantly at the level of transcription (Hiromi et al., 1985; Edgar et al., 1986). It is mediated through interactions between *cis*-acting regulatory elements and *trans*-acting proteins. These regulatory elements and their *trans*-acting factors are quite well characterized (see below).

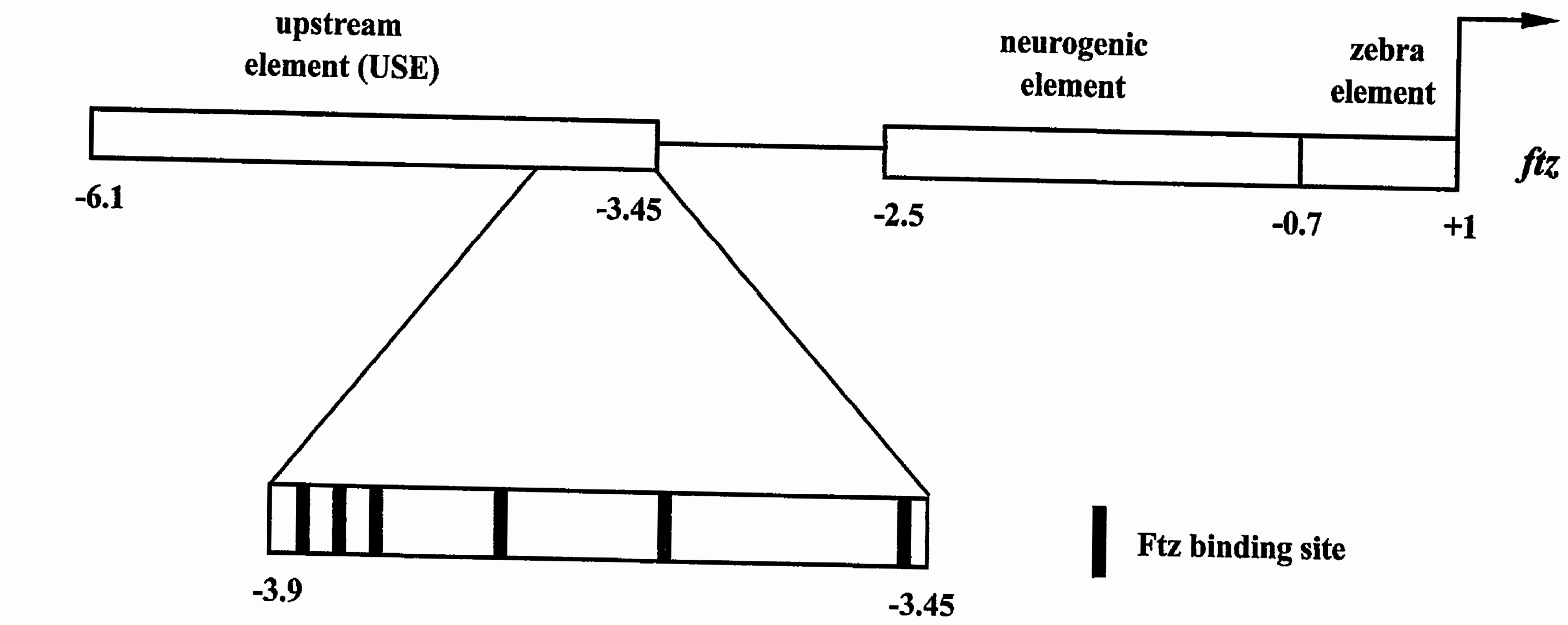
1.3.2.1 *Cis*-acting Transcriptional Elements

A 10 kb *ftz* genomic fragment, containing 6.1 kb of 5', 1.9 kb of 3', and coding sequence, is sufficient for proper *ftz* expression and rescue of *ftz*$^-$ embryos (Hiromi et al., 1985; Hiromi and Gehring, 1987). The 6.1 kb 5' region can be subdivided into three elements, the proximal "zebra element" (-0.7 kb to +1), the "neurogenic element" (-2.5 kb to –0.7 kb), and the "upstream element" (USE) (-6.1 kb to –3.45 kb) (Hiromi et al., 1985; Hiromi and Gehring, 1987;

Doe et al., 1988a; Dearolf et al., 1989a; Pick et al., 1990). These *cis*-acting elements are depicted in Figure 9.

The characteristic seven-striped expression pattern of *ftz* in the blastoderm requires two control elements, the zebra element and the USE (Hiromi et al., 1985; Hiromi and Gehring, 1987). The zebra element is believed to respond to maternal, gap and earlier acting pair-rule genes for general *ftz* activation and interstripe repression (Dearolf et al., 1990). Deletion analysis of this promoter region indicates that it is a mosaic of individual positive and negative elements (Dearolf et al., 1989a, 1989b, 1990). A *ftz* transgene, under the control of the zebra element alone has very little *ftz* rescuing activity (Hiromi et al., 1985; Hiromi and Gehring 1987). The USE plays roles in both stripe establishment and stripe maintenance. It acts as an enhancer of the zebra element to ensure high levels of *ftz* expression, but can also activate a heterologous promoter in *ftz*-like stripes. Systematic deletion analyses of the USE have identified several independent and redundant *cis*-acting elements, each capable of directing *ftz-lacZ* fusion gene expression in seven stripes (Pick et al., 1990; Schier and Gehring, 1993b). A proximal portion of the enhancer drives reporter gene expression in seven stripes in both presumptive mesoderm and ectoderm. A distal portion of the enhancer directs *lacZ* expression in seven stripes primarily in the presumptive mesoderm. To identify binding sites of transacting factors, the 2.6 kb USE has been analyzed by DNase I footprinting and EMSA analyses using staged embryonic nuclear extracts (Harrison and Travers, 1988). Over 50% of this region is bound by nuclear proteins, suggesting complicated protein-DNA interactions in the USE. Another study shows that the proximal enhancer of the USE contains at least nine protein binding sites that are bound by ten different DNA-binding complexes (Han et al., 1993). A 323-bp DNA fragment within this region was shown to contain all of the necessary protein binding sites required to direct reporter gene expression in seven

Figure 9. Schematic of the 5' *cis* elements that regulate *ftz* transcription. The 6.1 kb 5' *ftz* promoter contains three *cis*-acting regions, "the zebra, the neurogenic and the upstream elements" (Hiromi et al., 1985; Hiromi and Gehring, 1987). They are required to support the dynamic patterns of *ftz* expression in embryos. Each region is quite complex and contains multiple regulatory sites. The "zebra element" is required for seven-striped expression in the blastoderm, the "neurogenic element" for *ftz* expression in the CNS. The "upstream element" (USE) enhances the *ftz* seven-striped expression in the blastoderm and is necessary for *ftz* autoregulation. The USE does not play a major role in *ftz* expression in the CNS (see 1.3.2.1 for detailed description of these elements). The six Ftz-binding sites within the proximal portion of the "upstream element" are drawn in small bars. These sites have been shown to mediate Ftz autoregulation (Schier and Gehring, 1992).



upstream
element (USE)
neurogenic
element
zebra
element
ftz
-6.1
-3.45
-2.5
-0.7
+1
-3.9
-3.45
Ftz binding site

The 5' most 660 bp of the *ftz* USE is AT rich (Harrison and Travers, 1988). This region contains a putative scaffold attachment region (SAR), which buffers against the effects of external enhancers and chromosomal conformation. Another SAR is found in the distal 3' sequence of the 10 kb *ftz* rescuing fragment (Hiromi et al., 1985; Gasser and Laemmli, 1986). Deletion of these SARs results in severe position effects in *ftz* transgenes inserted at different genomic locations (Hiromi et al., 1985; Gasser and Laemmli, 1986).

The entire 6.1 kb of the *ftz* 5' promoter has considerable homology to the analogous *ftz* promoter regions found in two other *Drosophila* species, *D. hydei, and D. virilis.* Not only is the sequence conserved, but so are the relative positions, orientation, and distances between the zebra, neurogenic and upstream elements (Maier et al., 1990; 1993). Both of the *D. hydei* and *D. virilis ftz* 5' promoters are functional in *D. melanogaster* (Maier et al., 1990; Schier and Gehring, 1993b). The USE is the most conserved element amongst the three *Drosophila ftz* genes (Maier et al., 1990, 1993). There are several highly conserved short DNA sections in the USE (Harrison and Travers, 1988; Maier et al., 1990; Schier and Gehring, 1993b). Since the three *Drosophila* species diverged about 60 million years ago, and only functionally relevant DNA sequences should remain conserved (Perler et al., 1980), these short DNA sequences may represent important targets of *trans*-regulatory factors.

The neurogenic element is an independent region of the 5' promoter, essential for *ftz* expression in the developing CNS (Hiromi et al., 1985; Hiromi and Gehring 1987; Doe et al., 1988a). Deletion of this element does not substantially alter segmental expression, but removes all *ftz* expression in the CNS (Hiromi et al., 1985; Hiromi and Gehring 1987; Doe et al., 1988a The *cis*-acting element(s) necessary for *ftz* expression in the gut has not been identified.

1.3.2.2 Trans-acting Factors

DNA binding studies indicate that a large number of regulators are involved in regulating the striped pattern of *ftz* expression (Harrison and Travers, 1988, 1990; Dearolf et al., 1989a, 1989b; Pick et al., 1990; Ueda et al., 1990; Brown et al., 1991; Lavorgna et al., 1991; Topol et al., 1991; Han et al., 1993; Tsai and Gergen, 1995; Han et al., 1998). The following are well-studied transcription factors that directly interact with *ftz cis* elements *in vitro* and appear to regulate *ftz* transcription *in vivo*.

Ftz

Ftz plays an important role in maintaining quantitative levels of its own expression through direct interaction with the upstream element (USE) (Hiromi and Gehring, 1987; Pick et al., 1990; Schier and Gehring, 1992; Han et al., 1998) and probably other sites as well. The USE-*lacZ* seven-striped expression pattern is undetectable in *ftz* mutants (Hiromi and Gehring, 1987). DNase I protection analyses identified 2 high (GCAATTA-containing), 13 medium and 6 low affinity potential Ftz binding sites in the USE (medium and low affinity sites show little sequence similarity with high affinity sites) (Pick et al., 1990). Individual deletions of the 21 potential Ftz binding sites had no effect on USE-*lacZ* expression. However, deletion of all the Ftz binding sites abolished *ftz-lacZ* expression, suggesting functional redundancy (Schier and Gehring, 1993b). When some of these binding sites were changed to those recognized by Bcd (GGATTA), fusion gene expression was dramatically reduced. This decreased *ftz* expression can be specifically suppressed in the embryo by a DNA-binding specificity mutation in the Ftz homeodomain, FtzQ50K (Schier and Gehring, 1992). In FtzQ50K, the ninth residue in helix III of the homeodomain glutamine, is switched to lysine, thereby switching specificity from Ftz- to

Bcd-type binding sites (Hanes and Brent, 1989, 1991; Treisman et al., 1989; Wilson et al., 1996). This result is compelling evidence that Ftz enhances its own transcription through direct binding to its own enhancer.

Other experiments, however, suggest that Ftz autoregulation requires more than a simple protein-DNA interaction. First, Ftz without a homeodomain is capable of activating the endogenous *ftz* gene (Copeland et al., 1996; Hyduk and Percival-Smith, 1996). Second, Ftz autoregulates through interactions with other proteins bound to the USE (Schier and Gehring, 1993a; Yu et al., 1997; Han et al., 1998). For example Ftz-F1 (Ftz factor 1) is a cofactor required for Ftz autoregulation of USE reporter genes (Yu et al., 1997). However, Ftz-F1 is not necessary for endogenous *ftz* gene autoregulation (Guichet et al., 1997), indicating the presence of additional cofactors that act in a redundant fashion. Third, autoregulation is restricted to early embryogenesis up to the gastrulation stage. *ftz* USE-*lacZ* genes are not detectable in the CNS or in the hindgut (Hiromi and Gehring, 1987; Pick et al., 1990), even though high levels of Ftz are expressed there (Krause et al., 1988; Doe et al., 1988a). Apparently, Ftz alone is not sufficient to activate autoregulatory elements in specific neural precursor cells and in the hindgut. Therefore, the cofactors that interact with Ftz may be restricted in their temporal expression profile (or their activity) to the blastoderm and gastrula stages, and Ftz can only activate autoregulatory elements during these stages.

Ftz-F1

Ftz-F1 is a protein of the nuclear hormone receptor superfamily (Ueda et al., 1990; Lavorgna et al., 1991; Mangelsdorf et al., 1995). The *Ftz-F1* gene encodes α and β spliced forms, αFtz-F1 and βFtz-F1, that differ in their temporal expression patterns during embryogenesis

(Ueda et al., 1990; Lavorgna et al., 1991, 1993). The α isoform is maternally expressed with high levels in 0-4 hr embryos (Ueda et al., 1990; Lavorgna et al., 1991), overlapping both temporally and spatially with *ftz* blastoderm stripes. The β isoform is expressed during later embryogenesis and pupal development (Lavorgna et al., 1993).

Ftz-F1 was initially thought to be a positive regulator of *ftz* expression in the early embryo (Ueda et al., 1990; Lavorgna et al., 1991, Topol, 1991; Han et al., 1993). It has been shown to bind DNA cooperatively with Ftz on the USE (Yu et al., 1997; Han et al., 1998). The *ftz* zebra element has 2 and the USE has 3 potential Ftz-F1 binding sites (CAAGGC/TCA/GC) (Ueda, et al., 1990; Yu et al., 1997). Embryos that carry zebra element-*lacZ* and USE-*lacZ* constructs mutated in the Ftz-F1 binding sites show a dramatic overall decrease of β-galactosidase activity (Ueda et al., 1990; Han et al., 1998). Ftz and Ftz-F1 interact directly (Guichet et al., 1997; Yu et al., 1997; Han et al., 1998), and Ftz-F1 contact enables Ftz binding to a medium affinity site in the USE at Ftz concentrations that alone would normally bind only high affinity sites (Pick et al., 1990; Yu et al., 1997). Although these experiments all point to a role for Ftz-F1 in Ftz autoregulation, *ftz* expression is normal in *Ftz-F1* mutant embryos. It is possible that the effects of Ftz-F1 are redundant with those of other Ftz cofactors in the context of an intact *ftz* promoter.

Ttk

The *tramtrack* (*ttk*) protein is thought to be one of the repressors of *ftz* transcription in preblastoderm embryos (Brown et al., 1991; Brown and Wu, 1993). Ttk is a zinc-finger protein that was cloned based on its ability to bind to several sites in the *ftz* zebra element (Brown et al., 1991) and the USE element (Harrison and Travers, 1988, 1990). Its consensus binding sequence

is 5' CAGGACCT 3', which partially overlaps with those of Ftz-F1 sites. There are both maternal and zygotic components of *ttk* expression, and the pattern is essentially the temporal inverse to that of *ftz* (Harrison and Travers, 1990; Read and Manley, 1992; Brown and Wu, 1993). Initially, *ttk* is expressed homogeneously in preblastoderm embryos. By stage 4, when *ftz* mRNA is just apparent, *ttk* expression is disappearing. When *ftz* is expressed at its peak in seven stripes, there is no detectable *ttk* expression. Later, when *ftz* expression has decayed in the ectoderm and mesoderm, *ttk* expression reappears in 14 stripes (Harrison and Travers, 1990; Read and Manley, 1992; Brown and Wu, 1993). Point mutations of Ttk binding sites in the zebra element cause global derepression of a *ftz-lacZ* reporter in nuclear division cycle 10 embryos (Brown et al., 1991). Ectopic *ttk* expression in blastoderm embryos using a heat shock promoter causes near-complete repression of *ftz* blastoderm stripes (Brown and Wu, 1993). Thus Ttk may play an important role in preventing ectopic *ftz* expression prior and subsequent to the normal period of ectodermal expression.

It was also shown that *ttk* is expressed in cone cells but not photoreceptor cells of the *Drosophila* compound eye. Loss of Ttk activity results in ectopic formation of photoreceptor cells, whereas abnormal accumulation of Ttk in photoreceptor cells represses their differentiation (Lai, et al., 1996; Li et al., 1997).

Caudal

The maternal coordinate gene *caudal* (*cad*) is expressed in the posterior half of the embryo (Macdonald and Struhl, 1986; Mlodzik and Gehring, 1987) where it is thought to activate *ftz* expression (Macdonald and Struhl, 1986; Dearolf et al., 1989b). Cad interacts *in vitro* with several TTTATG sites in the *ftz* zebra element (Dearolf et al., 1989b). In *cad* mutant

embryos, *ftz* is eliminated in the posterior-most three stripes (MacDonald and Struhl, 1986). Mutations of the *in vitro* Cad binding sites in the zebra element also abolish *ftz* zebra element-*lacZ* expression in the posterior half of the embryo (Dearolf et al., 1989b). *cad* is thus an example of a maternal coordinate gene, expressed in an A-P concentration gradient, that most likely directly regulates pair-rule gene expression.

1.3.3 Regulation of *ftz* mRNA and Protein Stability

As described in the section on the *ftz* expression pattern, *ftz* mRNA and protein when first expressed in the blastoderm are uniformly distributed over much of the embryo and then rapidly evolve into seven-stripe patterns. To generate this normal striped pattern, *ftz* mRNA and protein in the anterior-most 35% and posterior-most 15% of the embryo, as well as in the interstripes, have to be rapidly removed. Ectopic expression of *ftz* throughout the embryo is lethal (Struhl, 1985), confirming that *ftz* expression must be restricted to particular domains to ensure correct development of the embryo. Because the *ftz* striped pattern evolves very rapidly, and because *ftz* acts autocatalytically, it is important that the *ftz* gene products be rapidly removed from regions where they should not be. The rapid degradation of *ftz* mRNA and protein provides one mechanism to ensure normal dynamic changes in *ftz* expression. The half-lifes of both *ftz* mRNA and protein have been shown to be in the range of 6-10 min in blastoderm stage embryos (Edgar et al., 1986, 1987; Kellerman et al., 1990). This would remove about 90% of the *ftz* products within 20 min after a shutdown of *ftz* transcription.

1.3.3.1 *ftz* mRNA Instability Elements

Riedl and Jacobs-Lorena (1996) mapped two destabilizing elements in the *ftz* mRNA, one located within the 5' third of the *ftz* mRNA and the other in the 3' untranslated region (UTR). Their results show that a 68 bp 3' *ftz* instability element (FIE3) is necessary for degradation of a hybrid *ftz* mRNA, and is sufficient to destabilize a heterologous stable mRNA. Since the same heterologous mRNA is stable in the ovary and is destabilized upon egg activation, FIE3 may function differently at different times or in different cell types in the embryo. Inhibition of protein synthesis stabilizes *ftz* mRNA (Edgar et al., 1986), suggesting that proteins, for example nucleases, are required for this process. Injection of *ftz* FIE3 RNA into early embryos results in delayed *ftz* stripe resolution and segmental defects (Riedl and Jacobs-Lorena, 1996). This may result from competition of the exogenous FIE3s for embryonic factors required for *ftz* mRNA degradation and stripe resolution. A *ftz* dominant allele, ftz^{Rpl}, lacks all sequences 3' to the homeobox, resulting in stabilization of the *ftz* mRNA (and protein) (Kellerman et al., 1990). The truncated transcripts may be stable because they lack the FIE3.

1.3.3.2 Ftz PEST Regions

The N-terminal part of Ftz is rich in proline (P), glutamic acid (E), aspartic acid (D), serine (S) and threonine (T) (PEST residues; Figure 6). PEST sequences are defined by stretches of amino acids that are rich in the above residues. They are flanked by lysine (K), arginine (R) or histidine (H). PEST sequences have been shown to target proteins for degradation (Rogers et al., 1986; Rechsteiner and Rogers, 1996). Three ftz^{Ual} (*Ultra-abdominal-like*) alleles, described by Kellerman et al. (1990), contain missense mutations that affect two proline residues located 5 amino acids apart in a Ftz PEST sequence (residues 207-221: Kellerman et al., 1990; Figure 6). Two are proline to leucine changes, and one a proline to serine change. The phenotypes of ftz^{Ual}

alleles range from a mild A1 to A3 homeotic transformation in adult flies to a severe gain-of-function "anti-*ftz*" cuticle (Kellerman et al., 1990). These effects are presumably due to the abnormally long half-lives of the mutant proteins and wider set of Ftz stripes that form as a consequence (Kellerman et al., 1990).

1.3.4 Regulation of Ftz Activity by Protein-protein Interactions

To identify functional domains within the *ftz* protein, Ftz deletion constructs have been expressed in cell culture and in *Drosophila* embryos. A deletion of much of the Ftz homeodomain destroyed the ability of Ftz to bind DNA and to activate transcription in cultured cells. However, the deleted protein was still capable of regulating many *ftz*-dependent activities in the embryo providing sufficient levels of the protein were present (Fitzpatrick et al., 1992). This result suggests that Ftz can regulate target genes through interactions with other DNA-bound proteins.

A number of proteins have been identified that interact with Ftz, including Paired (Prd) (Ananthan et al., 1993; Copeland et al., 1996), TFIIB (Colgan et al., 1993, 1995), Ftz-F1 (Guichet et al., 1997; Yu et al., 1997; Florence et al., 1997; Han et al., 1998), Hairy, and Sloppy-paired2 (Copeland and Krause, unpublished results), 33-13 (Dietrich and Krause, unpublished results), Ttk (Han et al., 1998), and alcohol dehydrogenase gene distal factor-1 (Adf-1; Han et al., 1998). Among these proteins, biochemical and genetic studies show that the interactions with Prd and Ftz-F1 regulate Ftz transcriptional activity both *in vitro* and *in vivo* (Ananthan et al., 1993; Copeland et al., 1996; Guichet et al., 1997; Yu et al., 1997; Florence et al., 1997; Han et al., 1998).

1.3.4.1 Ftz and Prd

Prd is a member of the pair-rule class of segmentation proteins and contains two DNA binding domains, a homeodomain and a paired domain (Bopp, et al., 1986). A synergistic interaction between Prd and Ftz on an *en* promoter sequence (3K'-TATA) was demonstrated in cultured cells. The combination of Ftz and Prd activates transcription at levels 1-2 orders of magnitude higher than levels activated by the individual proteins (Han et al., 1989; Ananthan et al., 1993). The Ftz homeodomain and carboxy-terminal regions were not necessary for this synergistic interaction (Ananthan et al., 1993). Sequences required for the synergism were mapped to the first 171 residues of Ftz, and to the homeodomain and adjacent N-terminal 59 aa of the *prd* protein (Ananthan et al., 1993).

Ftz was also shown to interact with Prd *in vitro* in a direct and DNA-independent fashion (Copeland et al., 1996). Prd was specifically retained on Ftz affinity columns and bound to Ftz on Far Western blots. The Ftz homeodomain was not required for these interactions. Residues 100-150 aa and the last 50 aa of the Ftz polypeptide were required to bind Prd. Deleting either of the sequences alone reduced Prd binding by 50-80%, and together, abolished the interaction completely (Copeland et al., 1996).

The interaction between Prd and Ftz is required *in vivo* for proper expression of the segment polarity gene *wg* (Copeland et al., 1996). In blastoderm embryos, *prd* is expressed in broad stripes that overlap with *ftz* as well as *wg* stripes (Kilcherr et al., 1986; Ingham et al., 1988). All *wg* stripes fail to initiate properly in *prd* mutant embryos, and heat shock-induced *prd* expression causes rapid broadening of *wg* stripes, except in cells that express *ftz* (Copeland et al., 1996). These results suggest that Prd activates *wg* transcription and that Ftz is capable of overriding this activity. FtzΔHD, but not Ftz missing the Prd-binding domain (aa 100-150), also

represses Prd-dependent *wg* activation, suggesting that Ftz represses *wg* through a direct interaction with Prd (Copeland et al., 1996). Consistent with this hypothesis, ectopic Ftz was unable to repress residual *wg* expression in *prd* mutant embryos, whereas repression was complete in wild-type embryos (Copeland et al., 1996).

1.3.4.2 Ftz and Ftz-F1

Maternal *Ftz-F1* mutations generate cuticle defects identical to those of *ftz*. Changes in the expression patterns of *en* and *wg* are also identical in *Ftz-F1* and *ftz* mutant embryos (Guichet et al., 1997). Since Ftz-F1 binds to *ftz cis*-regulatory elements, and is required for *lacZ* reporter expression under the control of these elements (see 1.3.2.2), it was a surprise to see that *ftz* expression in *Ftz-F1* mutants was indistinguishable from wild type (Guichet et al., 1997). It was therefore proposed that Ftz-F1 might be a cofactor for Ftz, much as Exd is a cofactor for homeotic proteins. Affinity chromatography and Far Western experiments confirmed that Ftz and Ftz-F1 do interact directly *in vitro*. This interaction does not require the Ftz homeodomain. However, the Ftz N-terminal residues 101-150 were shown to be necessary and residues 1-150 were sufficient for binding to Ftz-F1 (Guichet et al., 1997). The significance of this interaction was tested in the embryo. The expression of FtzΔ101-150 in the embryos gave similar *ftz*, *en* and *wg* expression patterns to *Ftz-F1* mutant embryos, indicating that Ftz and Ftz-F1 probably interact on the *en* and *wg* promoters but that the interaction is not required for *ftz* expression (Guichet et al., 1997).

A Ftz homeodomain-containing construct and Ftz-F1 were also shown to bind cooperatively on an *en* enhancer element. Expression of the binding element-*lacZ* reporter in

embryos also requires the presence of both Ftz and Ftz-F1 as well as the DNA binding sites of both proteins (Florence et al., 1997).

As presented in section 1.3.2.2, Ftz-F1 cooperates with Ftz to promote autoregulation on isolated portions of the *ftz* enhancer. This interaction increases the apparent affinity of Ftz for DNA (Pick et al., 1990; Yu et al., 1997; Han et al., 1998). Ftz and Ftz-F1 have also been shown to interact in yeast cells (Yu et al., 1997; Han et al., 1998).

1.4 Regulation of Transcription Factors by Phosphorylation

Phosphorylation provides a rapid and reversible mechanism to modulate the activity of proteins. It has been shown that phosphorylation can modulate transcription factor activities by a variety of different mechanisms. These include protein conformation, stability, subcellular localization, DNA binding activity and protein-protein interactions (Hunter and Karin, 1992; Karin, 1994; Hunter, 1995). The following are some well studied examples.

1.4.1 NF-κB/IκB and Dorsal/Cactus

The mammalian transcription factors nuclear factor kappa B (NF-κB) and inhibitor of NF-κB (IκB) and their *Drosophila* homologues Dorsal and Cactus share similar regulatory mechanisms. NF-κB and Dorsal are controlled by partition between the nucleus and the cytoplasm. When localized in the cytosol, NF-κB and Dorsal are inactive. Signal-dependent phosphorylation and degradation of the anchoring proteins IκB and Cactus release NF-κB and Dorsal to the nucleus where they can regulate target genes (reviewed in Verma et al., 1995; Baeuerle and Baltimore, 1996). NF-κB and Dorsal share a conserved amino-terminal Rel homology domain (RHD) and a nuclear localization signal (NLS). The RHD is a DNA-binding

and dimerization motif. IκB and Cactus proteins contain ankyrin repeats and PEST sequences (reviewed in Verma et al., 1995).

NF-κB is a nuclear transcriptional activator in B lymphocytes and mature macrophages, but is found in the cytoplasm of many other cells in an inactive form (Sen and Baltimore, 1986). The cytoplasmic form is bound to IκB (Baeuerle and Baltimore, 1989; Nolan et al., 1991). Signal induced phosphorylation of cytoplasmic IκB on two serine residues, located in the amino-terminal part of the protein, triggers its ubiquitination and proteasome-dependent degradation (Palombella et a., 1994; Brown et al., 1995; Traenckner et al., 1995; Thanos and Maniatis, 1995; Scherer et al., 1995; Aoki et al., 1996). Two related kinases, IKK-α and IKK-β (IκB kinase α and IκB kinase β), have been shown to phosphorylate these two serine residues in response to cytokines, such as tumor necrosis factor α (TNF-α) and interleukin-1 (IL-1) (Didonato et al., 1997; Mercurio et al., 1997; Zandi et al., 1997). The degradation of IκB unmasks the NLS of NF-κB, thereby allowing its migration to the nucleus (Ghosh and Baltimore, 1990; Verma et al., 1995; Baeuerle and Baltimore, 1996).

dorsal and *cactus* RNAs are synthesized during oogenesis and are uniformly distributed in the cytoplasm of the freshly laid egg (Steward et al., 1988; Steward, 1989; Roth et al., 1989; Rushlow et al., 1989). The dorso-ventral axis in the *Drosophila* embryo is established by a maternally encoded signaling pathway that directs ventral degradation of cactus and nuclear translocation of Dorsal on the ventral side of the embryo. This results in a nuclear-cytoplasmic concentration gradient of Dorsal along the ventral-dorsal axis (reviewed in St. Johnston and Nüsslein-Volhard, 1992; Morisato and Anderson, 1995; Belvin et al., 1995; Reach et al., 1996).

In the absence of signal, Cactus retains Dorsal in the cytoplasm by a direct interaction, and phosphorylation of Cactus is required to release Dorsal to the nucleus (Wasserman, 1993;

Baeuerle and Baltimore, 1996). Signal transduction to Cactus requires two pairs of N-terminal serine residues in a similar sequence context to IκB phosphorylation sites. Mutation of these four serines to alanines blocks signal-mediated degradation of Cactus (Reach et al., 1996). The *Drosophila* homologue of IKK has not been identified.

The structural and functional similarities between Cactus and IκB extend to their carboxy-terminal PEST domains. Deletion of the PEST-containing regions from IκB and Cactus leads to a substantial increase in steady-state protein levels, suggesting that these PEST sequences are required for constitutive turnover of the proteins (Lin et al., 1996; Van Antwerp and Verma, 1996; Liu et al., 1997). However, PEST-deleted forms of both Cactus and IκB are still rapidly degraded in response to signaling. This signal-dependent degradation is mediated by the N-terminal serine residues. Deletion of the PEST domains, however, reduces the efficiency of signal transduction (Belvin et al., 1995; Baeuerle and Baltimore, 1996; Reach et al., 1996; Liu et al., 1997). All the three serines in the Cactus PEST domain are phosphorylated in the *Drosophila* embryo and are required for normal embryogenesis. CKII can phosphorylate these sites *in vitro* (Liu et al., 1997).

1.4.2 CREB and CBP

The cAMP-response element binding protein (CREB) is a well studied signal-regulated transcription factor (reviewed in Lalli and Sassone-Corsi, 1994; Montminy, 1997). CREB contains a basic domain-leucine zipper (bZip) structure. The leucine zipper is required for dimerization, and the adjacent basic domain is involved in direct DNA contact. In non-stimulated cells, CREB binds constitutively to a palindromic response element, TGACGTCA, known as the cAMP-response element (CRE), but fails to activate downstream promoters. Activation of PKA

leads to the phosphorylation of CRE-bound CREB at serine 133 and results in a large increase in CREB transcriptional activity, without a significant effect on CREB DNA-binding activity (reviewed in Lalli and Sassone-Corsi, 1994; Montminy, 1997). Phosphorylation of S133 enables CREB to recruit CREB-binding protein (CBP) (Chrivia et al., 1993; Kwok et al., 1994; Arias et al., 1994; Parker et al., 1996; Radhakrishnan et al., 1997). CBP is a cellular coactivator for several transcription factors (reviewed in Janknecht and Hunter, 1996; Shikama et al., 1997). CBP functions as a mediator that links CRE-bound CREB with TFIIB (Kwok et al., 1994) and TBP (Swope et al., 1996; Yuan et al., 1996). CBP also has histone acetyltransferase (HAT) activity and can recruit other HAT proteins (e.g. PCAF, p300/CBP associated factor). The acetylation of nucleosomes increases the accessibility of the DNA to the transcriptional machinery (Bannister and Kouzarides, 1996; Ogryzko et al., 1996; Martinez-Balbas et al., 1998; Korzus et al., 1998; Utley et al., 1998).

CREB S133 phosphorylation can also be brought about independently by calmodulin-dependent protein kinase (Cam PK), or nerve growth factor-induced kinases and is thus a convergence point for different signaling pathways (reviewed in Lalli and Sassone-Corsi, 1994). Transgenic mice expressing CREB S133A under the control of the growth hormone promoter develop as dwarfs, due to an atrophic pituitary and an absence of the somatotroph cell lineage (Borrelli et al., 1989). This strongly suggests a requirement for CREB S133 phosphorylation in somatotroph cell differentiation.

1.4.3 Pit-1

The mammalian protein pituitary factor 1 (Pit-1) is another well-studied phosphoprotein. Pit-1 is a tissue-specific POU domain transcription factor (the POU domain contains a POU-

specific domain as well as a POU homeodomain) (Bodner and Karin, 1987; Bodner et al., 1988; Herr et al., 1988). It is expressed in and required for the specification and maintenance of lactotroph, somatotroph, and thyrotroph cells in the anterior pituitary (Ingraham et al., 1988; Li et al., 1990; Simmons et al., 1990, Castrillo et al. 1991; Radovick et al., 1992; Pfaffle et al., 1992; Lin et al., 1994). Pit-1 targets include genes encoding growth hormone and prolactin (Ingraham et al., 1990; Radovick et al., 1992), as well as its own promoter (McCormick et al, 1990).

Pit-1 is regulated by many environmental cues (McCormick et al, 1990); for example, epidermal growth factor, cAMP, and phobol ester are some of the factors shown to regulate Pit-1 (Elsholtz et al., 1986; Dana and Karin, 1989; Kapiloff et al., 1991; Okimura et al., 1994; Fischberg et al., 1994). Threonine 220, in the N-terminus of the Pit-1 homeodomain, is phosphorylated *in vitro* by PKA (Kapiloff et al., 1991; Caelles et al., 1995) and by an M-phase specific protein kinase (Caelles et al., 1995).

Phosphorylation of T220 has variable effects on Pit-1 DNA binding, depending on the target DNA binding site (Kapiloff et al., 1991). It causes a 3-fold increase in affinity for the proximal prolactin promoter, no change in binding to the Pit-1 5' autoregulatory site, and a 10-fold decrease in affinity for its binding site in the growth hormone promoter (Kapiloff et al., 1991). The differential influence of T220 phosphorylation on Pit-1 DNA binding may allow it to switch amongst these different target genes in response to different signals. Phosphorylation of this site also enhances the dimerization of Pit-1 on promoter binding sites of the prolactin and growth hormone genes (Kapiloff et al., 1991). Interestingly, T220 phosphorylation does not affect Pit-1 transcriptional activity in cultured cells (Fischberg et al., 1994; Okimura et al., 1994; Caelles et al., 1995). It is possible that the cells used in the assay do not have the components required to mediate the effects of T220 phosphorylation.

1.4.4 Phosphorylation of Ftz

Ftz is heavily phosphorylated on serine and threonine residues (Krause and Gehring, 1989). Two-dimensional Western blots show that Ftz exists as a mixture of phosphoisoforms with as many as sixteen phosphates per molecule (Krause et al. 1988; Krause and Gehring, 1989). These modifications occur in a tissue- and stage-specific fashion: when Ftz is expressed in blastoderm stripes, it exists as a different set of phosphoisoforms than when it is expressed in the central nervous system (CNS; Krause and Gehring, 1989). Phosphopeptide mapping and phospho-amino acid analysis, using the baculovirus expression system and purified kinases, have identified 18 potential phosphorylation sites out of a total of 41 serine and 41 threonine residues (Figure 6, Strome and Krause, unpublished results). The majority of these sites are also phosphorylated by staged *Drosophila* embryo extracts. The kinases tested that can phosphorylate Ftz include casein kinase 1 (CK1), protein kinase C (PKC), mitogen-activated protein kinase (MAPK), glycogen synthase kinase-3 (GSK-3), S6 kinase and PKA. Each of these kinases has been shown to modify certain transcription factors and has at least one *Drosophila* homologue (e.g. Kalderon and Rubin, 1988; Siegfried et al., 1990a; Siegfried et al., 1990b; Peverali et al., 1996; Jaffe et al., 1997). Figure 6 summarizes the mapped Ftz phosphorylation sites (Strome and Krause, unpublished results).

Phosphorylation affects a number of biochemical properties of Ftz (Krause and Gehring, 1989; Copeland, 1997). For example, Ftz protein purified from a baculovirus expression system, where it is heavily phosphorylated, is soluble whereas bacterially expressed Ftz is completely insoluble in the absence of denaturants. Phosphorylated and unphosphorylated Ftz proteins also yield different proteolysis profiles when treated with proteases and analyzed in a DNA binding

mobility shift assay, indicating that the proteins are in different conformations and expose different domains to the proteases (Copeland, 1997). Different phosphoisoforms also appear to have different DNA binding affinities when assayed by a two-dimensional Southwestern blot (Copeland, 1997). Phosphorylation may also affect the ability of Ftz to interact with other proteins since several sites are in the vicinity of mapped protein-protein interaction domains (Figure 6). However, this has yet to be demonstrated.

In summary, Ftz regulates a number of target genes, some of which are regulated in a stage-specific fashion. Rapid and reversible phosphorylation may serve to generate multiple Ftz isoforms, each with distinct regulatory functions. Thus, phosphorylation may provide an economic way of modifying the activities of Ftz, allowing it to serve several different functions during embryonic development.

1.5 Objectives and Overview of this Dissertation

The overall objective of this dissertation is to test the hypothesis that phosphorylation regulates Ftz activity. The experiments have been designed to test the importance of single phosphorylation events on Ftz activity *in vivo*. The overall flow of the experiments is outlined in Figure 10.

This objective has been met for one particular phosphorylation site, as demonstrated in Chapter 2. This site, Ftz T263, is shown to be phosphorylated by *Drosophila* embryo extracts and by purified PKA. Mutations causing T263 to be replaced by alanine or by aspartate were then made and the mutant proteins analyzed in embryos. The results show that phosphorylation of this site is critical for the function of *ftz* in the blastoderm. Additional analyses were carried out *in*

vitro to understand the molecular mechanisms underlying the mutant defects. Ftz DNA binding and general transactivation activities do not seem to be affected by the mutations.

Chapter 3 of this thesis demonstrates that, although important for segmentation, the phosphorylation states of Ftz T263 appear to be irrelevant for directing *eve* expression in the nervous system. This suggests a stage-specific requirement for T263 phosphorylation. Threonine 263 is residue 7 of the Ftz homeodomain, which is in the N-terminal arm, analogous to the position of T220 in Pit-1 (discussed in 1.4.3). Many other homeodomains have serine/threonine or alanine residues at this position, and the same subclass of homeodomains often share the same residue at position 7 (Scott et al., 1989; Gehring et al., 1994a; Manak and Scott, 1994). NMR analysis of a Ftz homeodomain-DNA complex reveals that T263 does not contact DNA (Qian et al., 1994). Consistent with this observation, the T263A and T263D mutations do not seem to affect Ftz homeodomain-DNA interactions (section 2.2.6). This residue may be on the surface of the Ftz molecule that faces away from the DNA molecule and that potentially contacts other proteins. Several protein-protein interactions have been tested for modulation by T263 mutations, and the results are presented in Appendix I. The Ftz homeodomain is shown to interact with a *Drosophila* high mobility group protein, HMG-D, and the interaction is affected by the Ftz T263D mutation. HMG-D also affects Ftz T263D homeodomain-DNA binding.

In summary, this dissertation provides a detailed analysis of mutations in a Ftz phosphorylation site in both *in vivo* and *in vitro* settings, and provides further insight into the regulation of transcription factors by phosphorylation. It should serve as a model for the regulation of other homeodomain proteins by phosphorylation.

Figure 10. Experimental approach. Site-directed mutagenesis and rescue experiments are diagrammed. First, mapped phosphorylation sites are mutated to both Ala and Asp, one at a time, to mimic the unphosphorylated and phosphorylated states respectively. The mutations are introduced into a 10 kb *ftz* rescue construct, and introduced into the *Drosophila* germ line by P element-mediated transformation. Flies carrying the transgenic constructs on the second chromosome are crossed to flies carrying protein null mutations in the *ftz* gene. The ability of the mutated genes to rescue viability (Survival Index: SI), segmentation, target gene expression and molecular activities are then assessed. The asterisk (*) indicated a mutated phosphorylation site. P: P-element inverted repeat. *ry*: *rosy* marker gene.

SKRTRQTYTR
P

Ftz T263 is phosphorylated *in vitro*

↓

ACG (T263) to GCG (A) and GAT (D)

site-directed mutagenesis

↓

N — * — C
homeobox

regenerate *ftz* transcription unit

↓

P *ry* 5 Kb *ftz* 3 Kb P

construct P-element vector

↓

P [*ftz**]; *ftz*+

make transgenic flies

↓

P[*ftz**] / P[*ftz**] ; *ftz*⁻ / *ftz*⁻

cross out endogenous *ftz*

↓

- lethal?
- phenotype?

analyze transgenic lines

↓

in vitro analysis of the mutants

Chapter 2:

A Phosphorylation Site in the Ftz Homeodomain is Required for Segmentation

Published by Jianli Dong, Ling-Hong Hung, Robert Strome, and Henry M. Krause. 1998. *EMBO J.* 17:2308-2318.

2.0 Abstract

The *Drosophila* homeodomain-containing protein Fushi tarazu (Ftz) is expressed sequentially in the embryo, first in alternate segments, then in specific neuroblasts and neurons in the central nervous system and finally in parts of the gut. During these different developmental stages, the protein is heavily phosphorylated on different subsets of Ser and Thr residues. This stage-specific phosphorylation suggests possible roles for signal transduction pathways in directing tissue-specific Ftz activities. Here we show that one of the Ftz phosphorylation sites, T263 in the N-terminus of the Ftz homeodomain, is phosphorylated *in vitro* by *Drosophila* embryo extracts and protein kinase A. In the embryo, mutagenesis of this site to the non-phosphorylatable residue Ala resulted in loss of *ftz*-dependent segments. Conversely, substitution of T263 with Asp, which is also non-phosphorylatable, but which successfully mimics phosphorylated residues in a number of proteins, rescued the mutant phenotype. This suggests that T263 is in the phosphorylated state when functioning normally *in vivo*. We also demonstrate that the T263 substitutions of Ala and Asp do not affect Ftz DNA binding activity *in vitro*, nor do they affect stability or transcriptional activity in transfected S2 cells. This suggests that T263 phosphorylation is most likely required for a homeodomain-mediated interaction with an embryonically expressed protein.

2.1 Introduction

Homeodomain proteins constitute a large family of eukaryotic transcription factors that share a common 60 amino acid DNA binding domain referred to as the homeodomain (reviewed in Gehring et al., 1994a; McGinnis, 1994; Sharkey et al., 1997). In all higher eukaryotes, homeodomain proteins play a major role in patterning the embryonic body plan. Loss-of-function or gain-of-function mutations cause transformations in regional identity that can be quite spectacular. In *Drosophila,* for example, antennae can be transformed into legs, eyes or mouth parts (Schneuwly et al., 1987; Mann and Hogness, 1990; Lin and McGinnis, 1992; Zeng et al., 1993; Halder et al., 1995; Aplin and Kaufman, 1997). These same antennal transformations can be induced by mis-expressing the vertebrate homologues of the same genes (Malicki et al., 1990; McGinnis et al., 1990; Zhao et al., 1993; Halder et al., 1995), emphasizing the functional as well as structural conservation of these proteins.

Homeodomain proteins are thought to control patterning processes by coordinating the expression of specific sets of target genes. Indeed, the ability of homeodomain proteins to bind specific DNA sequences *in vitro*, and to regulate gene expression in cultured cells, has been well documented (Jaynes and O'Farrell, 1988; Krasnow et al., 1989; Winslow et al., 1989; Han et al., 1989; Gehring et al., 1994b). The specificity of action that each homeodomain protein exhibits *in vivo*, however, has not yet been duplicated *in vitro*. In fact, homeodomain proteins tend to bind short A/T rich consensus sites that are often indistinguishable from one another (reviewed in: Hayashi and Scott, 1990; Kalionis and O'Farrell, 1993; Gehring et al., 1994b). A concerted effort has been made in the past few years to determine how specificity of action is achieved *in vivo*.

One potential source of specificity is through interactions with other DNA binding transcription factors. Indeed a number of DNA binding partners and cofactors have been

identified in the past few years (reviewed in Mann and Chan, 1996). Some of these partners change DNA binding specificity whereas others alter the ability to activate or repress adjoining promoters. Another potential source of specificity is the covalent addition of phosphate groups. Phosphorylation has been shown to affect a variety of transcription factor properties including structure, sub-cellular localization, DNA binding affinity and specificity, and the ability to activate transcription (reviewed in Hunter and Karin, 1992; Karin, 1994; Hill and Treisman, 1995). All homeodomain proteins examined thus far are phosphorylated (Krause et al., 1988; Gay et al., 1988; Krause and Gehring, 1989; Odenwald et al., 1989; Tanaka and Herr, 1990; Gavis and Hogness, 1991; Lopez and Hogness, 1991; Segil et al., 1991; Kapiloff et al., 1991; Wall et al., 1992; Ronchi et al., 1993; Bourbon et al., 1995; Caelles et al., 1995; Coqueret et al., 1996; Zannini et al., 1996; Zwilling et al., 1997; Jaffe et al., 1997), indicating a likelihood for similar types of phosphorylation induced changes in activity.

Here, we use the *Drosophila* homeodomain protein Fushi tarazu (Ftz) as a model to examine the importance of phosphorylation on homeodomain protein function and specificity. Ftz is expressed during three stages of embryogenesis: first in alternating segmental primordia, then in differentiating neurons in the central nervous system, and finally in portions of the gut and posterior epidermis (Hafen et al., 1984; Carroll and Scott, 1985; Krause et al., 1988). *ftz* mutant embryos lack alternate segmental regions (Wakimoto et al., 1984) and exhibit transformations in neuronal identities (Doe et al., 1988a).

Ftz was one of the first homeodomain proteins shown to be phosphorylated (Krause et al., 1988; Krause and Gehring, 1989). Phosphorylation occurs on equivalent numbers of serine and threonine residues, with as many as 16 phosphates per molecule. Interestingly, phosphorylation also occurs in a tissue- and stage-specific fashion (Krause and Gehring, 1989).

In this study, we report that Ftz residue threonine 263 (T263), which is in the N-terminal arm of the homeodomain, is phosphorylated *in vitro* in a cAMP-dependent fashion by *Drosophila* embryo extracts and by purified cAMP-dependent protein kinase (PKA). To test whether this phosphorylation event is required for Ftz activity, we mutated T263 to Ala and Asp to mimic the unphosphorylated and constitutively phosphorylated states, and then subcloned the mutant DNAs into a 10 kb fragment of *ftz* genomic DNA, capable of rescuing *ftz⁻* embryos to adulthood (Hiromi et al., 1985). The reconstituted genes were transformed into flies by P-element mediated germ line transformation and tested for their ability to rescue *ftz* mutant embryos. Ftz T263A mutant constructs failed to rescue *ftz⁻* flies to adulthood whereas wild-type and T263D Ftz constructs did. Phenotypic defects caused by the T263A mutation are described. The results indicate that T263 is phosphorylated when Ftz is in its active form, and that phosphorylation of T263 probably affects a protein-protein interaction.

2.2 Results

2.2.1 Ftz Phosphorylation by Embryo Extracts

The Ftz protein, expressed in embryos, is heavily phosphorylated (Krause and Gehring, 1989). In order to label and map sites, and to identify the kinases responsible, we added bacterially expressed Ftz to staged *Drosophila* extracts in the presence of [γ-^{32}P]ATP. Figure 11A shows labeling of Ftz by extracts under a variety of conditions. Moderate labeling was achieved in extracts adjusted to pH7.2 and 25mM $MgCl_2$ (lane 2 vs. lane 1). This low level labeling was largely blocked by the addition of inhibitors of protein kinase C (PKC) and calmodulin-dependent protein kinase (Cam PK) (lane 4). However, enhanced labeling could once again be achieved by the addition of cAMP (lane 6), indicating the presence of a PKA type

kinase with specificity for Ftz. Using a series of deleted Ftz polypeptides, the sites of cAMP-dependent phosphorylation were mapped to the Ftz homeodomain (Figure 11B). Deletion of the Ftz homeodomain removed labeling (lane 3), whereas the homeodomain by itself (lane 4) incorporated label as efficiently as the intact Ftz protein.

Figure 12 shows that homeodomain-specific labeling could also be achieved with purified PKA. As with the cAMP-dependent extract labeling, the homeodomain was found to be both required and sufficient for efficient labeling. In order to map the phosphorylated residue(s), extract and PKA labeled homeodomains were subjected to tryptic digestion, thin layer chromatography and solid phase Edman degradation of isolated peptides (Figure 13). Ftz homeodomain labeled using embryo extract yielded two spots in the tryptic fingerprint (Figure 12A). The same two spots were labeled by PKA (Figure 13B and C).

Edman degradation of the two eluted peptides yielded ^{32}P-labeled amino acid peaks at cycles 2 (Figure 13E) and 3 (not shown) for spots one and two respectively. A tryptic peptide near the N-terminus of the Ftz homeodomain is the only peptide that has a Ser or Thr residue at the second position (T263). Indeed, phosphorylation of T263 was confirmed by Edman degradation of undigested homeodomain and release of label at cycle 11 as expected (Figure 13F). Further confirmation of phosphorylation at T263 was obtained by changing the site to an alanine residue. Figure 11B (lane 5) shows that labeling of the mutant T263A homeodomain by embryo extract was markedly reduced, and Figure 13D shows that spot #1 in the tryptic fingerprint of the T263A homeodomain disappeared as expected. The same results were obtained when labeling was performed using purified PKA (Figure 12, lane 5, and data not shown).

Edman degradation analysis suggests that spot #2 corresponds to the homeodomain residue T269. Both sites, T263 and T269, are within PKA consensus sites. Notably, T263 is also

Figure 11. Labeling of Ftz by embryo extracts. 0-6 hr embryo extracts were used to phosphorylate Ftz polypeptides *in vitro* in the presence of [γ-^{32}P]ATP. Following labeling, proteins were electrophoresed on PAGE gels and the gels autoradiographed.

A) Stimulation of Ftz phosphorylation by cAMP. Lane 1: extract alone. Lane 2: extract + Ftz. Lane 3: extract + PKC/Cam PK inhibitors. Lane 4: extract + PKC/Cam PK inhibitors + Ftz. Lane 5: extract + PKC/Cam PK inhibitors + cAMP. Lane 6: extract + PKC/Cam PK inhibitors + Ftz + cAMP.

B) Mapping of Ftz cAMP-dependent phosphorylation sites. Full-length, HD deleted and T263A Ftz polypeptides were labeled in the presence of embryo extract, PKC and Cam PK inhibitors and cAMP. Lane 1: - Ftz. Lane 2: + Ftz. Lane 3: + FtzΔHD (homeodomain deleted). The protein migrates just below the full-length protein and above the major labeled band just below (determined by Western, not shown). Lane 4: Ftz homeodomain (aa 254-314). Lane 5: Ftz homeodomain with T263A substitution. Arrows indicate the positions of full-length and homeodomain Ftz polypeptides.

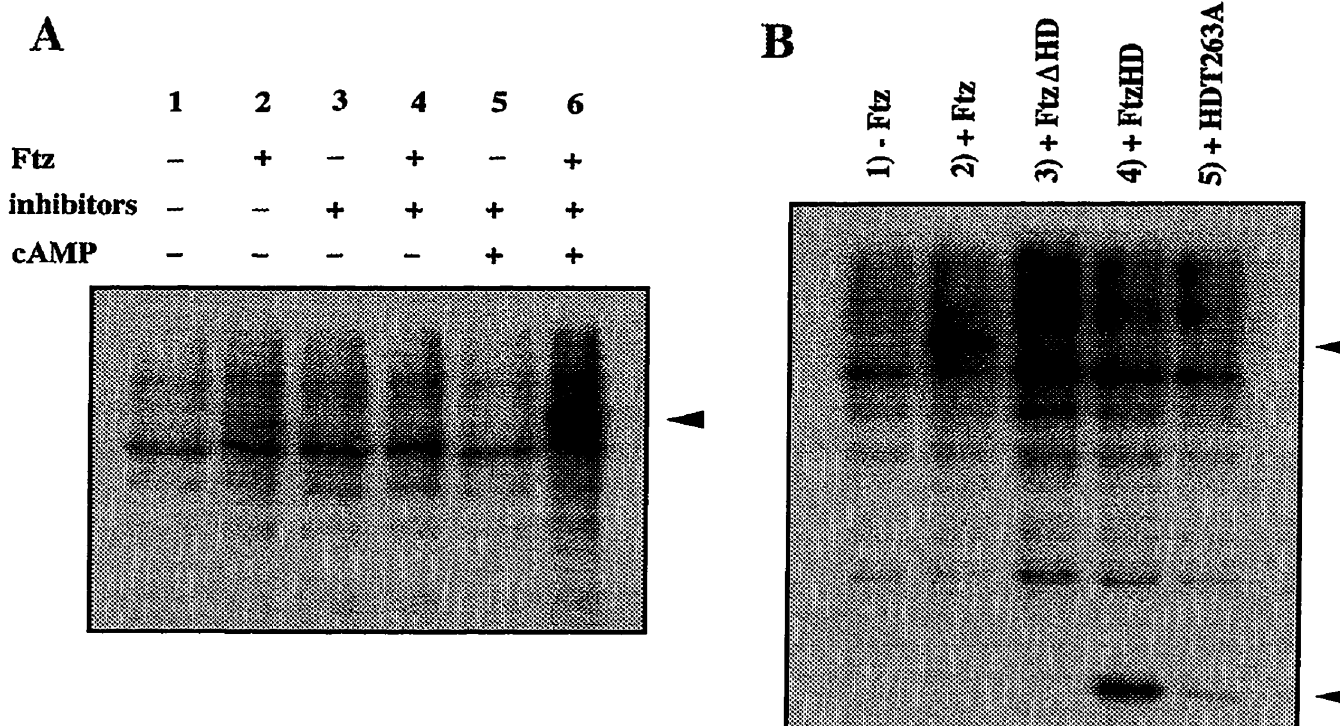
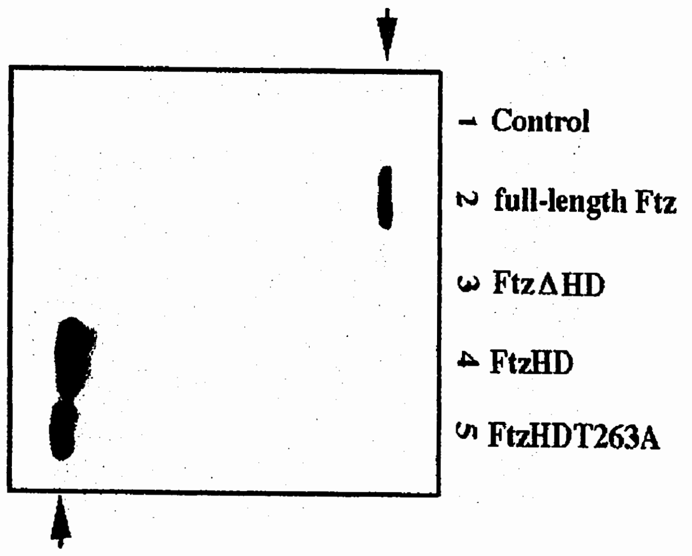

A
1
2
3
4
5
6
Ftz
–
+
–
+
–
+
inhibitors
–
–
+
+
+
+
cAMP
–
–
–
–
+
+
B
1) - Ftz
2) + Ftz
3) + FtzΔHD
4) + FtzHD
5) + HDT263A

Figure 12. Phosphorylation of Ftz by PKA. Full-length and deleted Ftz polypeptides were phosphorylated *in vitro* in the presence of purified protein kinase A. Labeled polypeptides were separated on PAGE gels and autoradiographed. Lane 1: no Ftz added. Lane 2: full-length Ftz. Lane 3: FtzΔHD. Lane 4: Ftz homeodomain. Lane 5: Ftz homeodomain with T263A substitution. Arrows indicate the positions of the full-length and homeodomain Ftz polypeptides.



1 Control
2 full-length Ftz
3 FtzΔHD
4 FtzHD
5 FtzHDT263A

Figure 13. Mapping cAMP- and PKA-dependent phosphorylation sites. Ftz homeodomain (HD), labeled *in vitro* by embryo extracts or purified PKA, was subjected to trypsin digestion, separated by thin layer chromatography and autoradiographed. A) Ftz HD labeled by embryo extract. B) Ftz HD labeled by PKA. C) equal mix of extract and PKA labeled proteins. Spot #1 from A) comigrates with spot #1 from B), as do the two #2 spots. D) T263A HD labeled by embryo extract. Spot #1 is absent. E) Manual sequencing of tryptic peptide #1. Label eluted at cycle 2. T263 is the only phosphorylatable residue within a tryptic peptide of the Ftz homeodomain that should be released at cycle 2. F) Manual sequencing of undigested Ftz homeodomain. Label eluted at cycle 11. T263 is the seventh residue of the homeodomain, and the homeodomain construct used has four additional amino acids at its N-terminal end (confirmed by DNA sequencing; data not shown). Strome and I did the experiment B. Experiments E and F are done by R. Strome and L. Hung (unless otherwise stated, the experiments in this thesis are done by myself).

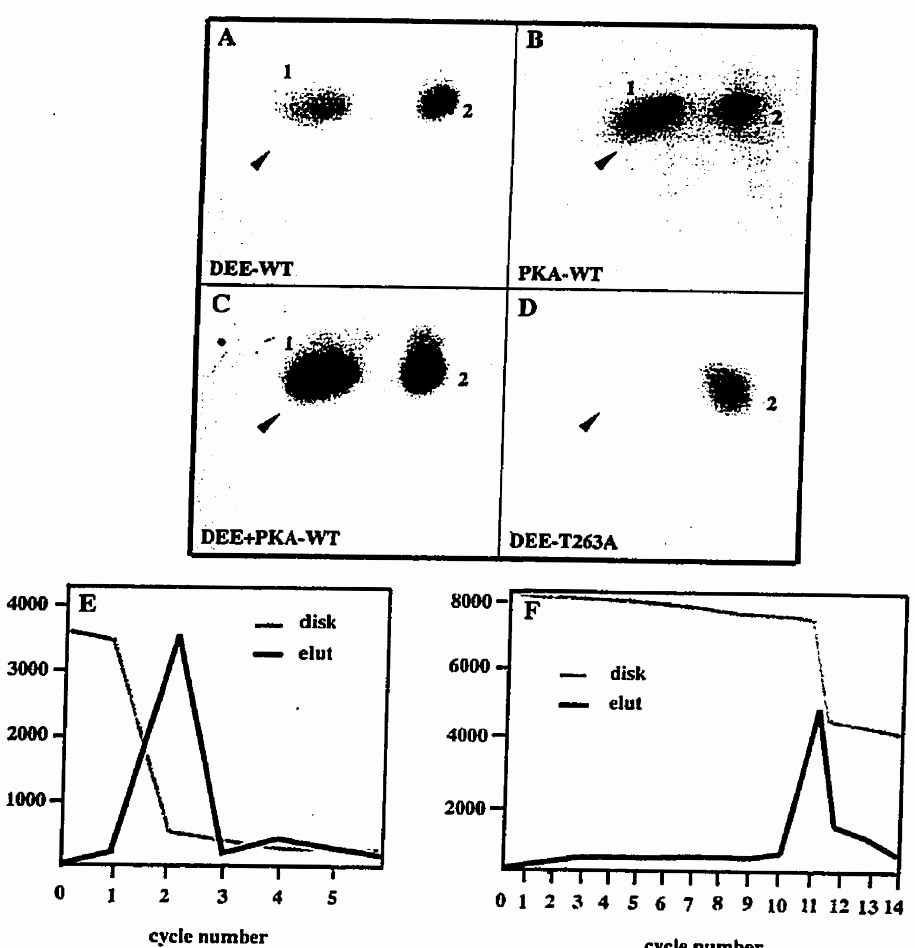

A
1
2
DEE-WT
B
1
2
PKA-WT
C
1
2
DEE+PKA-WT
D
2
DEE-T263A
E
disk
elut
4000
3000
2000
1000
0
1
2
3
4
5
cycle number
F
disk
elut
8000
6000
4000
2000
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
cycle number

Table 1. Survival indices of Ftz T263 mutant offspring. SI values (#flies with appropriate phenotype/# expected) are given for flies homozygous mutant for the endogenous *ftz* gene and carrying two copies of either a wild-type, T263A or T263D transgenic *ftz* gene. For each transgene, four independent lines were examined, all with inserts on the second chromosome (linkage group II). n= # flies scored.

	line	#rescued	SI
WT	1	132; n=559	0.708
	2	36; n=521	0.207
	3	65; n=531	0.367
	4	75; n=470	0.479
T263A	1	0; n=278	0
	2	0; n=278	0
	3	0; n=495	0
	4	0; n=443	0
T263D	1	3; n=401	0.022
	2	16; n=379	0.126
	3	63; n=744	0.254
	4	19; n=477	0.119

in the analogous position of mapped PKA kinase sites in the POU class homeodomains of Pit-1 and Oct-1 (Kapiloff et al., 1991; Segil et al., 1991). Other protein kinases, including PKC, casein kinase I (CKI), casein kinase II (CKII), mitogen activated protein kinase (MAPK), S6 protein kinase (S6K) and glycogen synthase kinase-3 (GSK-3), failed to phosphorylate this site. Phosphorylation by PKA, on the other hand, was equal in efficiency to phosphorylation of a PKA consensus site (heart muscle kinase peptide), by measuring the labeled bands on PAGE gels (data not shown).

2.2.2 *In vivo* Analysis of T263 Mutants

In order to test whether T263 is phosphorylated *in vivo*, and to determine the role of phosphorylation, Ftz T263A and T263D mutant expression constructs were injected into flies, and the transgenic constructs tested for their ability to functionally replace the endogenous *ftz* gene. The *ftz* open reading frame, along with 6.1 kb of 5' and 1.9 kb of 3' DNA, is sufficient to direct normal *ftz* expression and to fully rescue *ftz* mutant embryos (Hiromi et al., 1985). Rescue constructs were introduced into the genome by P-element-mediated germ line transformation (Spradling and Rubin, 1982). For each mutation, four transformant lines, all on the second chromosome, were used for subsequent analyses. These, along with lines carrying wild-type *ftz* transgenes, were crossed to flies carrying the *ftz* alleles ftz^{9H34} and ftz^{w20}, both of which are protein null. The resultant lines, P[*ftz*]; ftz^{9H34}/ftz^{w20}, were then tested for viability.

Table 1 shows the survival index (SI= No. of rescued flies obtained/No. of rescued flies expected) values for flies carrying wild-type, T263A and T263D transgenic *ftz* genes in the ftz^{9H34}/ftz^{w20} mutant background. The wild-type control construct rescued flies to adulthood

with SI values ranging from 21 to 71%. These values are in the same range as those found in previous studies (Hiromi et al., 1985; Furukubo-Tokunaga et al., 1992). These observed variations in rescuing capacity are likely to be due to reduced or ectopic *ftz* expression brought about by adjoining sequences at the various sites of P-element insertion.

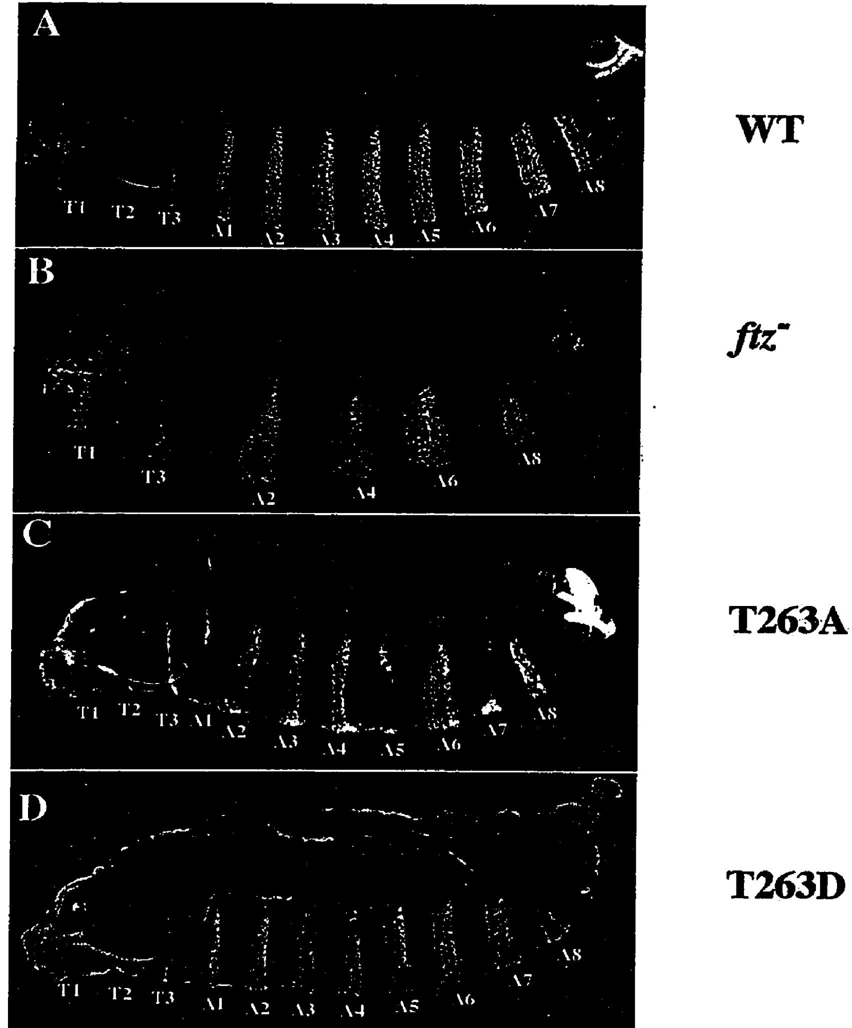
In contrast to the wild-type *ftz* construct, the T263A mutant construct showed no ability to rescue: all four transformant lines gave SI values of zero. On the other hand, the T263D construct was able to rescue mutant flies to adulthood, although less effectively than the wild-type construct (SI = 2-25% versus 21-71% for wild-type). Thus, the T263D protein probably exhibits minor irregularities in function or expression.

2.2.3 Cuticle Patterns

The rescue of flies to adulthood is a comprehensive measure of all *ftz* gene activities. Because *ftz* has several stage-specific developmental roles, reductions in SI values could result from either general or stage-specific defects. In order to test for segmentation-specific defects, we looked at the cuticles of larvae obtained from rescued embryos. We also examined the expression patterns of putative target genes required for *ftz*-dependent segmentation.

ftz mutant cuticles exhibit pairwise deletions of even-numbered parasegments (Furukubo-Tokunaga et al., 1992, Schier and Gehring, 1993a; and Figure 14B). Cuticle preparations of T263A embryos also exhibited pairwise deletions of even-numbered parasegments (Figure 14C). Interestingly, however, the penetrance of the phenotype was quite variable, with some cuticles exhibiting complete deletion of *ftz*-dependent parasegments, while others showed few or no detectable defects. Although variable from cuticle to cuticle, defects in each *ftz*-dependent parasegment occurred with a characteristic frequency. These ranged from the most to least

Figure 14. Cuticle preparations of T263 mutant larvae. Cuticles were prepared from P[*ftz* T263] rescued larvae and visualized by dark field microscopy. A) wild-type larval cuticle. B) homozygous *ftz* mutant cuticle. C) typical Ftz T263A rescued cuticle. D) typical Ftz T263D rescued cuticle.


A
T1
T2
T3
A1
A2
A3
A4
A5
A6
A7
A8
WT
B
T1
T3
A2
A4
A6
A8
ftz⁻
C
T1
T2
T3
A1
A2
A3
A4
A5
A6
A7
A8
T263A
D
T1
T2
T3
A1
A2
A3
A4
A5
A6
A7
A8
T263D

frequently affected as follows: parasegment (PS) 2 (97% affected)> PS6 (82%)> PS10,4 (70%)> PS12,14 (58%)> PS8 (36%) (n= 482).

In contrast to T263A cuticles, cuticles from T263D flies were indistinguishable from wild-type (Figure 14D). A few cuticles (<1%) had minor abnormalities, but these were variable and also seen when wild-type rescue constructs were used. The ability of the T263D protein, and not the T263A protein, to rescue *ftz*-dependent segmentation suggests that T263 is normally in the phosphorylated state when the protein is active.

2.2.4 *ftz* Autoregulation

To trace the causes of cuticular defects observed with T263A mutant embryos, we examined the expression of *ftz* target genes. The *ftz* gene itself is a well characterized target of Ftz activity. Ftz facilitates its own expression via binding and activation of an upstream enhancer element (Hiromi et al., 1985; Hiromi and Gehring, 1987; Pick et al., 1990; Schier and Gehring, 1992). Figure 15A and B show typical patterns of *ftz* transcripts at two different stages of embryogenesis. In the T263A mutants, stripes of expression were weaker and narrower than normal (Figure 15E and F), except perhaps at the earliest stages of initiation (not shown) which are thought to be *ftz*-independent. The frequency and severity of defects were similar at the two later stages shown. Patterns of protein expression (not shown) were similar to those of the transcripts, with the same variations seen at the same stages. Protein was localized properly to the nucleus in all transgenic lines. These observations suggest, but do not prove, that it is the ability of Ftz to autoregulate its own promoter, and not its synthesis, stability or localization, that is affected by the mutation.

Figure 15. ***ftz*** **gene autoregulation in Ftz T263 mutants.** *ftz* gene expression was examined by *in situ* hybridization at two different stages to test for defects in autoregulation. (A, B) Wild-type *ftz* expression. (C, D) Homozygous *ftz* mutant embryo. (E, F) Typical *ftzT263A* expression. (G, H) Typical *ftzT263D* expression. In E-H, homozygous *ftz*$^-$ embryos were identified based on lack of β-galactosidase expression (see Methods).

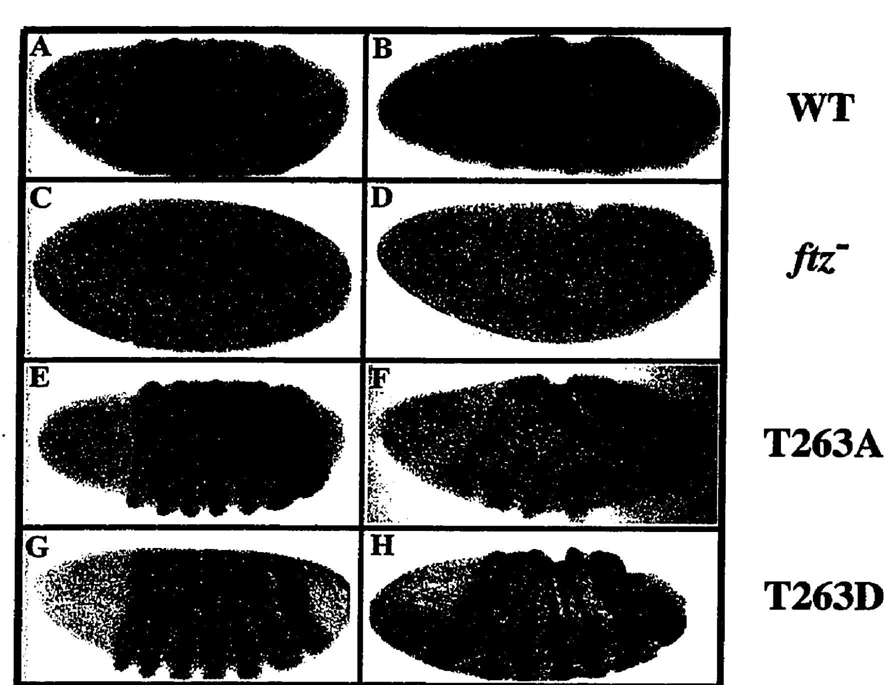
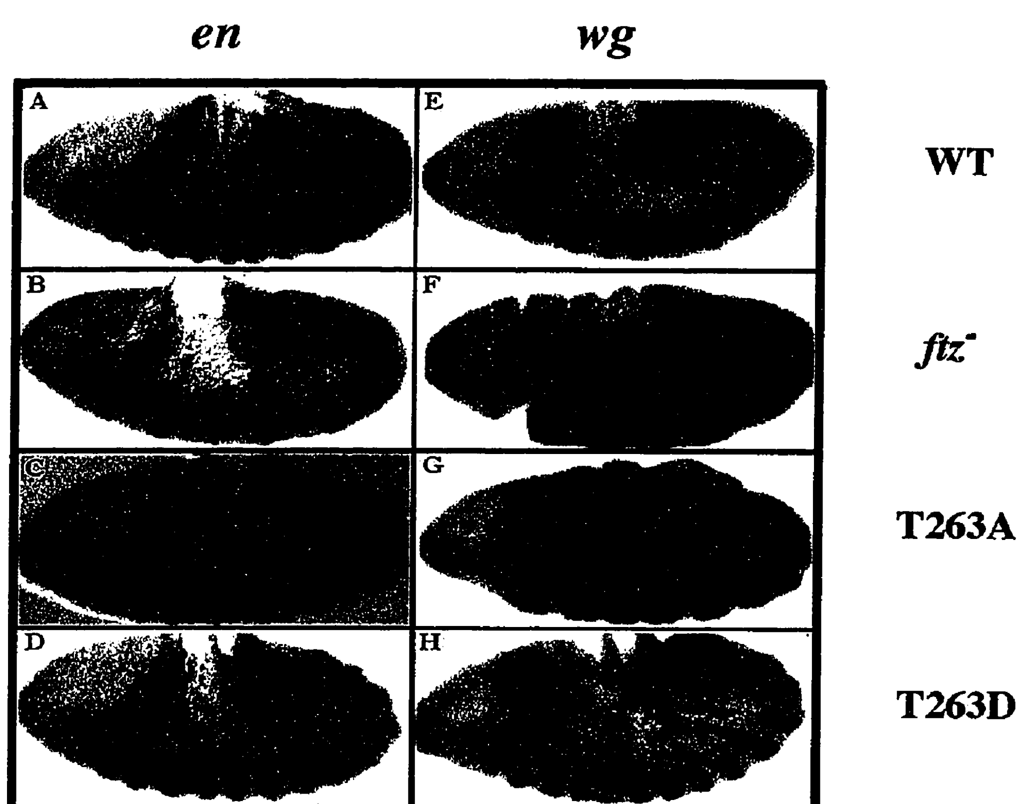

A
B
WT
C
D
ftz⁻
E
F
T263A
G
H
T263D

Figure 16. *en* and *wg* expression in Ftz T263 mutants. Patterns of *en* mRNA expression are shown on the left (A-D) and patterns of *wg* mRNA expression on the right (E-H). From top to bottom, embryos shown are wild-type (A, E), *ftz*$^{-}$ (B, F), Ftz T263A (C, G) and Ftz T263D (D, H). *ftz*-dependent *en* stripes are partially missing in the T263A mutant embryo and *wg* stripes are partially expanded. In contrast *en* and *wg* stripes are normal in the T263D embryo.



en
wg
A
B
C
D
E
F
G
H
WT
ftz-
T263A
T263D

As with the cuticle preparations, the severity of defects in *ftz* expression patterns was highly variable. Some embryos were devoid of detectable expression while others were essentially normal. Certain stripes were more prone to defects than others. Although stripe defects varied from embryo to embryo, the average frequency of defects occurred in the following order: stripe 1 > 3 > 5, 2 > 6, 7 > 4. This correlates well with the prevalence of defective *ftz* dependent parasegments in the T263A cuticle preparations (i.e. PS 2 > PS6 > PS10, 4 > PS12, 14 > PS8). The T263D embryos, in contrast, produced normal patterns of *ftz* mRNA (Figure 15G and H) and protein (not shown).

2.2.5 *en* and *wg* Expression

Ftz is thought to be a direct transcriptional activator of alternate stripes of expression of the segment polarity gene *engrailed* (*en*) (DiNardo and O'Farrell, 1987; Kassis, 1990; Florence et al., 1997). Conversely, transcription of alternate *wingless* (*wg)* stripes appears to be repressed by Ftz (Ingham et al., 1988; Copeland et al., 1996). Altered expression patterns of *en* and *wg* in T263A mutant embryos (Figure 16) correlated well with the defects observed in *ftz* expression patterns (Figure 15). *ftz*-dependent *en* stripes were generally weak or missing. Conversely, the *wg* stripes that are normally repressed by Ftz were expanded. As with *ftz* expression, the penetrance of these defects varied greatly from embryo to embryo and showed similar frequencies of segment-specific defects. No defects in *en* and *wg* stripes were observed in T263D embryos.

2.2.6 DNA-binding Activity

The results thus far indicate that the T263A mutation reduced Ftz activity, and that the effects of the T263D substitution were extremely minor. Since T263 resides in the DNA-binding

homeodomain, we tested whether DNA-binding activity was affected by the mutations. Electrophoretic mobility shift assays (EMSAs) were performed using consensus Ftz-binding sites (Percival-Smith et al., 1990; Pick et al., 1990; Florence et al., 1991). Wild-type, T263A and T263D homeodomains were expressed in reticulocyte lysates. Figure 17 shows that inherent DNA-binding activity of the homeodomain was not affected by either of the two mutations. The same result was achieved using a different Ftz-binding site and different concentrations of protein (data not shown). Thus, the effect of the T263A mutation on Ftz activity does not appear to be exerted at the level of protein-DNA recognition.

2.2.7 Transcriptional Activity in Cultured S2 Cells

A tissue culture co-transfection assay was used to further characterize the functional properties of Ftz T263 mutants. Wild-type, T263A and T263D Ftz expression constructs were co-transfected individually into *Drosophila* S2 cells together with the Ftz-responsive reporter plasmid 3'K TATA-CAT, which contains concatamerized Ftz-binding sites derived from a portion of the *en* promoter (Han et al., 1989). All three Ftz expression constructs were able to activate the reporter gene with similar efficiency: ~15- to 16-fold over basal levels (Figure 18). Furthermore, co-transfection of the catalytic subunit of PKA (hatched columns in Figure 18), or treatment of cells with PKA stimulators or inhibitors (not shown), had no effect on the activities of the three Ftz proteins. Other Ftz-responsive reporter plasmids, such as Ubx-CAT (Krasnow et al., 1989) and NP6-CAT (Jaynes and O'Farrell, 1988) also gave equivalent responses to the wild-type and mutant Ftz polypeptides (not shown). These results indicate that T263 phosphorylation does not affect the general ability of Ftz to bind and activate simple reporter genes in S2 cells.

Figure 17. Binding of Ftz T263 mutant homeodomains to DNA.

A) Wild-type and T263 mutant Ftz homeodomains were expressed in a reticulocyte lysate system and bound to a ^{32}P-labeled Ftz consensus DNA binding site GGGAAGCAATTAAGGAT (Percival-Smith et al. 1990; Pick et al. 1990). Complexes were resolved on non-denaturing polyacrylamide gels and visualized by autoradiography. Lane 1: no protein added. Lane 2: wild-type homeodomain. Lane 3: T263A homeodomain. Lane 4: T263D homeodomain. All three polypeptides showed equivalent DNA binding activity.

B) Western blot of *in vitro* expressed homeodomain polypeptides used in A). Proteins were separated on 15% PAGE gels, transferred to nitrocellulose and detected using a polyclonal Ftz antiserum. Lane 1: no protein added. Lane 2: wild-type homeodomain. Lane 3: T263A homeodomain. Lane 4: T263D homeodomain. All three polypeptides were expressed at similar levels.

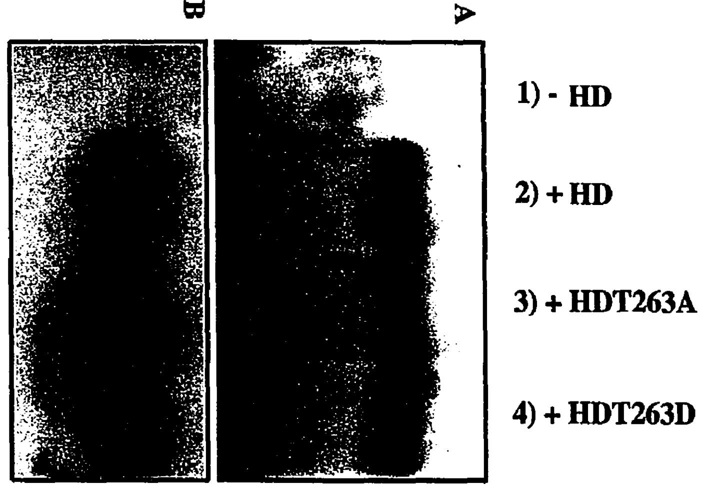
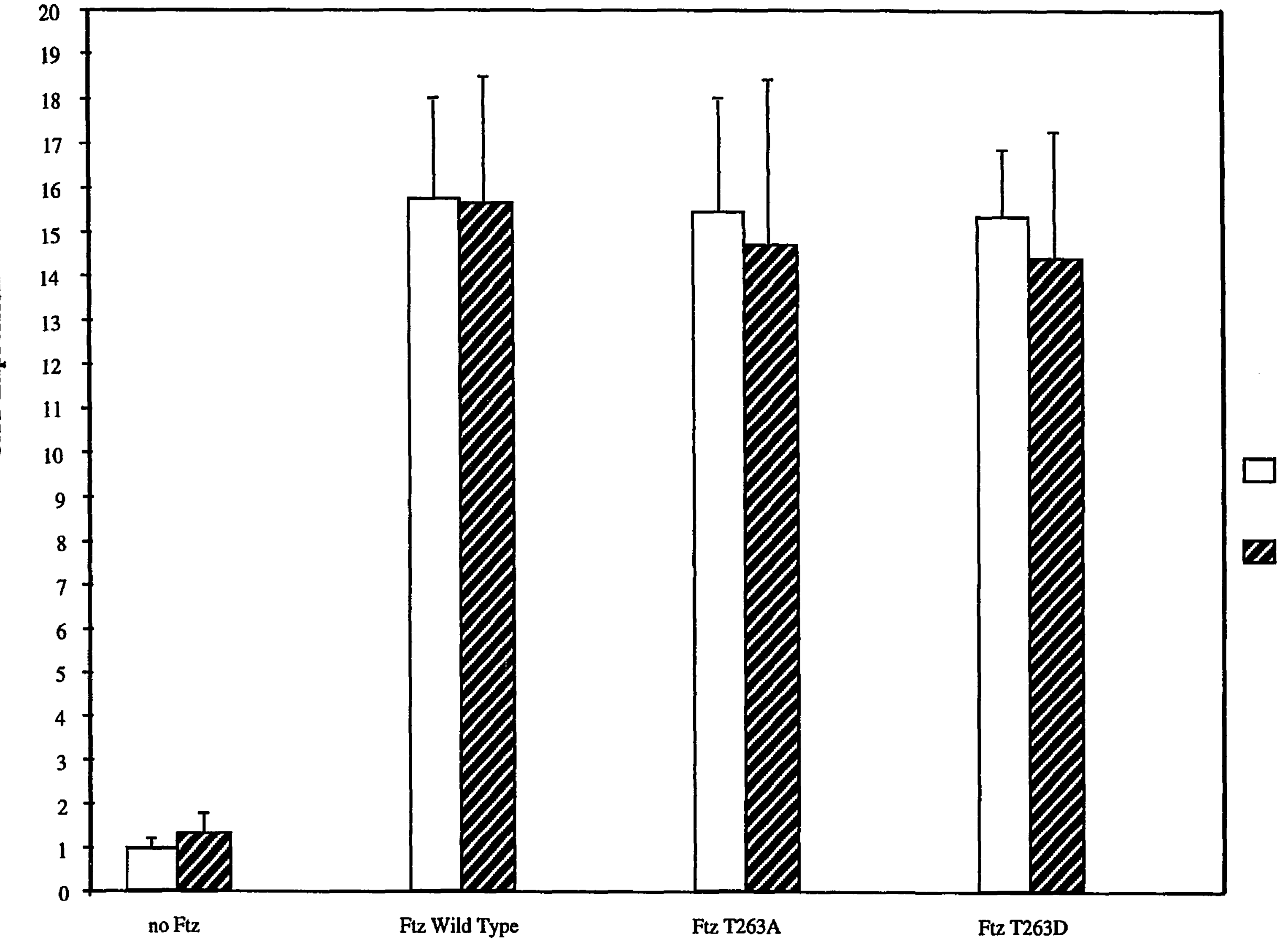

A
B
1) - HD
2) + HD
3) + HDT263A
4) + HDT263D

Figure 18. Transcriptional activity of Ftz T263 mutants in cultured cells. Wild-type and T263 mutant *ftz* cDNAs were placed under control of the actin 5C promoter and transfected into cultured *Drosophila* S2 cells together with the Ftz dependent reporter gene 3k' TATA-CAT (open columns). Duplicate transformations contained in addition a construct that expresses the catalytic subunit of PKA (cross-hatched columns). Levels of CAT expression were determined immunologically and then corrected for minor variations in levels of Ftz expression. Levels of CAT expression are given as levels relative to the level of basal promoter activity, which was assigned a value of one. All three Ftz proteins had equivalent levels of activity, and activity was not altered by coexpression of PKA. The average levels from 3 experiments are 1.0±0.2, 15.9±2.4, 15.7±2.6, and 15.6±1.6 for control, Ftz T263, T263A and T263D without coexpression of PKA; 1.3±0.4, 15.8±2.9, 14.9±3.8, and 14.6±2.8 for control, Ftz T263, T263A and T263D with PKA.


CAT Expression
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
no Ftz
Ftz Wild Type
Ftz T263A
Ftz T263D
Ftz expression construct
-DC0
+DC0

Rather, the effects of T263 phosphorylation are likely to depend on factors specifically expressed in the *Drosophila* embryo.

2.3 Discussion

2.3.1 Evidence for T263 Phosphorylation *in vitro* and *in vivo*

We have shown that T263, in the N-terminal arm of the Ftz homeodomain, is phosphorylated specifically and efficiently *in vitro* by embryo extracts and PKA. Mutation of this site to alanine, which is generally a conservative substitution (Cunningham and Wells, 1989), resulted in complete loss of Ftz rescuing activity *in vivo*. This lethality was attributed mainly to defects in *ftz*-dependent segmentation. In contrast, mutation of T263 to aspartate, a far less conservative substitution than alanine, but one which has been shown in functional and structural studies effectively to mimic threonine phosphate (see, for example, Cowley et al., 1994; Kowlessur et al., 1995; Napper et al., 1996; Peverali et al., 1996; Boekhoff et al., 1997; Jaffe et al., 1997), returned Ftz activities to near wild-type levels. This strongly suggests that T263 is phosphorylated *in vivo*, and that phosphorylation of this site is required for normal protein activity.

2.3.2 Stage Specificity of T263 Mutant Defects

The majority of T263A mutants died as embryos. Segmental defects, most prevalent in the head, are probably responsible for this lethality, as defects were observed in close to a quarter of the cuticles (97% of homozygous larvae). However, it is also possible that pattern abnormalities in the developing CNS or gut, where *ftz* is expressed later (Carrol and Scott, 1985; Krause et al., 1988), also contributed to lethality. Defects incurred at these later stages could also

account for the reduced rescuing capacity of the T263D mutant construct relative to a wild-type construct, since cuticle patterns of these animals appeared to be normal, yet SI values were significantly lower than for wild-type *ftz* constructs. Both gain-of-function and loss-of-function defects are possible, as cofactors and DNA targets may vary from tissue to tissue in their preference for phosphorylated or unphosphorylated Ftz. The phosphorylation state of T263 may also vary from tissue to tissue.

2.3.3 Variability in the T263A Phenotype

An interesting aspect of the T263A segmental phenotype was its variability. Cuticle patterns and Ftz target gene patterns ranged from wild-type in appearance to patterns that resembled *ftz* nulls. Also, while certain *ftz*-dependent segments were more likely to be affected than others, the segments affected within any given embryo were highly variable. Similar observations were made by Furukubo-Tokunaga *et al.* (1992) in a study where multiple mutations were introduced into the Ftz homeodomain. This variability suggests that there must be considerable variation within individual embryos in terms of *ftz* gene regulators and/or Ftz target gene co-regulators. This possibility is consistent with variations that we have observed in the normal initiating patterns of *ftz* and Ftz target genes: the intensities and widths of initiating stripes tend to vary from embryo to embryo, and yet normally end up equal in intensity and width later on (unpublished data). This variability may be unimportant in wild-type embryos, but may be critical when Ftz activity drops below threshold levels.

Interestingly, the T263A substitution caused a more severe phenotype than those caused by many of the previously generated homeodomain mutations, even though all of the latter included multiple substitutions. Often, those substitutions included residues in helix 3 which

makes key contacts with DNA. The relative severity of our T263 substitution suggests that this residue plays a particularly important role in Ftz homeodomain function.

2.3.4 Activities Affected by T263

The autoregulatory nature of *ftz* gene expression during its segmental phase of expression (Hiromi and Gehring, 1985; Pick et al., 1990; Schier and Gehring, 1992) makes it difficult to determine which aspects of Ftz activity are compromised. When Ftz activity is lowered, expression levels are lowered. This, in turn, would lead to reduced effects on other target genes such as *en* and *wg*. Hence, the T263A effects could be due to either a general reduction in overall activity, or to a specific defect in autoregulation.

For the same reason, a possibility that we cannot rule out is that the T263A defect is simply one of reduced stability. However, three points argue against this likelihood. First, no differences in stability were observed when mutant and wild-type Ftz proteins were expressed in cultured cells under the control of a heterologous promoter. Second, patterns of mRNA and protein in the embryo were similar. Third, protein expression levels approaching wild-type occasionally were achieved, and subsequent stability was normal.

Another possibility is that the T263A mutation affects DNA-binding properties. Homeodomain proteins make most of their base-specific contacts within the DNA major groove using helix 3 of the homeodomain. However, the N-terminal arm of the homeodomain makes additional DNA contacts, most often non-specifically, with phosphate groups on the minor groove side (Gehring et al., 1994b). In Ftz, these non-specific contacts are made by Arg residues at positions 3 and 5 of the N-terminal arm (Percival-Smith et al., 1990; Qian et al., 1994). T263 is at position 7, and does not appear to contact the DNA molecule. However, it is conceivable

that the addition of a large, negatively charged phosphate group could affect the homeodomain-DNA interaction. This appeared not to be the case, however, as the presence of either Thr, Ala or Asp at this position made no difference in binding of Ftz to consensus binding sites *in vitro* or in cultured cells. Nevertheless, the possibility still exists that interactions with sites other than those tested might be affected, as phosphorylation at this position does alter the DNA binding properties of Pit-1 and Oct-1 (Kapiloff et al., 1991; Segil et al., 1991; Caelles et al., 1995). Alternatively, binding site specificity could be affected indirectly by altering the ability of Ftz to interact with a cofactor that confers specificity.

2.3.5 Proteins that Interact with Ftz

The fact that T263A and T263D mutations had no observable effect on Ftz DNA binding or general transactivation activities suggests that phosphorylation of this site may modulate a protein-protein interaction. Indeed, the N-terminal arms of several homeodomain proteins are thought to serve as contact points that confer specificity with other proteins (reviewed in Mann and Chan, 1996). We have shown previously that, when expressed at sufficient levels from a heterologous promoter, a Ftz polypeptide that lacks DNA-binding activity is still capable of regulating target genes *in vivo* (Fitzpatrick et al., 1992; Copeland et al., 1996). This DNA-binding-independent activity is lower when the Ftz homeodomain is completely deleted, versus partially (middle of helix 1 to middle of helix 3) deleted (HMK and A. Percival-Smith, unpublished observations). These results suggest that protein-protein interactions play a particularly important role in recruiting Ftz to specific sites on DNA, and that portions of the homeodomain may serve as contact points. If so, then phosphorylation of T263 could affect these interactions.

Two Ftz-interacting proteins previously have been shown to interact specifically with Ftz; Paired (Prd) (Copeland et al., 1996) and the α spliced form of Ftz-F1, α Ftz-F1 (Guichet at al., 1997; Yu et al., 1997). It has also been observed that αFtz-F1 and a Ftz polypeptide containing the homeodomain, along with short stretches of flanking amino acids, bind cooperatively to adjacent sites on DNA (Florence et al., 1997). However, preliminary experiments with our Ftz T263 mutants suggest that the phosphorylation state of T263 does not affect either the Ftz-Prd or the Ftz-αFtz-F1 interaction. Hence, a yet to be identified protein expressed in embryos is likely to be the relevant target.

2.3.6 Conservation of Thr263

Figure 19 shows the N-terminal sequences of several homeodomain proteins. Homeodomains can be assigned to different subclasses based on amino acid sequence similarities. In approximately half of all HOX proteins (Groups 5-9), residue 7 of the homeodomain (analogous to Ftz T263) is either a Thr or Ser residue, while position 5 is an Arg residue (Gehring et al., 1994a; Sharkey et al., 1997). This conserves the PKA recognition site, and suggests that each of these proteins probably shares the ability to be phosphorylated at this position. This includes the more divergent POU class homeodomain proteins Pit-1 and Oct-1, which like Ftz are phosphorylated efficiently by PKA, or a PKA-like kinase *in vitro* (Segil et al., 1991; Kapiloff et al., 1991; Caelles et al., 1995). It is also worth noting that some homeodomain proteins, such as En and Even-skipped (Eve), normally possess alanines at position 7. Hence, our substitution of Thr 263 with Ala is a conservative one that is unlikely to exert its effect at the level of general homeodomain structure. This is consistent with our findings that this substitution

Figure 19. Comparison of homeodomain N-terminal sequences. Amino acid sequences at the beginning of several homeodomains are shown for comparison. The PKA consensus ($RX_{1-2}T/S$), including T263, is found in several other homeodomains. This includes the POU class where this residue has been shown to be a substrate for PKA. In a more divergent homeodomain subclass, the corresponding residue is an alanine, further demonstrating the conservative nature of this substitution.

Ftz	DSKRTRQ**T**YTR
Antp	ERKRGRQ**T**YTR
Ubx	LRRRGRQ**T**YTR
Prd	KQRRCRT**T**FSA
Scr	ETKRQRT**S**YTR
I-POU	AGEKKRT**S**IAA
Pit-1	RKRKRRT**T**ISI
Oct-1	RRRKKRT**S**IET
En	DEKRPRT**A**FSS
Eve	SVRRYRT**A**FTR

has no apparent effect on DNA-binding activity, transactivating activity (in S2 cells), protein stability or subcellular localization.

2.3.7 Identity of the T263 Kinase

For Pit-1, the serine in the analogous position of Ftz T263 could be phosphorylated *in vitro*, either by PKA or an M-phase-specific kinase (Kapiloff et al., 1991, Caelles et al., 1995). However, PKA-dependent phosphorylation could not be verified in cultured cells (Okimura et al., 1994; Fischberg et al., 1994). As with Pit-1, phosphorylation of Ftz T263 may or may not be mediated by PKA *in vivo*. Biochemical and genetic approaches to test this relationship (data not shown) have provided ambiguous results: cultured cells failed to provide enough metabolically labeled Ftz for analysis, and results obtained with mutations in the PKA catalytic subunit gene *DCO* (Kalderon and Rubin, 1988; Lane and Kalderon, 1993) have been difficult to interpret, due in part to the pleiotropic nature of the *DCO* mutant phenotype.

2.3.8 Homeodomain Proteins and Phosphorylation

All homeodomain proteins tested thus far are phosphorylated (Krause et al., 1988; Gay et al., 1988; Krause and Gehring, 1989; Odenwald et al., 1989; Tanaka and Herr, 1990; Gavis and Hogness, 1991; Lopez and Hogness, 1991; Segil et al., 1991; Kapiloff et al., 1991; Wall et al., 1992; Ronchi et al., 1993; Rosfjord et al., 1995; Bourbon et al., 1995; Caelles et al., 1995; Coqueret et al., 1996; Zannin et al., 1996; Zwilling et al., 1997; Jaffe et al., 1997). However, studies testing the relevance of these phosphorylation events are relatively few. For the vertebrate homeodomain proteins (Segil et al., 1991; Kapiloff et al., 1991; Fischberg et al., 1994; Okimura et al., 1994; Van Renterghem et al., 1995; Caelles et al., 1995; Coqueret et al., 1996; Zannini et

al., 1996; Yan and Whitsett, 1997; Poleev et al., 1997) tests have only been conducted *in vitro* and in cultured cells, often with conflicting results (Kapiloff et al., 1991; Fischberg et al., 1994; Okimura et al., 1994; Caelles et al., 1995). In the case of *Drosophila* homeodomain proteins, four have been tested for the effects of phosphorylation. Ultrabithorax (Ubx) was shown to exist as multiple-phosphate isoforms *in vivo*, but functional significance could not be ascertained (Gavis and Hogness, 1991; Lopez and Hogness, 1991). Bicoid phosphorylation levels were shown to correlate with activity of the *torso/D-raf* signaling cascade (Ronchi et al., 1993). Phosphorylation of sites mapped on the En protein enhanced DNA binding affinity by ~2-fold *in vitro* (Bourbon et al., 1995). More recently, several CKII sites on the Antp protein were found to affect activity both *in vitro* and *in vivo* (Jaffe et al., 1997). These sites were distributed outside of the homeodomain.

This is the first study where the functional relevance of a phosphorylation site within the highly conserved homeodomain has been investigated *in vivo*. We have shown that T263 is crucial for Ftz function in developing embryos and, unlike other homeodomain protein sites, is likely to be phosphorylated when the protein is active. The conservation of this site in other homeodomain proteins suggests that many of these sites will also be phosphorylated, and that phosphorylation at this position may be a general mechanism for modulating homeodomain protein activities.

2.4 Materials and Methods

2.4.1 Site-directed Mutagenesis and Construction of Ftz Expression Vectors

Site-directed mutagenesis was performed using *Pfu* DNA polymerase (Stratagene) following the instructions provided by the manufacturer, and using the template plasmid pGemF1

(Krause et al., 1988). The T263A and T263D mutations were introduced in by two step PCR mutagenesis. The T263A mutation was generated using the primer HK9 (CCCGTCAGGCGTACACC) together with a T3 primer (Pharmacia Biotech Inc.), and the T263D mutation using primers HK16 (CGGGTGTAATCCTGACGGG) and HK17 (GCCTCCGAGGATGTCGACTACTTGG). In the second step, T3 or HK17 primers were used as appropriate, with the first round PCR products and the same DNA template to generate DNA encoding the amino two thirds of the protein. Amplified fragments were gel-isolated, digested with *Sal* I and subcloned into pBlue*ftz* (Furukubo-Tokunaga et al., 1992). Orientation and sequences were verified by dideoxynucleotide sequencing. *ftz* containing *Xmn*I fragments were then removed from the recombinant pBlue*ftz* plasmids and subcloned into pBKMftzGΔX (Furukubo-Tokunaga et al., 1992) to reconstitute the full-length *ftz* open reading frame together with necessary 5' and 3' promoter regions. L. Hung made the T263A P-element construct.

ftz full-length and ΔHD bacterial expression constructs have been previously described (Krause et al., 1988; Hyduk and Percival-Smith, 1996). To construct T263 mutant homeodomain constructs, PCR was used to amplify the homeodomain portions of the wild type and mutant pBKM*ftz*G plasmids described above. The 5' primer used was GATGGATCCCGACTCGAAACGCACCCG and the 3' primer was GCCGGATCCTACGTGCGATCCTTCTTCG. The products contain *Bam*H1 sites at each end and encode four non-homeodomain residues, Gly-Leu-Asp-Pro, at amino terminal end of the homeodomain. The PCR products were treated with *Bam*H1 and then placed under T7 promoter control in the vector pET-3b. Positive clones were verified by DNA sequencing.

2.4.2 Expression and Partial Purification of Ftz Polypeptides

Ftz full-length, ΔHD and homeodomain polypeptides were expressed in BL21:DE3 pLysS cells (Rosenberg et al., 1987). Cells were grown at 37°C to an A_{600} of 0.6 to 0.8, induced by addition of 0.4 mM IPTG and grown for a further 2-3hr. Cells were pelleted, resuspended in PBS + 0.1% Triton X100 and sonicated. Pellets were resuspended in guanidine buffer (6M Guanidine HCl, 50 mM Tris pH 8.0), sonicated and centrifuged once again. An equal volume of cold isopropanol was added dropwise to the supernatant, and the sample was spun again to remove nucleic acids. 4 volumes of cold methanol were then added to precipitate Ftz polypeptides. Pellets were dissolved in guanidine buffer and then run over Centricon size exclusion columns (Amicon, Inc.). Full-length protein was run over a Centricon 30 column equilibrated in 20mM Tris, pH 8.0, 0.5 M NaCl, 1mM EDTA and 10% glycerol. Concentrated protein was diluted in the same buffer (2 ml) and spun two more times prior to recovery and addition of glycerol to 20% final concentration. Homeodomain polypeptides were purified similarly, except that they were run over Centricon 10 columns first, and then the flow-throughs concentrated on Centricon 3 columns. Final protein concentrations were approximately 0.5 mg/ml, and proteins were 50-90% pure.

2.4.3 *In vitro* Kinase Assays

Drosophila 0-6 hr embryo whole cell extract (kindly provided by S. Mason in D. Bentley's lab, Amgen, Toronto) was prepared according to the method of Kamakaka et al. (1993). Kinase assays were performed following protocols provided by Upstate Biotechnology, Inc. 30μl reactions contained 5mM MOPS, pH7.2, 8 mM β-glycerol phosphate, 1.5mM EGTA, 0.3mM Sodium orthovanadate, 0.3mM dithiothreitol, 25mM $MgCl_2$, 150 μM ATP, 10μCi [γ-^{32}P]ATP (4500 Ci/mmol, ICN radiochemicals) and 1μl embryo extract (5mg/ml). Where

indicated, 100 ng full-length or deleted Ftz polypeptide, 10μl inhibitor cocktail (6μM PKC inhibitor peptide, 6μM Cam PK inhibitor) and/or 10μM cAMP were added. For PKA reactions, 0.5μg PKA catalytic subunit cocktail (Upstate Biotechnology, Inc) was added in the place of embryo extract. Reactions were incubated at 30°C for 10 min and stopped by the addition of SDS PAGE loading buffer and boiled for 5 min. Proteins were resolved on a 12% polyacrylamide gel, transferred to nitrocellulose and detected by autoradiography.

2.4.4 Phosphopeptide Mapping and Phosphoaminoacid Analysis

Wild-type and mutant ^{32}P-labeled Ftz homeodomains were separated on 15% PAGE gels and blotted to nitrocellulose. Labeled bands were excised from the membrane and tryptic fingerprinting performed according to the method of Boyle et al. (1991). Labeled phosphopeptide spots were scraped from the thin layer chromatography plates and the peptide eluted with 100-200μl fresh 0.05M NH_4HCO_3. Peptides were then immobilized on a Sequelon disk and subjected to manual Edman degradation using a Millipore protein sequencing kit (Sequelon AA) as described by the manufacturer.

2.4.5 Construction of P-element Rescue Vectors

ftz containing *Kpn* I fragments from wild-type and T263 mutant pBKMftzG plasmids were subcloned into the P-element construct pC20K (Rubin and Spradling, 1983; Hiromi et al., 1985; Furukubo-Tokunaga et al., 1992) which contains the marker *rosy*. P-element mediated germ line transformation was as described (Spradling and Rubin, 1982; Rubin and Spradling, 1982). Four independent, homozygous viable, second chromosome insertion lines were isolated

for each construct. These were crossed to *ftz*^{9H34}/TM3 and *ftz*^{w20}/TM3 flies to generate P[ftz];*ftz*^{9H34}/TM3 and P[*ftz*];*ftz*^{w20}/TM3 stocks, which in turn were mated to give P[*ftz*]; *ftz*^{9H34}/*ftz*^{w20} offspring. The TM3 balancer used was marked with a *hunchback-lacZ* containing reporter gene to allow unambiguous identification of homozygous mutant offspring (Driever et al., 1989).

2.4.6 Cuticle Preparations, *In situ* Hybridization and Immunolocalization

Cuticle preparations were as described by Manoukian and Krause (1992). Double *in situ* hybridization (Lehmann and Tautz, 1994)/antibody detection, to visualize *ftz, en* or *wg* transcripts together with *hunchback* directed β-galactosidase expression, was performed as described in Manoukian and Krause (1992). Vectors and probes for *ftz, en* and *wg in situ* hybridization were as described (Copeland et al., 1996; Guichet et al., 1997). Ftz protein was detected as previously described (Krause et al., 1988) using a Ftz polyclonal antiserum and the ABC Kit from Vectastain.

2.4.7 Electrophoretic Mobility Shift Assays

In vitro translations of wild type and mutant Ftz homeodomains were performed using the TNT T7 Coupled Reticulocyte Lysate System (Promega) and unlabeled amino acids. After translation, proteins were separated on a 15% PAGE gel and quantitated by enhanced chemiluminescence (ECL)-Western blotting and Phosphorimager analysis. Equal amounts of protein were used for the electrophoretic mobility shift assays (EMSAs), performed as described by Percival-Smith et al (1990) using the double-strand DNA substrate GGGAAGCAATTAAGGATCCC (Percival-Smith et al., 1990; Pick et al., 1990).

2.4.8 Transient Transfection Assays

For cell culture transfection assays, cDNAs encoding *ftz* wild-type and mutant proteins were subcloned into the expression vector pPac (Krasnow et al., 1989). PKA expression was achieved using the plasmid pAct-DC0 described by Norris and Manley (1992). Schneider line-2 (S2) cells were grown at 25°C in Schneider medium (Gibco) containing 5% fetal bovine serum (heat inactivated, BRL). DNA transfections were performed using a CellPhect transfection kit (Pharmacia) and the reporter 3K'-TATA-CAT (Han et al., 1989). DNA precipitates contained 0.5-2 μg pPacFtz, as indicated, 1 μg reporter plasmid, 1 μg pAct-DC0 where indicated and enough pPac plasmid to make 3 μg total DNA. Following transfection and cell growth, as described in the CellPhect kit directions, cell extracts were prepared by freezing and thawing three times and pelleting cell debris using a 1 min centrifugation at 12,000 rpm. Levels of CAT expression were determined by Western blotting with an anti-CAT monoclonal antibody (5 Prime-3 Prime, Inc), detection using an enhanced chemiluminescence kit (Boehringer Mannheim) and quantitation using a BioRad Phosphorimager. Levels of Ftz in the same samples were determined similarly except that anti-Ftz antiserum (Krause et al., 1988) was used instead of anti-CAT antibodies.

Chapter 3: Tissue-specific requirements for a phosphorylation site in the Fushi tarazu homeodomain

Submitted by Jianli Dong and Henry M. Krause to *Development, Gene, and evolution.*

3.0 Abstract

The homeodomain protein Fushi tarazu (Ftz) is required for several embryonic patterning processes including segmentation and neurogenesis. During the stages that these processes are regulated, the protein is differentially phosphorylated, suggesting that phosphorylation may be playing a role in helping the protein to regulate different functions in different tissues. I showed in Chapter 2 that one of the Ftz phosphorylation sites, a protein kinase A-type site in the N-terminal arm of the homeodomain, is required for normal Ftz-dependent segmentation. Here, I test if phosphorylation of this site (Thr263) is also required in the developing central nervous system (CNS). A well-established role for Ftz in the CNS is for the differentiation of neurons referred to as RP2 neurons. Absence of Ftz expression in these cells causes a failure of certain target genes to be expressed and subsequent defects in RP2 differentiation. In contrast to their effects on segmentation, I find that mutation of Thr263 to either Ala or Asp has no effect on these functions. This suggests that the phosphorylation state of this site is irrelevant for Ftz function in the CNS, and that the requirement for Thr263 phosphorylation is tissue-specific.

3.1 Introduction

The *Drosophila* Fushi tarazu protein (Ftz) is a homeodomain-containing transcription factor (Jaynes and O'Farrell, 1988; Laughon and Scott, 1984). In the developing embryo, it acts as both an activator and repressor of transcription (Ingham, et al., 1988; Copeland, et al., 1996). The protein is expressed during three different stages of embryogenesis (Carroll and Scott, 1985; Krause, et al., 1988); first in seven zebra-like stripes that encircle the embryo, then in a subset of differentiating neurons of the central nervous system (CNS) and finally in parts of the gut and posterior ectoderm. During its first phase of expression, Ftz defines alternate segmental regions referred to as even-numbered parasegments (Wakimoto, et al., 1984; Lawrence, et al., 1987). In the second phase, it controls the differentiation of a subset of neurons (Doe, et al., 1988a). Whether the protein has an important function during its last phase of expression remains to be tested.

During the three phases of Ftz expression, the protein is heavily phosphorylated, with isoforms containing an average of 8-9 phosphates per molecule, and as many as 16-18 phosphates per molecule (Krause, et al., 1988; Krause and Gehring, 1989). Interestingly, it has been shown that the number and position of phosphates varies during the first two phases of Ftz expression (Krause and Gehring, 1989). This has prompted the suggestion that this tissue-specific phosphorylation may play an important role in changing the regulatory properties and roles of the protein during its three different phases of expression (Krause and Gehring, 1989).

The ability of phosphate groups to change transcription factor activities is now well documented. Examples of altered properties include changes in stability, subcellular localization, DNA binding affinity and the ability to interact with other proteins (reviewed in: Hunter and

Karin, 1992; Karin, 1994). Virtually all homeodomain proteins tested exist as phosphoproteins. However, very few studies have investigated the roles of these post-translational modifications, and only two have carried out analyses *in vivo* (Jaffe, et al., 1997; Dong, et al., 1998). As shown in Chapter 2, a site in the Ftz homeodomain could be phosphorylated by cyclic AMP-dependent protein kinase (PKA) *in vitro*, and that phosphorylation of this site appeared to be required for function during the first phase of Ftz expression. This was shown by changing the phosphorylated site, threonine 263 (Thr263), to either an alanine (Ala) or an aspartate (Asp) residue. Ala substitutions are conservative mutations for sites that are not phosphorylated, whereas Asp substitutions are excellent mimics of constitutively phosphorylated sites (see for example: Jaffe, et al., 1997; Kowlessur, et al., 1995; Napper, et al., 1996). When tested for rescuing activity, the Ala-substituted Ftz proteins yielded loss of function segmental phenotypes, whereas the Asp-substituted proteins yielded segmentally normal embryos and viable adults (Dong, et al., 1998).

My previous study (Dong, et al., 1998) did not address whether the effects of the Thr263Ala mutation also cause defects in the developing nervous system or gut. It is possible that the requirement for phosphorylation of this site is tissue-specific, in which case the Ala mutation might have no effect in these other tissues. It is also possible that phosphorylation of Thr263 may be detrimental to the activity of the protein at these later stages, since the Asp mutated protein did not rescue embryos as well as the wild-type protein (Dong, et al., 1998).

This study addresses the role of Thr263 phosphorylation in the CNS. I use the differentiation of RP2 neurons as our assay of Ftz activity. These neurons are the only Ftz-expressing neurons that have been shown to be affected by mutations in the *ftz* gene (Doe, et al., 1988a). In *ftz* mutant embryos, they fail to express the segmentation genes *even-skipped* and

Ultrabithorax, and consequently, their axons are routed in the wrong direction (Doe, et al., 1988a). Here, I find that both the Thr263Ala and Thr263Asp mutations have no effect on this Ftz activity, suggesting that the role of Thr263 phosphorylation is tissue-specific.

3.2 Results and Discussion

3.2.1 Ftz Thr263Ala Embryos Have Normal Eve Staining in RP2 Neurons

Segmentation defects caused by loss of *ftz* expression in the blastoderm lead to indirect patterning defects in the CNS (Doe, et al., 1988a). These indirect effects make it difficult to interpret defects that may be due specifically to loss of Ftz expression in the CNS. Fortunately, the Thr263Ala phenotype varies significantly in terms of the penetrance and severity of segmental defects (Dong, et al., 1998). Most progeny are missing several *ftz*-dependent segments, but the number and identity vary from individual to individual. At the extremes, a small percentage of embryos develop normally, while a few resemble *ftz* nulls. Thus, the ability of the protein to function normally appears to be dependent on genetic or epigentic factors that vary from segment to segment and embryo to embryo. This variability allowed me to examine the ability of Ftz Thr263 mutant proteins to rescue CNS development in regions of the embryo where segmentation was normal. If phosphorylation of Thr263 is required in the CNS as it is in the blastoderm, then we would expect to find neurons in regions that are segmentally normal that fail to differentiate normally when rescued with the Ftz Thr263Ala mutant protein. Conversely, the protein may have to be unphosphorylated to mediate RP2 differentiation, in which case the Ftz Thr263Asp protein would fail to rescue the RP2 phenotype. Consistent with this possibility, Ftz Thr263Asp-rescued embryos fail to produce adults with the same efficiency as animals rescued

by a wild-type Ftz protein, even though they have no detectable defects in segmentation (Dong, et al., 1998).

En staining was used to assess embryo segmentation in Thr263Ala- and Thr263Asp-rescued embryos. Embryos that exhibited minor segmental defects were examined in regions that were segmentally normal for irregularities in the patterning of *ftz*-dependent RP2 neurons. RP2 neurons were identified by immunofluorescence using Eve-specific antibodies. Figure 20 shows En and Eve expression patterns in wild-type and Ftz Thr263Ala-rescued embryos. Panel A shows the 14-stripe pattern of En in the ectoderm of a stage 16 (16 hour old) wild-type embryo. Panel B shows the pattern of Eve expression in the developing CNS of the same embryo. Arrows in the double-exposure (C), and the close-up (D), indicate the positions of RP2 neurons. Two RP2 neurons are present along the ventral midline of each segment. Approximately one quarter of the embryos derived from the Thr263Ala; ftz^- cross (150/642) were ftz^-, exhibiting variable defects in Ftz-dependent (even-numbered) En stripes, as previously reported (Dong, et al., 1998). The remaining three-quarters (492/642) were normal in segmentation and in CNS patterning.

Of the embryos that did have segmental defects, no RP2 defects were observed in the regions where segmentation was normal. Panel E) shows a typical Ftz Thr263Ala-rescued embryo stained for En. The arrowhead shows a disruption in En stripe #8. Segmentation in the rest of the embryo is essentially normal. In these regions, the Eve-marked RP2 cells are all present and positioned normally (panels F-H). Forty Ftz Thr263Ala partially rescued embryos, ranging in severity of segmental defects, were carefully examined in this manner, and none were found with abnormal RP2 neurons in regions that were segmentally normal. Indeed, normal RP2 neurons were often observed in *ftz*-dependent segments containing partial deletions, further

suggesting that the two *ftz*-dependent processes are functionally distinct, and that Ftz T263 phosphorylation is not required in the CNS.

3.2.2 RP2 Neurons Are Unaffected in Ftz T263D-rescued Embryos

Embryos rescued with Ftz Thr263Asp have fewer survivors than embryos rescued by the wild-type protein (Dong, et al., 1998). However, they exhibit no obvious segmental defects, suggesting the possibility that it may be the CNS-specific roles of the protein that are disrupted in these embryos. Figure 21B shows a homozygous ftz^{9H34} mutant embryo from the *ftz* Thr263Asp rescue cross, stained for expression of Eve in the CNS. Homozygous *ftz* mutant embryos were identified by lack of β-Galactosidase expression (see Materials and Methods). All of the RP2 neurons in the embryo shown are present and normally positioned (indicated by arrows). 340 embryos were examined in total. Although 2/340 embryos did show loss of Eve staining in either an aCC/pCC or RP2 neuron, this frequency of aberrant staining (<1%) was also observed in wild-type embryos.

3.2.3 Tissue-specific Requirements for T263 Phosphorylation

My previous analysis (Dong, et al., 1998) showed that replacement of Thr263 with an alanine residue results in loss of the ability of Ftz to regulate target genes required for *ftz*-dependent segmentation, resulting in the loss of *ftz*-dependent segments. The ability of a Ftz protein with a non-conservative aspartate residue at the same position, to fully rescue this phenotype, suggested that this site is normally phosphorylated when the protein carries out these functions. In contrast, the results provided here suggest that Ftz does not need to be phosphorylated at this position for function in the CNS. Indeed, the lack of an effect by either an

alanine or aspartate substitution suggests that the phosphorylation status of this site is irrelevant for Ftz function in this tissue.

There are, however, several caveats to consider with this finding. First, relatively little is known about the requirements of Ftz in the CNS. Although Ftz is expressed in many different types of differentiating neurons, and over the course of about 6 hours, its only known role thus far is for the proper differentiation of RP2 neurons (Doe, et al., 1988a). It is quite likely that Ftz is required for the differentiation of at least a subset of the other Ftz-expressing neurons, and that appropriate assays to detect these roles have yet to be developed. If so, then Ftz Thr263 phosphorylation may be important for these processes. Nevertheless, I can say that at least one CNS function, the differentiation of RP2 neurons, does not appear to require phosphorylation of this site.

Another possible caveat is that the random ability of the Ftz Thr263Ala mutant protein to rescue some segments in some embryos is somehow connected to the later functions of the protein in the CNS. Variations in the cellular environment that facilitate function in the blastoderm may be propagated to analogous regions of the developing CNS, ensuring function there as well. However, the time separating these different processes, and the differences in Ftz activities and environments in the two very different tissues, make this possibility seem somewhat unlikely.

Finally, I cannot exclude the possibility that we are examining processes with slightly different thresholds or sensitivity levels. For example, the earlier actions of Ftz depend on an ability of the protein to autoregulate the transcription of its own gene, beginning with low levels of initial expression. In contrast, Ftz expression in the CNS is not dependent on autoregulation. If the Thr263Ala mutant protein had a somewhat reduced level of activity, it may be unable to

autoregulate at low levels, but may be capable of many other functions such as the regulation of RP2 differentiation.

3.2.4 What is the Cause of T263D Lethality?

The lower survival numbers of Ftz Thr263Asp-rescued embryos, versus embryos rescued with a wild-type protein (2-25% versus 20-70%; Dong, et al., 1998), raises the question of when and why the Thr263Asp-rescued flies are dying. One possibility is that the defects early in development are outwardly quite minor, and that I have simply been unable to detect them. Minor defects may have significant ramifications as the developing flies progress through three larval instars and metamorphosis. As discussed above, defects may occur in other differentiating Ftz-expressing neurons that I was not able to follow due to lack of known functions and assays. Another possibility is that phosphorylation of this site, and/or the aspartate substitution, may be detrimental for other Ftz-dependent functions at other stages of development. For example, Ftz is expressed once again after expression in the CNS in parts of the developing gut and posterior ectoderm. It is possible that Thr263 must be unphosphorylated for proper Ftz function in these tissues.

3.2.5 Phosphorylation as a Means of Switching Tissue-specific Homeoprotein Activities

Phosphorylation would be an ideal mechanism for switching a protein's activities so that it could perform different functions in different cell types. However, there is no formal proof, of which we are aware, that phosphorylation can change the activity of a regulatory protein in a tissue- or developmentally-specific manner. My results suggest that phosphorylation of Ftz Thr263 does have a tissue- and stage-specific function, although it does not appear to be acting as

a simple switch. Phosphorylation appears to be required for the early segmental role of Ftz, but appears to be irrelevant for Ftz function in the CNS. The lethality of the Thr263Asp mutant protein, however, suggests that the unphosphorylated state is also important for function somewhere or sometime. Ftz is also phosphorylated at many other sites (perhaps as many as 20), also in a tissue- and stage-specific fashion (Krause and Gehring, 1989). It is quite possible that some of these other sites will also function as switches of protein activity and functions.

3.2.6 Molecular Functions of Ftz Affected by Thr263 Phosphorylation

Threonine 263 is located in the N-terminal extension of the Ftz homeodomain. This region has been shown to be important for other homeodomain-protein interactions (Lin and McGinnis, 1992). Indeed, the results of our previous analysis (Dong, et al., 1998) suggested that phosphorylation of Thr263 is required for Ftz to interact with an embryonically expressed cofactor. However, this cofactor has yet to be identified. The results of *in vitro* studies suggest that interactions with two previously identified cofactors, Paired (Copeland, et al., 1996) and Ftz-F1 (Guichet, et al., 1997), are not affected by phosphorylation of this site (J.D. and H.M.K., unpublished data). However, these studies were performed with the homeodomain alone, which by itself showed no ability to interact with these proteins. It is therefore still possible that phosphorylation of Thr263, in the context of a full-length Ftz protein, could affect these interactions. Phosphorylation would be a rapid and convenient mechanism for regulating these or other protein-protein interactions. These regulated interactions, in turn, may control the protein's activity or specificity in a tissue- or stage-specific fashion.

3.3 Materials and Methods

3.3.1 Fly Strains

Homozygous lines carrying the wild-type, Thr263Ala or Thr263Asp *ftz* rescue constructs on second chromosome have been previously described (Dong et al, 1998). These lines were maintained as strains carrying the *ftz* null allele, ftz^{9H34}, on the third chromosome balanced over an *actin-lacZ-* marked TM3 balancer chromosome. One quarter of the progeny are ftz^{9H34} homozygotes, with *ftz* activity provided by homozygous *ftz* trans-genes located on the second chromosome. Three transgenic lines were tested for each of the three *ftz* constructs (wild-type, Thr263Ala and Thr263Asp). Flies were maintained at 25°C.

3.3.2 Embryo Collection, Fixation and Immunostaining

Eggs were collected for 4 hr and aged for 13 hr at 25°C prior to fixation. Immunofluorescent staining was performed using anti-Eve antibodies (provided by M. Frasch) at a dilution of 1:5000 and an anti-En monoclonal antibody (provided by N. Patel) at a dilution of 1:5. Secondary antibodies (both from Jackson laboratories) were Cy3-conjugated goat anti-rabbit IgG diluted to 1:400 and FITC-conjugated goat anti-mouse IgG diluted to 1:100. Homozygous ftz^{9H34} embryos were identified by lack of β-galactosidase expressed from an *actin* promoter-driven lacZ gene located on a marked TM3 balancer chromosome. β-Gal was detected using an anti-β-Gal monoclonal antibody (Promega) diluted to 1:2000. Images were captured using a Zeiss Axioplan microscope and a Princeton CCD camera, and then processed using Adobe PhotoShop.

Figure 20. Eve expression in wild-type and Thr263Ala mutant embryos. The 16 hour-old wild-type (A-D) and Ftz Thr263Ala-rescued (E-H) embryos are stained for either En (A, E), Eve (B, D, F, H) or both (C, G). The wild-type embryo in (A) shows the normal pattern of En stripes. The white arrow heads in E, G) show abnormal En expression in the Ftz Thr263Ala-rescued embryo. Eve expression in (B) shows the normal pattern of RP2 (white arrows) aCC/pCC and other Eve-expressing neurons. Panel (D) shows a close-up of the region boxed in (B). Panel H) shows that RP2 neurons are also present and located normally in the analogous region of the Ftz Thr263Ala embryo.

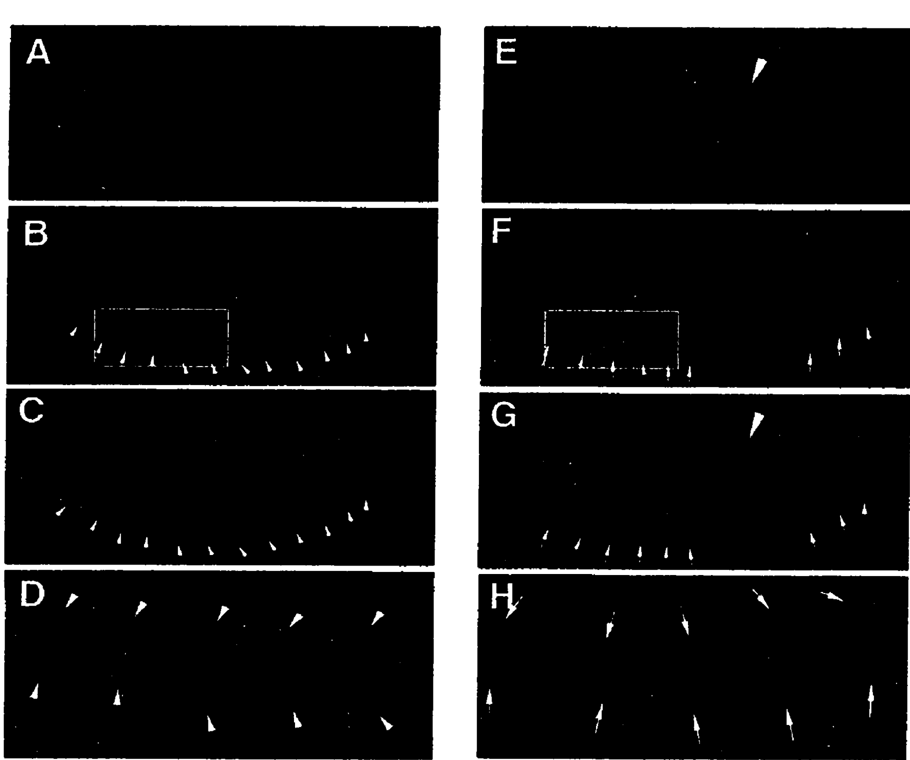
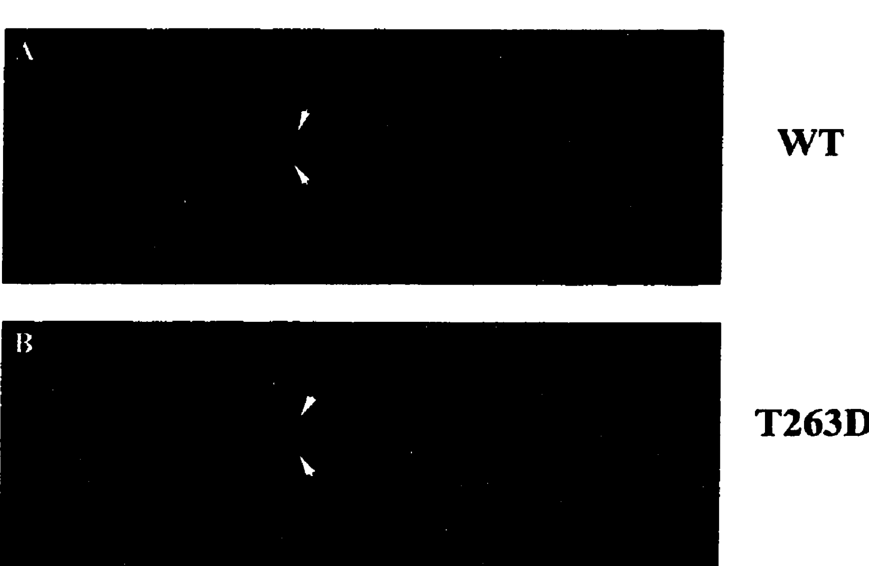

A
B
C
D
E
F
G
H

Figure 21. Eve expression in wild-type and Thr263Asp mutant embryos. Eve expression is shown in a wild-type (A) and a Thr263Asp-rescued *ftz* mutant (B) embryo. As shown in Figure 20, two RP2 neurons are present near the ventral midline in each segment of the wild-type embryo (A). The same is true of the Ftz Thr263Asp-rescued embryo shown in B).


A
WT
B
T263D

Chapter 4: Discussion and Future Directions

4.1 Summary

The homeobox gene *fushi tarazu* is temporally expressed in three different tissues during *Drosophila* embryogenesis: the blastoderm, CNS and hindgut. The *ftz* gene is required for the formation of alternate segments in the blastoderm and for the differentiation of RP2 neurons in the CNS. Its function in gut development is unclear. Ftz is heavily phosphorylated in the embryo; as many as 16-20 phosphoisoforms exist. This phosphorylation is stage-specific: different phosphoisoforms are formed in the blastoderm and in the CNS (Krause et al. 1988; Krause and Gehring, 1989). The correlation between stage-specific activities and differential phosphorylation suggests that phosphorylation may provide a rapid and reversible mechanism to modulate tissue-specific Ftz activities.

I have provided the first experimental support for the hypothesis that phosphorylation is important for tissue-specific modulation of Ftz activity. Threonine 263, in the N-terminus of the Ftz homeodomain, is phosphorylated *in vitro* by *Drosophila* embryo extracts and by PKA. The importance of this phosphorylation site was examined by mutating it to Ala and Asp to mimic the unphosphorylated and constitutively phosphorylated states. The activities of these mutated proteins were tested in a *ftz*$^-$ rescue experiment in developing embryos. This *in vivo* rescue assay is the most relevant and comprehensive test of Ftz functions possible. The results show that the *ftz*^{T263A} allele cannot rescue *ftz*$^-$ flies to adulthood. It generates a range of hypomorphic pair-rule defects that vary in severity. The *ftz*^{T263D} allele, on the other hand, rescues up to 25.4% of *ftz*$^-$ flies to adulthood and has no effect on segmental patterning.

As a homeodomain-containing protein, Ftz functions as a transcriptional regulator of various target genes. I analyzed the expression of three known *ftz* pair-rule targets, *ftz*, *en*, and *wg*. The expression patterns of these genes were consistent with T263A acting as a hypomorphic

mutation and with T263D functioning normally. These results suggest that Ftz T263 is phosphorylated *in vivo* and that the phosphorylated form is required for the function of Ftz in the blastoderm. Both mutations affecting T263, however, showed normal Ftz activity in RP2 neurons in the CNS, indicating that T263 phosphorylation may be irrelevant for Ftz function in the CNS and suggesting a stage-specific requirement for phosphorylation of Ftz T263.

The post-translational modification of transcription factors by phosphorylation can affect their activities through a variety of diverse mechanisms (Hunter and Karin, 1992; Karin, 1994). Biochemical analysis of T263 mutant proteins showed that protein stability, subcellular localization, DNA binding activity and basic transcriptional activity are all unaffected. I therefore propose that T263 phosphorylation acts by modulating Ftz interactions with a blastoderm-expressed cofactor. This cofactor has yet to be identified.

4.2 Discussion

4.2.1 Ftz T263A is Defective in Segmentation

As discussed in 2.3.4, it seems that one of the mutant defects in the Ftz T263A protein is an inability to autoregulate. Ftz regulates its own expression by binding to its upstream enhancer. This activity also involves Ftz cofactors (Pick et al., 1990, Schier and Gehring, 1992, 1993b, Guichet et al., 1997; Yu, et al., 1997; Han et al., 1998). The phenotypes of T263A mutant embryos are remarkably similar to those of embryos homozygous for mutations in the Ftz homeodomain that were described by Furukubo-Tokunaga et al. (1992) and by Schier and Gehring (1992). In those embryos, the hypomorphic defects were attributed to decreases in *ftz* autocatalysis. Hence, these phenotypes appear to have a common basis, although the molecular mechanisms involved need not necessarily be the same (e.g. DNA binding versus protein-protein

interactions). It is worth mentioning that the DNA sequences involved in *ftz* autoregulation (Schier and Gehring, 1992, 1993b) can be found in other genes, for instance, in the first intron of *en*, which is also a regulatory target of *ftz* (Kassis, 1990; Florence et al., 1997). Therefore, we cannot exclude the possibility that other *ftz* target genes are affected directly by the T263A mutation, and that these effects are not just secondary effects resulting from decreased levels of Ftz.

In Ftz T263A mutant embryos, the absolute intensity of *ftz* expression is generally decreased (Figure 15). Neither the alanine nor the aspartate mutation caused *ftz* expression outside its normal domains (e.g. the anterior and posterior ends of the embryos and interstripes), indicating that T263 phosphorylation regulates the level of *ftz* expression, not its spatial pattern. This is consistent with the requirement of Ftz for enhancement and maintenance of *ftz* striped expression, and not for stripe initiation and definition (Dearolf et al., 1990; Yu and Pick, 1995). Thus, T263 phosphorylation appears to control quantitative rather than qualitative differences in *ftz* expression in blastoderm embryos.

4.2.2 Ftz T263 Phosphorylation is not Required in the CNS

There are several differences in *ftz* regulation and activity in the blastoderm versus in the CNS. First, the spatial expression patterns are different; 7 stripes in the blastoderm, and 14 stripes in the CNS (Krause et al., 1988). Second, the *cis*-regulatory elements for *ftz* transcription in the two tissues are different: the neurogenic element is required in the CNS but not in the blastoderm, whereas the upstream element is required in the blastoderm and not the CNS. Notably, autoregulation does not occur in the CNS (Hiromi et al., 1985; Hiromi and Gehring, 1987; Doe et al., 1988a). Third, Ftz activities in the two tissues are different. For instance, in

segmentation, Ftz and Eve repress each other to define the segmental boundaries, whereas in the CNS Ftz activates Eve to support the differentiation of the RP2 neurons (Doe et al., 1988a, 1988b). Fourth, consistent with the above differences, the phosphoisoforms of Ftz are different in the two tissues (Krause and Gehring, 1989). Here, I have provided the first evidence of a stage-specific requirement for phosphorylation in the regulation of a homeodomain protein.

The existence of stage-specific Ftz phospho-isoforms may reflect another layer of control during development that has allowed fine turning of the homeobox regulatory system. By making use of the same *ftz* gene, and assigning unique functions through differential phosphorylation in each of the two tissues, different developmental processes can be controlled by the same gene product.

Cellular context is probably critical in determining the consequences of T263 phosphorylation. For example, a protein cofactor expressed in the blastoderm embryos may direct Ftz activities to unique sets of target genes. Interaction with this cofactor may depend on T263 phosphorylation. If such a cofactor is not present in the CNS, then T263 phosphorylation in the CNS might be irrelevant for Ftz function.

4.2.3 Molecular Consequences of Phosphorylation of Ftz T263

At this point, the molecular basis of the T263A defect is still unclear. However, my work does provide some insight and suggests that regulation of a Ftz cofactor interaction is the most likely explanation. As discussed in section 1.3.4, protein-protein interactions play an important role in the regulation of Ftz function. Prd and Ftz-F1 are the best studied Ftz cofactors (Ananthan et al., 1993; Copeland et al., 1996; Guichet et al., 1997; Yu et al., 1997; Florence et al., 1997; Han et al., 1998). However, both interactions involve Ftz domains other than the homeodomain

and do not require phosphorylation. It is possible that these interactions are affected by phosphorylation, but studies with mutant alleles that alter T263 indicated that this site is not involved (J.D. and H.M.K., unpublished observation). Other proteins that interact with Ftz could be affected by T263 phosphorylation. For instance, TFIIB (Colgan et al., 1993, 1995), Hairy, Eve and Sloppy-paired2 (Copeland and Krause, unpublished results), Ttk and Adf-1 (Han et al., 1998) are potential Ftz-interacting proteins, based on *in vitro* binding studies. These interactions may be important for Ftz activity and could be regulated by T263 phosphorylation.

T263 is in the N-terminus of the Ftz homeodomain. The N-terminal arms have been shown to be important for the functions of the *Antp*, *Scr*, *Dfd* and *Ubx* homeodomain proteins *in vivo* (Lin and McGinnis, 1992; Chan and Mann, 1993; Furukubo-Tokunaga et al., 1993, Zeng et al., 1993). Residue 7 of these homeodomains, including Ftz T263, was disordered in the 3D structure and was not shown to make direct contacts with DNA (reviewed in Gehring et al., 1994b; for Ftz see Qian et al., 1994). However mutations in the N-terminal arms of several homeodomains have been reported to affect homeodomain-DNA binding affinity (Percival-Smith et al. 1990) and specificity (Damante et al., 1996). The N-terminal arms of homeodomains also contact other proteins, resulting in changes in homeodomain-DNA binding and transcriptional activities (Zappavigna et al., 1994, 1996; Zwilling et al., 1995). Homeodomain-interacting proteins include MATα2 and Mcm1/**a**1 (see section 1.2.4.1), Exd/Pbx and HOM/Hox (see section 1.2.4.2), TBP (Msx-1, Eve: Zhang et al., 1996; Um et al., 1995), TFIIE (Antp, Abd: Zhu and Kuziora, 1996), Unc-86 (Mec-3: Xue et al., 1993), and HMG2 (Oct-1, Oct-2 and Oct-6: Zwilling et al., 1995). Some of these homeodomain-interacting proteins, or their homologues, have the potential to interact with other homeodomains, and these interactions could be under the influence of homeodomain phosphorylation.

Ftz, like many other homeodomain proteins, shows a relaxed DNA-binding selectivity *in vitro*, suggesting that functional specificity is achieved *in vivo* through mechanisms other than simple DNA binding. Ftz may use its homeodomain to associate with cofactors similar to those listed above. T263 phosphorylation may affect these interactions. T263 phosphorylation, and affected cofactor interactions, may ensure the highly specific recognition and regulation of *ftz* targets. The candidate proteins influenced by Ftz T263 phosphorylation could be co-activators or co-repressors. The interaction(s) could occur in either the presence or absence of DNA, and could affect either DNA binding or transcriptional activity *in vivo* (e.g. affect Ftz TFIIB interaction). Finally, T263 phosphorylation could regulate the Ftz-cofactor interaction either positively or negatively.

4.2.4 Is PKA the T263 Kinase?

Is PKA the kinase responsible for phosphorylating T263 *in vivo*? So far, a genetic relationship between PKA and *ftz* has not been established. The *Drosophila* gene encoding the catalytic subunit of PKA, *DC0*, has been cloned and analyzed (Kalderon and Rubin, 1988; Lane and Kalderon, 1993). *DC0* is a maternal effect gene and is important in oogenesis, embryogenesis, and larval development (Lane and Kalderon, 1993). Female flies heterozygous for two weak *DC0* alleles are fertile but produce offspring with defects in embryogenesis, including a variety of alterations in cuticular patterning. Flies zygotic null for DC0 die as morphologically normal first-instar larvae (Lane and Kalderon, 1993).

I have pursued a genetic approach to see if DC0 is involved in Ftz phosphorylation in the embryo. This was done by crossing *ftz* gain-of-function (GOF; ftz^{Ual} alleles), or loss-of-function (LOF; ftz^{47} and ftz^{56}) mutant males, with *DC0* temperature sensitive (ts; *B10* and *B12*) mutant

females. *DCO* ts alleles exhibit segmental defects when raised at non-permissive temperatures early in development (Lane and Kalderon, 1993). If PKA were required for Ftz activity, then LOF *ftz* alleles should enhance these *DCO*-specific defects, while GOF *ftz* alleles should suppress them. The results, in fact, were quite the opposite. The LOF *ftz* alleles suppressed the *DCO* phenotype, whereas the GOF ftz^{Ual} alleles enhanced the phenotype (data not shown). These results argue against DCO being the kinase that phosphorylates Ftz T263 *in vivo*. However, the situation may be complicated by additional target sites for DCO on the Ftz polypeptide (e.g. T269, Figure 6), or by other effects of this kinase on proteins that regulate Ftz activities (e.g. other kinases and Ftz cofactors). If DCO is not the kinase that phosphorylates Ftz at this site, then a kinase with similar substrate specificity should be present in the early embryo. A number of genes encoding kinases with high homology to the PKA catalytic subunit have been identified in *Drosophila* (e.g. *DC1* and *DC2*: Kalderon and Rubin, 1988). Such kinases may have the potential to phosphorylate Ftz T263, but the majority of these putative kinase genes have not been characterized in detail.

4.2.5 Are Residues at Position 7 Phosphorylated in Other Homeodomains?

So far, the phosphorylation of homeodomain residue 7 has been demonstrated in three proteins, Ftz, Pit-1 and Oct-1. We have shown that phosphorylation at this site is crucial for Ftz function in the developing embryo. Phosphorylation of Pit-1 at this site affects its DNA binding activities and protein interactions (Kapiloff et al., 1991; Fischberg et al., 1994; Caelles et al., 1995). Oct-1 phosphorylation at this position regulates its DNA binding activity. This phosphorylation is under the control of cell cycle-related kinases (Segil et al., 1991; Roberts et al., 1991). Comparison of known homeodomains, interestingly, shows that the residues in this

position are mostly either Thr/Ser or Ala, as shown in Figure 19. PKA has a preference for basic residues preceding the phosphorylation site. Its optimal recognition sequences are Arg-Arg/Lys-X-Ser/Thr>Arg-X2-Ser/Thr=Arg-X-Ser/Thr, where X is any amino acid. Since homeodomain residue 5 is a conserved Arg, Arg-X-Thr/Ser (residue 7) would make a reasonably good PKA site. It is tempting to speculate that phosphorylation of this residue is a general mechanism in the modulation of the Thr/Ser-7 containing homeodomains. If so, our *in vivo* and *in vitro* studies with Ftz T263 mutations may serve as a good paradigm for the regulation of other such homeodomain proteins.

The four cloned *ftz* homologues, *D. melanogaster ftz* (Kuroiwa et al., 1984; Weiner et al., 1984; Laughon and Scott, 1984), *D. hydei ftz* (Maier et al., 1990, 1993), *Tribolium ftz* (Brown et al., 1994), and *Schistocerca Dax* (divergent *Antennapedia* class homeobox gene: Dawes et al., 1994), have divergent homeodomain sequences (e.g. 13/60 residues are different between *D. melanogaster ftz* and *Dax*), yet residues 2 to 10 in their N-terminal arms are identical (except residue 9 in *D. hydei ftz* is a Ser instead of Thr). It will be interesting to see if the threonines at position 7 in the other three *ftz* homeodomains are also phosphorylated and required for protein activity.

4.2.6 Multiple Levels of *ftz* Regulation

Because of its critical patterning roles in *Drosophila* embryogenesis, the *ftz* gene is tightly regulated during embryogenesis. This regulation occurs at multiple levels, including gene transcription, stability of the gene products, protein-protein interactions and protein phosphorylation. These different layers of regulation are overlapping and interactive.

ftz is primarily controlled at the level of gene transcription. Multiple stage-specific protein complexes bind to *ftz* regulatory elements, as elucidated by footprints generated using *ftz* 5' *cis*-regulatory elements (Harrison and Travers, 1988, 1990; Dearolf et al., 1989a, 90; Pick et al., 1990; Ueda et al., 1990; Brown et al., 1991; Lavorgna et al., 1991; Topol et al., 1991; Han et al., 1993, 1998; Tsai and Gergen, 1995). It is likely that these factors are required to bind and to vacate their binding sites at precise times, in order to initiate, maintain and repress *ftz* transcription within particular spatial domains and temporal windows. The *ftz* enhancer is likely operating as an "enhanceosome" (Carey, 1998) by the formation of higher-order nucleoprotein complexes. The enhanceosome is a network of protein-DNA and protein-protein interactions, assembled depending on the arrangement of *cis*-acting elements and the complementary binding of transcription factors on an enhancer (Carey, 1998).

The second level of regulation is in the stability of *ftz* mRNA and protein. *ftz* RNA and protein expression patterns are constantly changing. Small differences in Ftz concentration have the potential to disturb embryogenesis (Kellerman et al., 1990). Therefore, the rapid degradation of *ftz* mRNA and protein is essential to eliminate *ftz* products in regions that do not need *ftz* in order to avoid detrimental effects, caused by ectopic expression.

The third level of regulation is achieved through protein-protein interactions. For example, in the absence of its cofactors Prd and Ftz-F1, Ftz cannot regulate its target genes *wg* and *en* (Copeland et al., 1996; Guichet et al., 1997). The regulation of Ftz by its cofactors can occur in different manners. First, interaction with cofactors may change Ftz homeodomain conformation, resulting in different DNA binding specificity. The DNA binding site of a Ftz/cofactor dimer is probably larger than a monomer Ftz binding site, thus additional specificity is achieved by binding to a more complex sequence. Second, Ftz and its cofactors may have

relatively low DNA binding affinity on their own. However, when they bind in a heterodimeric fashion, affinity may increase considerably, as observed for Ftz and Ftz-F1 (Yan et al., 1997; Han et al., 1998). Third, protein-protein interactions may affect interactions with the general transcriptional machinery, possibly in a promoter-dependent fashion. These may also allow Ftz to function as a transcriptional activator, as well as a repressor. Ftz could use its different parts to interact with a number of different cofactors simultaneously.

Another level of regulation is phosphorylation, as exemplified in this dissertation. In the case of T263, the effect of phosphorylation is most likely exerted on particular protein-protein interactions. The molecular consequences of these interactions have yet to be determined. Other Ftz phosphorylation sites may affect other activities, such as protein stability, DNA binding, or all of the above.

4.3 Future Directions

4.3.1 Identification of Proteins that Interact with Ftz and the Effect of Ftz T263 Phosphorylation on the Interaction

Identification of other Ftz cofactors will help understanding the molecular mechanism of Ftz phosphorylation and T263 mutations. These studies should 1) identify proteins that interact with Ftz, 2) test whether these interactions are affected by T263 phosphorylation, and 3) analyze the molecular outcomes of these interactions (see preliminary results in Appendix I).

Phosphorylating T263 efficiently, and without labeling other Ftz phosphorylation sites, may be difficult. The heterogeneous nature of Ftz phosphoisoforms could obscure any effects that phosphorylation of Ftz T263 may have on Ftz function. Therefore, T263A and T263D derivatives of Ftz will provide useful tools to assess the effects of phosphorylation of T263 on

protein-protein interactions. Alternatively, the purified Ftz homeodomain could be radioactively labeled on T263 with purified PKA and used as a probe. T269, the other PKA phosphorylation site in the Ftz homeodomain, could be mutated to an unphosphorylatable form to avoid effects caused by T269 phosphorylation.

Proteins known to interact with Ftz or with other homeodomains should be tested first to see if the T263 mutations affect these interactions. Alternatively, a differential screening method, using wild type, T263A and T263D Ftz proteins as probes, could be used to identify novel Ftz-interacting proteins that interact in a "T263-dependent" fashion. Several techniques could be used, including affinity chromatography, Far Western assays, two-hybrid analysis, and expression library screening. These techniques have been used successfully to identify several Ftz-interacting proteins (Copeland, Ph.D. thesis, 1997; Dietrich and Krause, unpublished result).

The band-shift assay and transcriptional activity in S2 cells are convenient measures to determine, *in vitro,* how the second protein affects Ftz activities in DNA binding and gene transcription. Ftz wild type and T263 mutant derivatives should be tested to see the consequence of mutations affecting T263. For example, in a band shift assay, the second protein may increase or decrease Ftz DNA binding affinity or may form a stable complex with Ftz on DNA. These effects may be binding site-specific. T263A and T263D mutations should influence these interactions in opposite ways so that if one increases the interactions, the other should decrease the interactions.

For the cotransfection assay in cultured cells, Ftz wild-type and T263 mutants will be individually cotransfected with the second protein in the presence of a reporter gene. Co-expressing the second protein with Ftz should cause changes in Ftz-dependent transcriptional activation that are T263-dependent. Stimulation or cotransfection of PKA may further influence

the interaction between wild-type Ftz and the second protein, whereas the T263 mutants should not be affected. A number of Ftz-responding reporter genes could be used to test for sequence- or promoter-specific effects.

Genetic tests should be done to show if the identified protein is required *in vivo* for the regulation of Ftz activity in blastoderm embryos. This is the crucial test of the relevance of these protein interactions and their modulation by Ftz T263 phosphorylation. Some of the methods described above for identifying interacting proteins result in subcloning of the genes. For those that do not, the protein of interest must be partially sequenced to allow cloning of the corresponding gene. Once the gene is cloned and analyzed by sequencing and restriction digestion, its temporal and spatial expression pattern will be determined by Northern blot analysis and whole-mount *in situ* hybridization to see if it overlaps with *ftz* blastoderm expression. Then, its chromosomal location will be mapped by *in situ* hybridization on polytene chromosomes. Finally, mutant phenotypes of the gene will be searched for to see if any existing mutations affect the gene in question. If they exist, the phenotypes of the mutants will be analyzed to see if they have segmental roles.

The general strategy for genetic analysis will be as follows. First, if no mutants exist, mutations in the identified gene must be made. I would hop P-elements into the region where the gene in question maps, using a nearby P-element to start with and using PCR to identify local insertions. To get a null, I would hop the P-elements back out, and use PCR/Southern blots to identify appropriate deletions (Voelker et al., 1984; Cooley et al., 1988; Robertson et al., 1988; Tower et al., 1993; Zhang and Spradling 1993; Dalby et al., 1995). The generated alleles will be crossed with Ftz wild-type, T263A and T263D lines to generate combinations of double mutants in a *ftz* null background. Survival Index, cuticle preparation, and *ftz* target gene expression will

then be analyzed. These data will be compared with the results described in section 2.2 to see if the candidate gene affects T263 mutant phenotypes. If the second protein is indeed a Ftz cofactor, and its interaction with Ftz is affected by T263 phosphorylation, the T263A and T263D mutant phenotypes should be enhanced or suppressed by mutations in the second protein, the effect depending on the positive or negative outcome of the interaction.

It is possible that the effects of some interacting proteins may be redundant with the effects of others, thereby masking their potential importance. To avoid this problem, *ftz-lacZ* reporter genes could be used to analyze *ftz cis*-regulatory elements (Hiromi et al., 1985; Hiromi and Gehring, 1987; Pick et al., 1990; Schier and Gehring, 1993a, 1993b). Each of these *ftz-lacZ* constructs contains only a portion of the *ftz* promoter. By reducing the size of the element and the number of trans-acting factor binding sites, the complicating actions of redundant factors may be avoided.

The determination of ternary complex structures of Ftz wild type, T263A, T263D, along with cofactors bound to DNA will provide a detailed molecular mechanism of T263 effects. For this analysis, the minimal interaction domains of the proteins need to be identified first and expressed in soluble form. The three-dimensional crystal structure of the α2-**a1** homeodomain heterodimer bound to DNA is an example of a cooperative homeodomain protein-DNA interaction. A 16 aa tail extending C-terminal to the α2 homeodomain is disordered in the absence of **a1**. However, in the **a1**/α2-DNA complex, this 16 aa tail forms an amphipathic helix that interacts with helices I and II of the **a1** homeodomain (Li et al., 1995). Similar effects may occur on the Ftz N-terminal arm and its N-terminal extension, which is also unstructured (Qian et al., 1994). It will be interesting to see in the ternary complex a) if residue T263 is exposed on the surface of Ftz homeodomain away from the DNA molecule, and if it is involved in direct contact

with the cofactor, b) what effect the Ftz cofactor has on the configuration of T263 and its flanking residues, c) if there is difference in Ftz structure with T263, T263A and T263D without the cofactor, and if cofactor binding is required for restructuring. The purification and the crystallization of the Ftz homeodomain/cofactor-DNA complex, and analysis of the crystals that form can be performed following the methods described by Qian et al. (1994) and Li et al. (1995).

4.3.2 Analysis of the Effects of Mutations Affecting other Ftz Phosphorylation Sites

Ftz phospho-amino acid analyses have mapped 18 candidate sites for phosphorylation (Figure 6). Any of these phosphorylation sites may regulate Ftz activity by a variety of means. Five of these phosphorylation sites have already been individually converted to alanine and aspartate and subcloned into the 20 kb P-element transformation construct (Hiromi et al., 1985; also see section 2.4.2). The five sites are Ser^{100}, Thr^{164}, Thr^{198}, $Ser^{219\text{-}221}$ and Ser^{257}. Other mapped phosphorylation sites could also be mutated to Ala and Asp and studied similarly.

The studies of Ftz T263 should serve as a good model for the studies of these other sites. First the measurement of survival indices serves to determine the cumulative effects of mutations on all *ftz*-dependent processes. Second, cuticles, CNS and gut morphologies would be assayed to determine the effects of mutations on each of the *ftz*-dependent patterning processes. Mutant phenotypes may occur in any or all of the three tissues that Ftz is expressed in. Both hypomorphic and hypermorphic phenotypes are possible. Third, the expression patterns of *ftz* target genes, which reflect the transcriptional activities of Ftz mutants in the embryo, would be assayed. Depending upon particular mutant phenotypes, subsets of *ftz* target genes would be monitored by whole-mount *in situ* hybridization and/or immunological staining. Based upon the

mutant phenotypes, the mutant genes could be studied further to determine: 1) which developmental processes are affected; 2) how the sites normally come to be phosphorylated, and 3) how phosphorylation affects the molecular properties of the mutant proteins. For instance, *ftz* expression levels, subcellular localization, DNA and protein binding, and transcriptional activities in cultured cells could all be affected.

The analysis of these five sites, as well as other sites, should be in accordance with their potential function based on their location in the Ftz polypeptide, and with the kinases that phosphorylate them (Figure 6). For example, Ser^{100} is phosphorylated in baculovirus cells, and *in vitro* by CK1. It is just C-terminal to a putative nuclear localization signal (NLS) and could therefore regulate Ftz nuclear transport (Görlich, 1997). Ser^{100} is also followed by 33 aa that are 100% conserved between *Drosophila melanogaster* and *Drosophila hydei* Ftz proteins. This region is required for Ftz interactions with its cofactors Prd and Ftz-F1. In *Drosophila hydei*, the residue in the position of Ser^{100} is an Asp. Since CK1 appears to be ubiquitous, this site is likely constitutively phosphorylated in *D. melanogaster*, making the Asp residue in *D. hydei* a conservative substitution. If so, the S100D mutation in D. *melanogaster* Ftz should have no effect, whereas the S100A mutation should.

Thr^{164} is phosphorylated in baculovirus cells and *in vitro* by MAPK and GSK-3. Thr^{198} is also phosphorylated by MAPK. These residues are located in the middle of the Ftz protein. The function of this region is currently unknown. Ser^{219} and Ser^{221} are within a genetically verified PEST sequence that affects Ftz protein stability. Both sites are phosphorylated in baculovirus-infected cells, suggesting that these sites are also phosphorylated in the embryo. The phosphorylation of these sites might be required to ensure rapid turnover of Ftz protein in the embryo. Since very low levels of Ftz are expressed in the embryo, to facilitate the measuring of

the Ftz mutants in the embryo, a heat shock strategy as described in Krause et al. (1988), could be used to achieve higher levels of *ftz* expression in the embryo. Finally, Ser^{257} is phosphorylated by PKC *in vitro*, and like T263, is in the N-terminus of the Ftz homeodomain. Hence, it may regulate either a protein-DNA or protein-protein interaction that can be verified using similar assays proposed for T263.

4.4 Conclusion

My dissertation has lent substantial support to the hypothesis that phosphorylation affects Ftz activity and that this form of regulation is stage- and tissue-specific. My results establish the importance of T263 phosphorylation for the role of *ftz* in segmentation and have revealed an additional level of complexity in the regulation of Ftz activity. Interestingly, I have found that the same mutations have no effect on Ftz activity in the CNS, suggesting that the requirement for phosphorylation is stage-specific. Thus, the regulation of Ftz activity by T263 phosphorylation is dependent upon the *in vivo* cellular context. It will be interesting to identify the cofactor that appears to require this tissue-specific modification, and to characterize its expression, function, and its effects on Ftz. It may be possible to duplicate the effects of this interaction on Ftz and the effects of T263 phosphorylation *in vitro*, on or off DNA, and in cultured cells. Such studies would generate a detailed understanding of the molecular mechanisms underlying homeodomain protein function during development.

Appendix I:

Effects of Ftz T263 Mutations on Protein Interactions

Introduction of Proteins Tested

As discussed in sections 2.3.5 and 4.2.3, the most likely mechanism underlying the requirement for T263 phosphorylation is an altered ability to interact with an embryonically expressed Ftz cofactor. Several candidate proteins have been tested to see if they interact with the Ftz homeodomain, and if substitution of alanine or aspartate for T263 affects the interaction. These include HMG-D (*Drosophila* high mobility group protein), FISH-HOOK, Ftz-F1, 33-13, and TFIIEβ.

HMG-D and FISH-HOOK are both high mobility group (HMG) domain proteins (Grosschedl et al., 1994). HMG domain proteins are architectural proteins that can affect gene transcription by molding DNA conformation and by changing both protein-DNA and protein-protein interactions (Grosschedl et al., 1994; TenHarmsel and Biggin, 1995). HMG-D is one of the *Drosophila* counterparts of the vertebrate HMG1/HMG2 class of HMG proteins (Ner and Travers, 1994). It is expressed maternally and zygotically and is an abundant protein in early *Drosophila* embryos (Stroumbakis and Tolias, 1994). HMG-D contains three domains: an N-terminal HMG domain, a basic region, and an acidic C-terminal tail. It contacts AT-rich DNA in the minor groove via its HMG domain and changes DNA conformation through DNA bending (Ner and Travers, 1994; Payet and Travers, 1997). The vertebrate HMG2 protein was isolated by screening an expression library for proteins that directly interact with the homeodomain protein Oct-2. In a Far Western experiment, HMG2 interacted with the homeodomains of the POU homeodomain proteins Oct-2, Oct-1 and Oct-6 (Zwilling et al., 1995). Thus, an interaction between the homeodomain of Ftz and HMG-D is quite possible. Consistent with this possibility, the 5'-most 660 bp of the *ftz* upstream regulatory element is AT rich (Harrison and Travers, 1988) and contains binding sites for HMG-D (Gasser and Laemmli, 1986, Churchill et al., 1995),

as well as Ftz (Krause and Gehring, 1988). It is therefore possible that Ftz and HMG-D interact in some fashion on this portion of the *ftz* enhancer.

The *Drosophila* FISH-HOOK (Nambu and Nambu, 1996) protein belongs to the SOX subgroup of HMG domain proteins that exhibit similarity to the mammalian testis determining factor, sex-determining region of the Y chromosome (SRY). It is first expressed in the entire trunk of syncytial blastoderm embryos. The expression then resolves in a series of seven irregular ectodermal stripes in the cellular blastoderm that partially overlaps with *ftz* stripes (Nambu and Nambu, 1996). *fish-hook* null mutants exhibit severe segmentation abnormalities and defects in *ftz*, *eve* and *wg* expression, suggesting a potential function in modulating the activities of segmentation genes, including *ftz*.

Ftz-F1, as discussed above, is a protein of the nuclear receptor superfamily and regulates Ftz activity. 33-13 was identified using the yeast two-hybrid system to identify proteins that interact with Ftz (Dietrich and Krause, unpublished result). The bait used in this screen contained the Ftz homeodomain, without 12 residues in the C-terminus, but with an additional 17 residues N-terminal to the homeodomain. Far Western analysis has confirmed the interaction of 33-13 with the Ftz homeodomain. 33-13 is maternally expressed, binds DNA, and is localized in the nucleus (Dietrich and Krause, unpublished observations). All of these properties are consistent with possible function of 33-13 as a transcription factor.

In a glutathione bead pull down assay, TFIIEβ interacted with the homeodomains of Antp and Abd-B (Zhu and Kuziora, 1996). TFIIE is a component of the eukaryotic RNA polymerase II transcription apparatus. It consists of two subunits, α and β, which form a heterotetramer ($\alpha_2\beta_2$) in solution (Peterson et al., 1991). Interactions with TFIIEβ may be a common property of the Antp subclass of homeodomains, of which Ftz is a member. Interestingly, the Eve homeodomain

is unable to interact with TFIIEβ (Zhu and Kuziora, 1996). Eve is a general repressor and has an alanine at the position corresponding to Ftz T263, raising the possibility that this may be a residue that is involved in determining homeodomain-TFIIEβ interactions.

Results

Of the five proteins I tested by Far Western blot analysis, HMG-D and 33-13 had detectable levels of interaction with the Ftz homeodomain. Of these two, only the interaction with HMG-D is affected by mutations that alter T263. The results of these Ftz homeodomain-HMG-D interactions and the effects of the Ftz T263 mutations on the interaction are presented in Figure 22.

Ftz wild-type and T263A homeodomains exhibited similar affinity for the HMG-D protein. However, the HMG-D-Ftz homeodomain interaction was diminished by the Ftz T263D mutation, suggesting that phosphorylation of Ftz T263 may negatively regulate the HMG-D-Ftz interaction.

The possible significance of this interaction was tested further using an electrophoretic mobility assay (EMSA) to see if the interaction affected binding of Ftz to DNA. A Ftz consensus binding site, 5'GGAAGCAATTAAGGATCCC3' (Percival-Smith et al., 1990; Pick et al., 1990; Florence et al., 1991), was used for the assay. Ftz shifted the portion of labeled oligos while HMG-D alone did not bind. When added together with wild-type or T263A Ftz homeodomain, HMG-D had no effect on the Ftz shift. The intensity and position of the shifted bands were unchanged (Figure 23). However, the T263D Ftz homeodomain-DNA interaction was diminished in intensity in the presence of HMG-D protein. Thus, it seems that HMG-D has a

negative effect on T263D DNA binding, even though its interaction with Ftz is relatively weak in the absence of DNA.

Discussion

The preliminary results show that the T263D mutation affects the ability of the Ftz homeodomain to interact with HMG-D. Substitution of aspartate for T263 inhibits the interaction whereas replacement of T263 by alanine has no effect. T263D homeodomain DNA binding is also diminished in the presence of HMG-D. HMG-D and Ftz are co-expressed in the early embryo. They bind to the same region in the *ftz* upstream regulatory element. HMG-D binds DNA in the minor groove, as does the N-terminal arm of the Ftz homeodomain, where T263 resides. These observations suggest that Ftz and HMG-D may interact *in vivo* at the Ftz enhancer. However, given the fact that ftz^{T263D} mimics active ftz^{+} during segmentation, and that HMG-D appears to be having a negative effect on its interaction with DNA, this result is difficult to rationalize. Hence, if these two proteins do interact *in vivo*, the consequences may be complex, or may involve other factors.

Materials and Methods

Wild-type, T263A and T263D Ftz homeodomains were expressed in *E. coli* using the T7 expression plasmid pET-3b and partially purified, as described in section 2.4.2. Ftz homeodomains were separated on a 15% PAGE gel and quantitated by ECL Western blotting and Phosphorimager analysis (BioRad), and by Coomassie staining against BSA standards. Full-length HMG-D (Ner and Travers, 1994), Ftz-F1 (Guichet et al., 1997), 33-13 (Dietrich and Krause, unpublished), FISH-HOOK (Nambu and Nambu, 1996) and TFIIEβ (from the laboratory

of J. Greenblatt, University of Toronto) were expressed using a Promega TNT *in vitro* transcription/translation kit. Far Western and EMSA experiments were each done three times and the results were quantitated by Phosphorimager analysis (BioRad).

Far Western blot analysis was performed as previously described (Guichet et al., 1997). A 300 ng sample of each of the Ftz homeodomains was run out on PAGE gels. The homeodomains were transferred from the gel onto nitrocellulose. To make probes, 1 μg each of the full-length HMG-D, Ftz-F1, 33-13, FISH-HOOK and TFIIEβ proteins was individually labeled by transcription and translation of cDNA in the presence of ^{35}S-Met, using the TNT kit. The probes were tested by running out on PAGE gels and autoradiographed prior to use. The nitrocellulose blots were blocked, incubated with 50 μl ^{35}S-labeled proteins in 5 ml cocktail and washed, as previously described (Guichet et al., 1997). Bound signal was detected by autoradiography.

Equal amounts of Ftz T263 wild-type and mutant homeodomains were used for the EMSA assay, performed as described in section 2.4.8, using the double-stranded DNA substrate 5'GGGAAGCAATTAAGGATCCC3' (Percival-Smith et al., 1990; Pick et al., 1990; Florence et al., 1991). Full-length HMG-D was expressed *in vitro* using the TNT kit, as described above, without ^{35}S-labeling. 10 μl of the TNT reactions was included in a 20 μl protein-DNA binding reaction.

Figure 22. The Ftz homeodomain/HMG-D interaction is diminished by T263D. The interactions of Ftz wild-type, T263A and T263D homeodomains with HMG-D were analyzed by Far Western blot assay. A. Far Western analysis. Equal amounts of Ftz wild-type, T263A and T263D homeodomains were separated on SDS PAGE gels, and transferred to nitrocellulose. ^{35}S-labeled HMG-D protein was incubated with the nitrocellulose blot. Ftz wild-type and T263A mutant homeodomains interact with HMG-D protein. Ftz T263D, on the other hand, binds more weakly. The intensity of band with T263D was about 1/3 of those with wild-type and T263A (3.1 ± 0.6, 3.2 ± 0.2 and 1.0 ± 0.1 for wild-type, T263A and T263D respectively, $\alpha=0.05$. These numbers are intensity measurements of the Far Western nitrocellulose blots by Phosphorimager analysis). B. Coomassie blue staining shows the partially purified Ftz homeodomains separated by SDS-PAGE. The levels of all three homeodomain polypeptides are approximately equal. The experiment was performed three times with the same results.

-ve WT T263A T263D

A

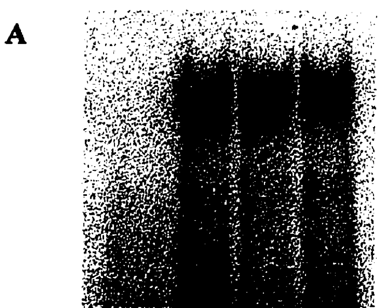
Far Western

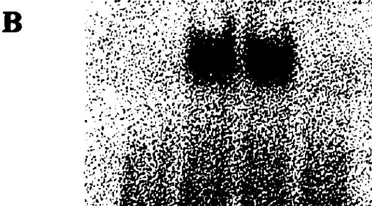
B

Coomassie staining

Figure 23. The Ftz T263D-DNA interaction is abolished by HMG-D. EMSA analysis of DNA binding by Ftz wild-type, T263A and T263D homeodomains is shown in the absence (A) and presence (B) of HMG-D protein. Equal amounts of Ftz wild-type, T263A and T263D homeodomains were individually incubated with the ^{32}P-labeled Ftz binding fragment 5'GGAAGCAATTAAGGATCCC3'. The bound complexes were then separated on native PAGE gels. In the presence of HMG-D, Ftz T263D homeodomain-DNA binding activity was diminished to background levels (n = 3). HMG-D did not affect wild-type and T263A homeodomain-DNA binding. The EMSA experiment was done three times and the results were the same.

-ve WT T263A T263D





Appendix II:

Sequence Alignment of Representative Homeodomain Classes

The amino acid sequences of several classes (paralog groups) of homeodomains, chosen mostly from *Drosophila melanogaster* and human, are aligned from position 1 to 60. The consensus sequence is based on the highest amino acid frequencies derived from 346 homeodomains (Gehring et al., 1994). Dashes indicate identity with the corresponding residue in the consensus homeodomain. The Xs in h Brn-1, h Brn-2 and MATa1 indicate the end of their homeodomains. h Brn-1 and h Brn-2: human Brain-1 and Brain-2, h Evx2: human Even-skipped homologue, h En-2: human Engrailed homologue, Otd: Orthodenticle, r Isl-1: rat Islet-1. The human α-fetoprotein enhancer-binding protein (ATBF1) has 4 homeodomains listed as ATBF1 HD1 to ATBF1 HD4. For more sequence alignment, see Scott et al. (1989) and Gehring et al. (1994).

```
                                10        20        30        40        50        60
     Class             RRRKRTAYTRYQLLELEKEFHFNRYLTRRRRIELAHSLNLTERQVKIWFQNRRMKWKKEN (consensus)
(Paralog group)

     Lab               NNSG--NF-NK--T--------------A----I-NT-Q-N-T------------Q--RV
     h HOXA1           PNAV--NF-TK--T---------K----A--V-I-A--Q-N-T------------Q--RE
     h HOXB1           PSGL--NF-TR--T---------K--S-A--V-I-AT-E-N-T------------Q--RE
     h HOXD1           SSAI--NFSTK--T---------K----A----I-NC-H-NDT------------Q--RE

     Pb                P--L-----NT------------K--C-P----I-A--D-------V--------H-RQT
     h HOXA2           S--L-----NT------------K--C-P--V-I-AL-D-------V--------H-RQT
     h HOXB2           A--L-----NT------------K--C-P--V-I-AL-D-------V--------H-RQT
     h HOXA3           SK-A-----SA--V------------V-P--V-M-NL-------I----------Y--DQ
     h HOXB3           SK-A-----SA--V------------C-P--V-M-NL---S---I----------Y--DQ
     h HOXD3           SK-V-----SA--V------------C-P--V-M-NL-------I----------Y--DQ

     Dfd               PK-Q------H-I--------Y-----------I--T-V-S---I-------------D-
     h HOXA4           PK-S------Q-V--------------------I--T-C-S-----------------DH
     h HOXB4           PK-S------Q-V--------Y---------V-I--A-C-S---I-------------DH
     h HOXC4           PK-S-A----Q-V--------Y-----------I----C-S---I-------------DH
     h HOXD4           PK-S------Q-V--------------------I--T-C-S---I-------------DH

     Scr               TK-Q-S------T--------------------I--A-C-----I----------L---H
     h HOXA5           GK-A--------T--------------------I--A-C-S---I-------------D-
     h HOXB5           GK-A--------T--------------------I--A-C-S---I-------------D-
     h HOXC5           GK-S--S-----T--------------------I-NN-C-N---I-------------DS

     Antp              -K-G-QT-----T--------------------I--A-C-----I---------------
     Abd-A             ---G-QT---F-T----------H---------I--A-C-----I----------L---L
     Ubx               ---G-QT-----T--------T-H---------M--A-C-----I----------L---I
     Ftz               SK-T-QT-----T------------I------DI-NA-S-S---I----------S--DR
     h HOXA6           G--G-QT-----T--------------------I-NA-C-----I---------------
     h HOXB6           G--G-QT-----T--------Y-----------I--A-C-----I--------------S
     h HOXC6           ---G-QT-S---T--------------------I-NA-C-----I--------------S
     h HOXA7           -K-G-QT-----T--------------------I--A-C-----I--------------H
     h HOXB7           -K-G-QT-----T--------Y-----------I--A-C-----I---------------
     h HOXB8           ---G-QT-S---T-------L--P----K----VS-A-G---------------------
     h HOXC8           --SG-QT-S---T-------L--P----K----VS-A-G---------------------
     h HOXD8           ---G-QT-S-F-T-------L--P----K----VS-A-A---------------------
```

```
                                10        20        30        40        50        60
Class              RRRKRTAYTRYQLLELEKEFHFNRYLTRRRRIELAHSLNLTERQVKIWFQNRRMKWKKEN (consensus)
(Paralog group)

Abd-B              V-K--KP-SKF-T-------L--A-VSKQK-W---RN-Q----------------N--NS
h HOXA9            T-K--CP--KH-T-------L--M----D--Y-V-RL------------------M--I-
h HOXB9            S-K--CP-SK--T-------L--M----D--H-V-RL---S--------------M--M-
h HOXC9            T-K--CP--K--T-------L--M----D--Y-V-RV------------------M--MS
h HOXD9            T-K--CP--K--T-------L-M-----D--Y-V-RV------------------M--MS
h HOXA10           G-K--CP--KH-T-------L-M-----E--L-ISR.VH--D-------------M--M-
h HOXC10           G-K--CP--KH-T-------L-M-----E--L-ISKTI---D-------------M--M-
h HOXD10           G-E--CP--KH-T-------L-M-----E--L-ISK.V---D-------------M--MS
h HOXA11           T-K--CP--K--IR---R--F-SV-INKEK-LQ-SRM----D-------------E--I-
h HOXC11           T-K--CP-SKF-IR---R--F--V-INKEK-LQ-SRM----D-------------E--LS
h HOXD11           S-K--CP--K--IR---R--F--V-INKEK-LQ-SRM----D-------------E--L-
h HOXC12           S-K--KP-SKL--A---G--LV-EFI--Q--R--SDR---SDQ------------K-RLL
h HOXD12           A-K--KP--KQ-IA---N--LV-EFIN-QK-K--SNR---SDQ------------K-RVV
h HOXA13           G-K--VP--KV--K---R-YAT-KFI-KDK-RRISATT--S----T-------V-E--VI
h HOXC13           G-K--VP--KV--K-----YAASKFI-KEK-RRISATT--S----T-------V-E--VV
h HOXD13           G-K--VP--KL--K---N-YAI-KFINKDK-RRISAAT--S----T-------V-D--IV

h Pit-1            K-KR--TISVAAKDA--RH-GEQNKPSSQEIMRM-EE---EKEV-RV--C---QRE-RVK
h Oct-1            --K---SIETNIRVA---S-LE-QKP-SEETIMI-DQ--MEKEVIRV--C---Q-E-RI-
h Oct-2            --K---SIETNVRFA---S-LA-QKP-SEEILLI-EQ-HMEKEVERV--C---Q-E-RI-
h Brn-1            K-K---SIEVSVKGA--SH-LKCPKPSSQEITN--D--Q-EKEV-RV--C---Q-E-RXX
h Brn-2            K-K---SIEVSVKGA--SH-LKCPKPSSQEITN--D--Q-EKEV-RV--C---Q-E-RXX


Eve                V--Y---F--D--GR-----YKEN-VS-P--C---AQ---P-STI-V--------D-RQR
h EVX2             V--Y---F--E-IAR-----YREN-VS-P--C---AA---P-TTI-V--------D-RQR

En                 EK-P---FSSE--AR-KR--NE-----E---QQ-SSE-G-N-A-I------K-A-I--ST
h En-2             DK-P---F-AE--QR-KA--QT-----EQ--QS--QE-S-N-S-I------K-A-I--AT

Prd                Q--C--TFSAS--D---RA-ERTQ-PDIYT-E---QRT----ARIQV--S---ARLR-QH
Pax6               LQ-N--SF-QE-IEA-----ERTH-PDVFA-ER--AKID-P-ARIQV--S---A--RR-E
Pax3               Q--S--TF-AE--E---RA-ERTH-PDIYT-E---QRAK---AR-QV--S---AR-R-QE

Otd                Q--E--TF--A--DV--AL-GKT--PDIFM-E-V-LKI--P-SR-QV--K---A-CRQQL
```

```
                            10        20        30        40        50        60
     Class         RRRKRTAYTRYQLLELEKEFHFNRYLTRRRRIELAHSLNLTERQVKIWFQNRRMKWKKEN (consensus)
(Paralog group)

     Bcd           P--T--TF-SS-IA---QH-LQG----AP-LAD-SAK-A-GTA------K---RRH-IQS

     r Isl-1       TT-V--VLNEK--HT-RTCYAA-PRPDALMKEQ-VEMTQ-SP-VIRV----K-C-D--RS

     TCL-3         KKKP--SF--L-IC----R--RQK--ASAE-AA--KA-KM-DA---T------T--RRQT

     Cad           KDKY-VV--DF-R------YCTS--I-I--KS---QT-S-S------------A-ERTS-

     ATBF1 HD1     NK-P--RI-DD--RV-RQY-DI-NSPSEEQIK-M-DKSG-PQKVI-H--R-TLF-ERQR-
     ATBF1 HD2     K-SS--RF-D---RV-QDF-DA-A-PKDDEFEQ-SNL---PT-VIVV----A-Q-AR-NY
     ATBF1 HD3     DK-L--TI-PE--EI-YQKYLLDSNP--KMLDHI--EVG-KK-V-QV----T-ARER-GD
     ATBF1 HD4     QK-F--QM-NL--KV-KSC-NDY-TP-MLECEV-GNDIG-PK-V-QV----A-A-E--SK

     Cut           SKKQ-VLFSEE-KEA-RLA-ALDP-PNVGTIEF--NE-G-AT-TITN--H-H--RL-QQV

     MATa1         SPKGKSSISPQARAF--QV-RRKQS-NSKEKE-V-KKCGI-PL--RV--I-K--RS-XXX
     MATα2         -GHRF-KENVRI-ESWFAKKNIEP--DTKGLEN-MKNTS-SRI---N-VS---R-E-TIT
```

References

Akam, M. 1989. Hox and HOM: homologous gene clusters in insects and vertebrates. *Cell* **57**:347-349.

Ananthan, J., R. Baler, D. Morrissey, J. Zuo, Y. Lan, M. Weir, and R. Voellmy. 1993. Synergistic activation of transcription is mediated by the N-terminal domain of *Drosophila fushi tarazu* homeodomain and can occur without DNA binding by the protein. *Mol. Cell. Biol.* **13**:1599-1609.

Aoki, T., Y. Sano, T. Yamamoto, and J. Inoue. 1996. The ankyrin repeats but not the PEST-like sequences are required for signal-dependent degradation of IκBa. *Oncogene* **12**:1159-1164.

Aplin, A.C., and T.C. Kaufman. 1997. Homeotic transformation of the legs to mouthparts by *proboscipedia* expression in *Drosophila* imaginal discs. *Mech. Dev.* **62**:51-60.

Arias, J., A.S. Alberts, P. Brindle, F.X. Claret, T. Smeal, M. Karin, J. Feramisco, and M. Montminy. 1994. Activation of cAMP and mitogen responsive genes relies on a common nuclear factor. *Nature* **370**:226-229.

Ashburner, M. 1989. *Drosophila*: A laboratory handbook, pp. 139-298. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Aspland, S.E., and R.A.H. White. 1997. Nucleocytoplasmic localisation of *extradenticle* protein is spatially regulated throughout development in *Drosophila. Development* **124**:741-747.

Azpiazu, N. and G. Morata. 1998. Functional and regulatory interactions between Hox and *extradenticle* genes. *Genes & Dev.* **12**:261-273.

Baeuerle, P.A., D. Baltimore. 1989. A 65-kD subunit of active NF-κB is required for inhibition of NF-κB by IκB. *Genes & Dev.* **3:**1689-1698.

Baeuerle, P.A., D. Baltimore. 1996. NF-κB: ten years after. *Cell* **87:**13-20.

Bannister, A. J. and T. Kouzarides. 1996. The CBP co-activator is a histone acetyltransferase. *Nature* **384:**641-643.

Belvin, M.P., Y. Jin, and K.V. Anderson. 1995. Cactus protein degradation mediates *Drosophila* dorsal-ventral signaling. *Genes Dev.* **9:**783-93.

Billeter, M., Y.Q. Qian, G. Otting, M. Müller, W. Gehring, and K. Wüthrich. 1990. Determination of the three-dimensional structure of the *Antennapedia* homeodomain from *Drosophila* in solution by ^{1}H nuclear magnetic resonance spectroscopy. *J. Mol. Biol.* **214:**183-197.

Billeter, M., Y.Q. Qian, G. Otting, M. Muller, W. Gehring, and K. Wüthrich. 1993. Determination of the NMR solution structure of an *Antennapedia* homeodomain-DNA complexes. *J. Mol. Biol.* **234:**1084-1093.

Bodner, M., and M. Karin. 1987. A pituitary specific trans-acting factor can stimulate transcription of the growth hormone gene in extracts of non-expressing cells. *Cell* **50:**267-275.

Bodner, M., J.L. Castrillo, L.E. Theill, T. Deerinck, M. Ellisman, and M. Karin. 1988. The pituitary specific transcription factor GHF-1 is a homeobox containing protein. *Cell* **55:**505-518.

Boekhoff,I., Touhara,K., Danner,S., Inglese,J., Lohse,M.J., Breer,H. and R. J. Lefkowitz. 1997. Phosducin, potential role in modulation of olfactory signaling. *J. Biol. Chem.*, **272:**4606-4612.

Boncinelli, E. 1997. Homeobox genes and disease. *Curr. Opin. Gen. Dev.* **7:**331-337.

Bopp, D., M. Burri, S. Baumgartner, G. Frigerio, and M. Noll. 1986. Conservation of a large protein domain in the segmentation gene *paired* and in functionally related genes of *Drosophila*. *Cell* **47:**1033-1040.

Borrelli, E., R. Heyman, C. Arias, P. Sawchenko, and R. M. Evans. 1989. Transgenic mice with inducible dwarfism. *Nature* **339:**538-541.

Bourbon, H-M, E. Martin-Blanco, D. Rosen, and T.B. Kornberg. 1995. phosphorylation of the *Drosophila* Engrailed protein at a site outside its homeodomain enhances DNA binding. *J. Biol. Chem.* **270:**11130-11139.

Boyle, W.J., P. van der Geer, and T. Hunter. 1991. Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. *Methods Enzymology* **201:**110-149.

Brody, T.B. 1996. The Interactive Fly, Stages of Development. http://flybase.bio.indiana.edu/allied-data/lk/interactive-fly/aimain/2stages.htm

Brown, J.L., S. Sonoda, H. Ueda, M.P. Scott, and C. Wu. 1991. Repression of the *Drosophila fushi tarazu (ftz)* segmentation gene. *EMBO J.* **10:**665-674.

Brown, J.L., and C. Wu. 1993. Repression of *Drosophila* pair-rule segmentation genes by ectopic expression of *tramtrack*. *Development* **117:**45-58.

Brown, K., S. Gerstberger, L. Carlson, G. Franzoso, and U. Siebenlist. 1995. Control of IκBa proteolysis by site-specific, signal-induced phosphorylation. *Science* **267:**1485-1488.

Brown, S.J. R.B. Hilgenfeld, and R.E. Denell. 1994. The beetle *Tribolium castaneum* has a *fushi tarazu* homolog expressed in stripes during segmentation. *Proc. Natl. Acad. Sci. USA.* **91:**12922-12926.

Buratowski, S. 1994. The basics of basal transcription by RNA polymerase II. *Cell* **77:**1-3.

Caelles, C., H. Hennemann, and M. Karin, 1995. M-phase-specific phosphorylation of the POU transcription factor GHF-1 by a cell cycle-regulated protein kinase inhibits DNA binding. *Mol. Cell. Bio.* **15:**6694-6701.

Carè, A., A. Silvani, E. Meccia, G. Mattia, A. Stoppacciaro, G. Parmiani, C. Peschle, and M.P. Colombo. 1996. HOXB7 constitutively activates basic fibroblast growth factor in melanomas. *Mol. Cell. Biol.* **9:**4842-4851.

Carey, M. 1998. The enhanceosome and transcriptional synergy. *Cell* **92:**5-8.

Carroll, S.B. and M.P. Scott. 1985. Localization of the *fushi tarazu* protein during *Drosophila* embryogenesis. *Cell* **43:**47-57.

Castrillo, J.L., L.E. Theill, and M. Karin. 1991. Function of the homeodomain protein GHF1 in pituitary cell proliferation. *Science* **253:**197-199.

Castronovo, V., M. Kusaka, A. Chariot, J. Gielen, and M. Sobel. 1994. Homeobox genes: potential candidates for the transcriptional control of the transformed and invasive phenotype. *Biochem. Pharm.* **47:**137-143.

Chan, S.K., and R.S. Mann. 1993. The segment identity functions of Ultrabithorax are contained within its homeo domain and carboxy-terminal sequences. *Genes & Dev.* **7:**796-811.

Chan, S.K., L. Jaffe, M. Capovilla, J. Botas, and R.S. Mann. 1994. The DNA binding specificity of *Ultrabithorax* is modulated by cooperative interactions with *extradenticle*, another homeoprotein. *Cell* **78:**603-615.

Chan, S.K., and R.S. Mann. 1996. A structural model for an extradenticle-Hox-DNA complex accounts for the choice of HOX protein in the heterodimer. *Proc. Natl. Acad. Sci. USA* **93:**5223-5228.

Chan, S.K., H. Pöpperl, R. Krumlauf, and R.S. Mann. 1996. An extradenticle-induced conformational change in a HOX protein overcomes an inhibitory function of the conserved hexapeptide motif. *EMBO J.* **15:**2476-2487.

Chan, S.K., H.D. Ryoo, A. Gould, R. Krumlauf, and R.S. Mann. 1997. Switching the *in vivo* specificity of a minimal Hox-responsive element. *Development* **124:**2007-2014.

Chang, C.P., W.F. Shen, S. Rozenfeld, H.J. Lawrence, C. Largman, and M. Cleary. 1995. Pbx proteins display hexapeptide-dependent cooperative DNA binding with a subset of Hox proteins. *Genes & Dev.* **9:**663-674.

Chang, C.P., L. Brocchieri, W.F. Shen, C. Largman, and M. Cleary. 1996. Pbx modulation of Hox homeodomain amino-terminal arms establishes different DNA-binding specificities across the HOX locus. *Mol. Cell Biol.* **16:**1734-1745.

Chawengsaksophak, K., R. James, V.E. Hammond, F. Köntgen, and F. Beck. 1997. Homeosis and intestinal tumours in *Cdx2* mutant mice. *Nature* **368:**84-87.

Chrivia, J. C., R. P. S. Kwok, N. lamb, M. Hagiwara, M. R. Montminy, and R. H. Goodman. 1993. Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature* **365:**855-859.

Churchill, M.E., D.N. Jones, T. Glaser, H. Hefner, M.A. Searles, and A.A. Travers. 1995. HMG-D is an architecture-specific protein that preferentially binds to DNA containing the dinucleotide TG. *EMBO J.* **14:**1264-75

Colgan, J., S. Wampler, and J.L. Manley. 1993. Interaction between a transcriptional activator and transcription factor IIB in vivo. *Nature* **362:**549-553.

Colgan, J., H. Ashali, and J.L. Manley. 1995. A direct interaction between glutamine-rich activator and the N terminus of TFIIB can mediate transcriptional activation in vivo. *Mol. Cell. Biol.* **15:**2311-2320.

Cooley, L., R. Kelley, and A. Spradling. 1988. Insertional mutagenesis of the *Drosophila* genome with single P elements. *Science* **239:**1121-1128.

Copeland, J.W.R., A. Nasiadka, B.H. Dietrich, and H.M. Krause. 1996. Patterning of the *Drosophila* embryo by a homeodomain-deleted Ftz polypeptide. *Nature* **379:**162-165.

Copeland, J.W.R. 1997. Ph.D. Thesis. University of Toronto.

Coqueret, O., G. Bérubé, and A. Nepveu. 1996. DNA binding by Cut homeodomain protein is down-regulated by protein kinase C. *J. Biol. Chem.* **271:**24862-24868.

Cowley, S., H. Paterson, P. Kemp, and C.J. Marshall. 1994. Activation of MAP kinase kinase is necessary for PC12 differentiation and for transformation of NIH 3T3 cells. *Cell* **77:**814-852.

Cunningham, B.C., and J.A. Wells. 1989. High-resolution epitope mapping of hGH-receptor interactions by alanine-scanning mutagenesis. *Science* **244:**1081-1085.

Dalby, B., A.J. Pereira, and L.S.B. Goldstein. 1995. An inverse PCR screen for the detection of P element insertions in cloned genomic intervals in *Drosophila melanogaster. Genetics* **139:**757-766.

Damante, G., L. Pellizzari, G. Esposito, F. Fogolari, P. Viglino, D. Fabbro, G. Tell, S. Formisano, and R. Di Lauro. 1996. A molecular code dictates sequence-specific DNA recognition by homeodomains. *EMBO J.* **15:** 4992-5000.

Dana, S., and M. Karin. 1989. Induction of human growth hormone promoter activity by the adenosine 3',5'-monophosphate pathway involves a novel responsive element. *Mol. Endocrinology* **3:**815-821.

Dawes, R., I. Dawson, F.Falciani, G. Tear, and M. Akam. 1994. Dax, a locust Hox gene related to *fushi-tarazu* but showing no pair-rule expression. *Development* **120:**1561-1572.

Dearolf, C.R., J. Topol, and C.S. Parker. 1989a. Transcriptional control of *Drosophila fushi tarazu* zebra stripe expression. *Genes & Dev.* **3:**384-389.

Dearolf, C.R., J. Topol, and C.S. Parker. 1989b. The *caudal* gene product is a direct activator of *fushi tarazu* transcription during *Drosophila* embryogenesis. *Nature* **341:**340-343.

Dearolf, C.R., J. Topol, and C.S. Parker. 1990. Transcriptional regulation of the *Drosophila* segmentation gene *fushi tarazu (ftz). BioEssay* **12:***109-113.*

Dedera, D.A., E.K. Waller, D.P. LeBrun, A. Sen-Majumdar, M.E. Stevens, G.S. Barsh, and M.L. Cleary. 1993. Chimeric homeobox gene *E2A-PBX1* induces proliferation, apoptosis, and malignant lymphomas in transgenic mice. *Cell* **74:**833-843.

Desplan, C., J. Theis, and P.H. O'Farrell. 1985. The *Drosophila* developmental gene, *engrailed,* encodes a sequence-specific DNA binding activity. *Nature* **318:**630-635.

Desplan, C., J. Theis, and P.H. O'Farrell. 1988. The sequence specificity of homeodomain-DNA interaction. *Cell* **54:**1081-1090.

Didonato, J.A., M. Hayakawa, D.M. Rothwarf, E. Zandi, and M. Karin. 1997. A cytokine-responsive IκB kinase that activates the transcription factor NF-κB. *Nature* **388***:*548-554.

DiNardo, S., and P.H. O'Farrell. 1987. Establishment and refinement of segmental pattern in the *Drosophila* embryo: spatial control of *engrailed* expression by pair-rule genes. *Genes & Dev.* **1:**1212-1225.

Doe, C.Q., Y. Hiromi, W.J. Gehring, and C.S. Goodman. 1988a. Expression and function of the segmentation gene *fushi tarazu* during *Drosophila* neurogenesis. *Science* **239:**170-175.

Doe, C.Q., D. Smouse, and C.S. Goodman. 1988b. Control of neuronal fate by the *Drosophila* segmentation gene *even-skipped*. *Nature* **333:**376-378.

Doe, C.Q., and M.P. Scott. 1988. Segmentation and homeotic gene function in the developing nervous system of *Drosophila*. *Trends Neurosci.* **11:**101-106.

Doe, C.Q. 1992. Molecular markers for identified neuroblasts and ganglion mother cells in the *Drosophila* central nervous system. *Development* **116**:855-863.

Doe C.Q. 1998 (updated). Hyper Neuroblast Map. Doe Lab homepage at http://www.life.uiuc.edu/doelab/nbmap.html

Dong, J., R. Strome, L.H. Hung, and H.M. Krause. 1998. A phosphorylation site in the Ftz homeodomain is required for activity. *EMBO J.* **17:**2308-2318.

Draganescu, A., J.R. Levin, and T.D. Tullius. 1995. Homeodomain proteins: what governs their ability to recognize specific DNA sequences? *J. Mol. Biol.* **250:**595-608.

Dranginis, A.M. 1990. Binding of yeast **a**1 and *a*2 as a heterodimer to the operator DNA of a haploid-specific gene. *Nature* **347:**682-685.

Driever, W., G. Thoma, and C. Nusslein-Volhard. 1989. Determination of spatial domains of zygotic gene expression in the *Drosophila* embryo by the affinity of binding sites for the *bicoid* morphogen. *Nature* **340:**363-7.

Dube, I.D., S. Kamel-Reid, C.C. Yuan, M. Lu, X. Wu, G. Corpus, S.C. Raimondi, W.M. Crist, A.J. Carroll, and J. Minowada. 1991. A novel human homeobox gene lies at the chromosome 10 breakpoint in lymphoid neoplasias with chromosomal translocation t(10;14). *Blood* **78**:2996-3003.

Duboule, D., and G. Morata. 1994. Colinearity and functional hierarchy among genes of the homeotic complexes. *Trends Genet.* **10**:358-364.

Duncan, I.M. 1986. Control of *bithorax* complex functions by the segmentation gene *fushi tarazu* of *Drosophila melanogaster*. *Cell* **47**:297-309.

Edgar, B.A., and G. Schubiger. 1986. Parameters controlling transcriptional activation during early *Drosophila* development. *Cell* **44**:871-877.

Edgar, B. A., M. P. Weir, G. Schubiger, and T. Kornberg. 1986. Repression and turnover pattern *fushi tarazu* RNA in the early *Drosophila* embryo. *Cell* **47**:745-754.

Edgar, B.A., G.M. Odell, and G. Schubiger. 1987. Cytoarchitecture and the patterning of *fushi tarazu* expression in the *Drosophila* blastoderm. *Genes & Dev.* **1**:1226-1237.

Elsholtz, H.P., H.J. Mangalam, E. Potter, V.R. Albert, S. Supowit, R.M. Evans, and M.G. Rosenfeld. 1986. Two different *cis*-active elements transfer the transcriptional effects of both EGF and phorbol esters. *Science* **234**:1552-1557.

Fischberg, D.J., X. Chen, and C. Bancroft. 1994. A Pit-1 phosphorylation mutant can mediate both basal and induced prolactin and growth hormone promoter activity. *Mol. Endocrinology* **8**:1566-1573.

Fitzpatrick, V.D., and C.J. Ingles. 1989. The *Drosophila fushi tarazu* polypeptide is a DNA-binding transcriptional activator in yeast cells. *Nature* **337**:666-668.

Fitzpatrick, V.D., A. Percival-Smith, C.J. Ingles, and H.M. Krause. 1992. Homeodomain-independent activity of the *fushi tarazu* polypeptide in *Drosophila* embryos. *Nature* **356:**610-612.

Florence, B., R. Handrow, and A. Laughon. 1991. DNA-binding specificity of the *fushi tarazu* homeodomain. *Mol. Cell. Biol.* **11:**3613-3623.

Florence, B., A. Guichet, A. Ephrussi, and A. Laughon. 1997. Ftz-F1 is a cofactor in Ftz activation of the *Drosophila engrailed* gene. *Development* **124:**839-847.

Frankel, A.D., and P.S. Kim. 1991. Modular structure of transcription factors: implications for gene regulation. *Cell* **65:**717-719.

Frasch, M., and M. Levine. 1987. Complementary patterns of *even-skipped* and *fushi tarazu* expression involve their differential regulation by a common set of segmentation genes in *Drosophila. Genes & Dev.* **1:**981-995.

Frasch, M., T. Hoey, C. Rushlow, H. Doyle, and M. Levine. 1987. Characterization and localization of the *even-skipped* protein of *Drosophila. EMBO J.* **6:**749-759.

Furukubo-Tokunaga, K., M. Muller, M. Affolter, L. Pick, U. Kloter, and W. J. Gehring. 1992. *In vivo* analysis of the helix-turn-helix motif of the *fushi tarazu* homeo domain of *Drosophila melanogaster. Genes & Dev.* **6:**1082-1096.

Gasser, S.M., and U.K. Laemmli. 1986. Cohabitation of scaffold binding regions with upstream/enhancer elements of three developmentally regulated genes of *D. melanogaster. Cell* **46:**521-530.

Gavis, E.R., and D.S. Hogness. 1991. Phosphorylation, expression and function of the Ultrabithorax protein family in *Drosophila melanogaster. Development* **112:**1077-1093.

Gay, N.J., S.J. Poole, and T.B. Kornberg. 1988. The *Drosophila engrailed* protein is phosphorylated by a serine-specific protein kinase. *Nucleic Acids Res.* **16:**6637-6647.

Gehring, W.J. 1987. Homeo boxes in the study of development. *Science* **236:**1245-1252.

Gehring, W.J, M. Affolter, and T. Bürglin. 1994a. Homeodomain proteins. *Annu. Rev. Biochem.* **63:**487-526.

Gehring, W.J., Y.Q. Qian, M. Billeter, K. Furukubo-Tokunaga, A.F. Schier, D. Resendez-Perez, M. Affolter, G. Otting, and K. Wüthrich. 1994b. Homeodomain-DNA recognition. *Cell* **78:**211-223.

Ghosh, S., and D. Baltimore. 1990. Activation in vitro of NF-κB by phosphorylation of its inhibitor IκB. *Nature* **344:**678-682.

Görlich, D. 1997. Nuclear protein import. *Curr. Opin. Cell Biol.* **9:** 412-419.

Goutte, C., and A.D. Johnson. 1988. **a1** protein alters the DNA binding specificity of *a*2 repressor. *Cell* **52:**875-882.

Grosschedl, R., K. Giese, and J. Pagel. 1994. HMG domain proteins: architectural elements in the assembly of nucleoprotein structures. *Trends Genet.* **10:**94-100.

Guichet, A., J.W.R. Copeland, M. Erdélyi, D. Hlousek, P. Závorszky, J. Ho, S. Brown, A. Percival-Smith, H.M. Krause, and A. Ephrussi. 1997. The nuclear receptor homologue Ftz-F1 and the homeodomain protein Ftz are mutually dependent cofactors. *Nature* **385:**548-52.

Hafen, E., A. Kuroiwa, and W.J. Gehring. 1984. Spatial distribution of transcripts from the segmentation gene *fushi tarazu* during *Drosophila* embryonic development. *Cell* **37**:833-41.

Halder,G., P. Callaerts, and W.J. Gehring. 1995. Induction of ectopic eyes by targeted expression of the *eyeless* gene in *Drosophila*. *Science* **267**:1788-1792.

Hall, M.N., and A.D. Johnson. 1987. Homeo domain of the yeast repressor α2 is a sequence-specific DNA-binding domain but is not sufficient for repression. *Science* **237**:1007-1012.

Han, K., M.S. Levine, and J.L. Manley. 1989. Synergistic activation and repression of transcription by *Drosophila* homeobox proteins. *Cell* **56**:573-583.

Han, W., Y. Yu, N. Altan, and L. Pick. 1993. Multiple proteins interact with the *fushi tarazu* proximal enhancer. *Mol. Cell. Biol.* **13**:5549-59.

Han, W., Y. Yu, K. Su, R.A. Kohanski, and L. Pick. 1998. A binding site for multiple transcriptional activators in the *fushi tarazu* proximal enhancer is essential for gene expression *in vivo*. *Mol. Cell. Biol.* **18**:3384-3394.

Hanes, S.D., and R. Brent. 1989. DNA specificity of the *bicoid* activator protein is determined by homeodomain recognition helix residue 9. *Cell* **57**:1275-1283.

Hanes, S.D., and R. Brent. 1991. A genetic model for interaction of the homeodomain recognition helix with DNA. *Science* **251**:426-430.

Harrison, S.D., and A.A. Travers. 1988. Identification of the binding sites for potential regulatory proteins in the upstream enhancer element of the *Drosophila fushi tarazu* gene. *Nucl. Acids Res.* **24**:11403-11416.

Harrison, S.D., and A.A. Travers. 1990. The *tramtrack* gene encodes a *Drosophila* finger protein that interacts with the *ftz* transcriptional regulatory region and shows a novel embryonic expression pattern. *EMBO J.* **9**:207-216.

Hatano, M., C.W. Roberts, M. Minden, W.M. Crist, and S.J. Korsmeyer.

1991. Deregulation of a homeobox gene, HOX11, by the t(10;14) in T cell leukemia. *Science* **253:**79-82.

Hatano, M., C.W.M. Roberts, T. Kawabe, J. Shutter, and S. J. Korsmeyer. 1992. Cell cycle progression, cell death and T cell lymphoma in HOX11 transgenic mice. *Blood* **80(suppl.):**1412.

Hayashi, S., and M.P. Scott. 1990. What determines the specificity of action of *Drosophila* homeodomain proteins? *Cell* **63:**883-894.

Herr, W., R.A. Sturm, R.G. Clerc, L.M. Corcoran, D. Baltimore, P.A. Sharp, H.A. Ingraham, M.G. Rosenfeld, M. Finney, G. Ruvkun, and R. Horvitz. 1988. The POU domain, a large conserved region in the mammalian pit-1, oct-1, oct-2, and *Caenorhabditis elegans unc-86* gene products. *Genes & Dev.* **2:**1513-1516.

Herskowitz, I. 1989. A regulatory hierarchy for cell specialization in yeast. *Nature* **342:**749-757.

Hill, C.S., and R. Treisman. 1995. Transcriptional regulation by extracellular signals: mechanisms and specificity. *Cell* **80:**199-211.

Hiromi, Y., A. Kuroiwa, and W.J. Gehring. 1985. Control elements of the *Drosophila* segmentation gene *fushi tarazu. Cell* **43:**603-613.

Hiromi, Y., and W.J. Gehring. 1987. Regulation and function of the *Drosophila* segmentation gene *fushi tarazu. Cell* **50:**963-974.

Hoey, T., and M. Levine. 1988. Divergent homeobox proteins recognize similar DNA sequences in *Drosophila. Nature* **332:**858-861.

Howard, K., and P. Ingham. 1986. Regulatory interactions between the segmentation genes *fushi tarazu*, *hairy*, and *engrailed* in the *Drosophila* blastoderm. *Cell* **44:**949-957.

Hunter, T., and M. Karin. 1992. The regulation of transcription by phosphorylation. *Cell* **70:**375-387.

Hunter, T. 1995. Protein kinases and phosphotases: the yin and yang of protein phosphorylation and signaling. *Cell* **80:**225-236.

Hyduk, D., and A. Percival-Smith. 1996. Genetic characterization of the homeodomain-independent activity of the *Drosophila fushi tarazu* gene product. *Genetics* **142:**481-492.

Ingham, P.W., and A. Martinez-Arias. 1986. The correct activation of *Antennapedia* and *bithorax* complex genes requires the *fushi tarazu* gene. *Nature* **324:**592-597;

Ingham, P.W. 1988. The molecular genetics of embryonic pattern formation in *Drosophila. Nature* **335:**25-34.

Ingham, P.W., N. Baker, and A. Martinez-Arias. 1988. Regulation of segment polarity genes in the *Drosophila* blastoderm by *fushi tarazu* and *even skipped. Nature* **331:**73-75.

Ingraham, H.A., R. Chen, H.J. Mangalam, H.P. Elsholtz, S.E. Flynn, C.R. Lin, D.M. Simmons, L. Swanson, and M.G. Rosenfeld. 1988. A tissue-specific transcription factor containing a homeodomain specific pituitary phenotype. *Cell* **55:**519-529.

Ingraham, H.A., S.E. Flynn, J.W. Voss, V.R. Albert, M.S. Kapiloff, L. Wilson and M.G. Rosenfeld. 1990. The POU-specific domain of Pit-1 is essential for sequence-specific, high affinity DNA binding and DNA-dependent Pit-1-Pit-1 interaction. *Cell* **61:**1021-1033.

Ish-Horowicz, D., S.M. Pinchin, P.W. Ingham, and H.G. Gyurkovics. 1989. Autocatalytic *ftz* activation and metameric instability induced by ectopic *ftz* expression. *Cell* **57:**223-232.

Jaffe, L., H. Ryoo, and R.S. Mann. 1997 A role for phosphorylation by casein kinase II in modulating *Antennapedia* activity in *Drosophila. Genes & Dev.* **11:**1327-1340.

Janknecht, R., and T. Hunter. 1996. A growing coactivator network. *Nature* **383:**22-23.

Jaynes, J.B., and P.H. O'Farrell. 1988. Activation and repression of transcription by homeodomain-containing proteins that bind a common site. *Nature* **336:**744-749.

Johnson, F.B. E. Parker, and M. Krasnow. 1995. Extradenticle protein is a selective cofactor for the *Drosophila* homeotics: role of the homeodomain and YPWM amino acid motif in the interaction. *Proc. Natl. Acad. Sci. USA* **92:**739-743.

Kalderon, D., and G. M. Rubin. 1988. Isolation and characterization of *Drosophila* cAMP-dependent protein kinases genes. *Genes & Dev.* **2:**1539-1556.

Kalionis, B., and P.H. O'Farrell. 1993. A universal sequence is bound *in vitro* by diverse homeodomains. *Mech. Dev.* **43:**57-70.

Kamps, M.P., A.T. Look, and D. Baltimore. 1991. The human t(1; 19) translocation in pre-B ALL produces multiple nuclear E2A-Pbx1 fusion proteins with differing transforming potentials. 1991. *Genes Dev.* **5:**358-368.

Kamakaka,R.T., M. Bulger, and J.T. Kadonaga.1993. Potentiation of RNA polymerase II transcription by Gal4-VP16 during but not after DNA replication and chromatin assembly. *Genes Dev.* **7:**1779-1795.

Kapiloff, M.S., Y. Farkash, M. Wegner, and M.G. Rosenfeld. 1991. Variable effects of phosphorylation of Pit-1 dictated by DNA response elements. *Science* **253:**786-789.

Karin, M. 1994. Signal transduction from the cell surface to the nucleus through the phosphorylation of transcription factors. *Curr. Opin. Cell Biology* **6:**415-424.

Karlsson, O., S. Thor, T. Norberg, H. Ohlsson, and T. Edlund. 1990. Insulin gene enhancer binding protein isl-1 is a member of a novel class of proteins containing both a homeo- and a Cys-His domain. *Nature* **344:**879-882.

Kassis, J.A. 1990. Spatial and temporal control elements of the *Drosophila engrailed* gene. *Genes Dev.* **3:**433-443.

Keleher, C.A., C. Goutte, and A.D. Johnson. 1988. The yeast cell-type-specific repressor *a*2 acts cooperatively with a non-cell-type-specific protein. *Cell* **53:**927-935.

Keleher, C.A., S. Passmore, and A.D. Johnson. 1989. Yeast repressor *a*2 binds to its operator cooperatively with yeast protein Mcm1. *Mol. Cell. Biol.* **9:**5228-5230.

Kellerman, K.A., D.M. Mattson, and I. Duncan. 1990. Mutations affecting the stability of the *fushi tarazu* protein of *Drosophila. Genes & Dev.* **4:**1936-1950.

Kilcherr, F., S. Baumgartner, D. Bopp, E. Frei, and M. Noll. 1986. Isolation of the *paired* gene of *Drosophila* and its spatial expression during early embryogenesis. *Nature* **321:**493-499.

Kissinger, C.R., B. Liu, E. Martin-Blanco, T.B. Kornberg, and C.O. Pabo. 1990. Crystal structure of an *engrailed* homeodomain-DNA complex at 2.8 A resolution: a framework for understanding homeodomain-DNA interactions. *Cell* **63:**579-590.

Klemm, J.D., M.A. Rould, R. Aurora, W. Herr, and C.O. Pabo. 1994. Crystal structure of the Oct-1 POU domain bound to an octamer site: DNA recognition with tethered DNA-binding modules. *Cell* **77:**21-32.

Kornberg, T.B., and T. Tabata. 1993. Segmentation of the *Drosophila* embryo. *Curr. Opin. Gen. Dev.* **3:**583-593.

Korzus, E., J. Torchia, D.W. Rose, L. Xu, R. Kurokawa, E.M. McInerney, T.M. Mullen, C.K.

Glass, and M.G. Rosenfeld. 1998. Transcription factor-specific requirements for coactivators and their acetyltransferase functions. *Science* **279:**703-707.

Kowlessur, D., X. Yang, and S. Kaufman. 1995. Further studies of the role of Ser-16 in the regulation of the activity of phenylalanine hydroxylase. *Proc. Natl. Acad. Sci. USA* **92:**4743-4747.

Krasnow, M.A., E.E. Saffman, K. Kornfeld, and D.S. Hogness. 1989. Transcriptional activation and repression by *Ultrabithorax* proteins in cultured *Drosophila* cells. *Cell* **57:**1031-1043.

Krause, H.M., R. Klemenz, and W.J. Gehring. 1988. Expression, modification, and localization of the *fushi tarazu* protein in *Drosophila* embryos. *Genes & Dev.* **2:**1021-1036.

Krause, H.M., and W.J. Gehring. 1988. The location, modification, and function of the *fushi tarazu* protein during *Drosophila* embryogenesis. *Progress in Clinical & Biological Research* **284:**105-123.

Krause, H.M., and W.J. Gehring. 1989. Stage-specific phosphorylation of the *fushi tarazu* protein during *Drosophila* development. *EMBO J.* **8:**1197-1204.

Kuroiwa, A., E. Hafen, and W.J. Gehring. 1984. Cloning and transcriptional analysis of the segmentation gene *fushi tarazu* of *Drosophila. Cell* **37:**825-831.

Kwok, R.P., J.R. Lundblad, J.C. Chrivia, J.P. Richards, H.P. Bachinger, R.G. Brennan, S.G. Roberts, M.R. Green, and R.H. Goodman. 1994. Nuclear protein CBP is a coactivator for the transcription factor CREB. *Nature* **370:**223-226.

LaCasse, E.C., and Y.A. Lefebvre. 1995. Nuclear localization signals overlap DNA- or RNA-binding domains in nucleic acid-binding proteins. *Nucleic Acids Res.* **23:**1647-1656.

Lai, Z., S.D. Harrison, F. Karim, Y. Li, and G.M. Rubin. 1996. Loss of *tramtrack* gene activity results in ectopic R7 cell formation, even in a *sina* mutant background. *Proc. Natl. Acad. Sci. USA* **93:**5025-5030.

Lalli, E., and P. Sassone-Corsi. 1994. Signal transduction and gene regulation: the nuclear response to cAMP. *J. Biol. Chem.* **269:**17359-17362.

Lane, M. E., and D. Kalderon. 1993. Genetic investigation of cAMP-dependent protein kinase function in *Drosophila* development. *Genes & Dev.* **7:**1229-1243.

Laughon, A., and M.P. Scott. 1984. Sequence of a *Drosophila* segmentation gene: Protein structure homology with DNA-binding proteins. *Nature* **310:**25-31.

Laughon, A. 1991. DNA binding specificity of homeodomains. *Biochemistry* **30:**11357-11367.

Lavorgna, G., H. Ueda, J. Clos, and C. Wu. 1991. FTZ-F1, a steroid hormone receptor-like protein implicated in the activation of *fushi tarazu*. *Science* **252:**848-851.

Lavorgna, G., F.D. Karim; C.S. Thummel, and C. Wu. 1993. Potential role for a FTZ-F1 steroid receptor superfamily member in the control of *Drosophila* metamorphosis. *Proc. Natl. Acad. Sci. USA* **90:**3004-3008.

Lawrence, P.A., and G. Morata. 1983. The elements of the *bithorax* complex. *Cell* **35:**595-601.

Lawrence, P.A., P. Johnston, P. Macdonald, and G. Struhl. 1987. Borders of parasegments in *Drosophila* embryos are delimited by the *fushi tarazu* and *even-skipped* gene. *Nature* **328:**440-442.

Lawrence, P.A. 1988. The present status of the parasegment. *Development* **104(suppl):**61-65.

Lawrence, P.A. 1992. The making of a fly. Blackwell Scientific Publications. Oxford.

Lehmann, R. and D. Tautz.1994. *In situ* hybridization to RNA. In Goldstein,L.S.B. and E.A. Fyrberg, E.A. (ed.), *Drosophila melanogaster: Practical uses in cell and molecular biology.* Academic Press, Inc., San Diego, USA, pp. 576-597.

Levine, M., and T. Hoey. 1988. Homeobox proteins as sequence-specific transcription factors. *Cell* **55:**537-540.

Lewis, E.B. 1978. A gene complex controlling segmentation in *Drosophila. Nature* **276:**565-570.

Li, S., E.B. Crenshaw, E.J. Rawson, D.M. Simmons, L.W. Swanson, and M.G. Rosenfeld. 1990. Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene pit-1. *Nature* **347:**528-533.

Li, S., Y. Li, R.W. Carthew, and Z. Lai. 1997. Photoreceptor cell differentiation requires regulated proteolysis of the transcriptional repressor Tramtrack. *Cell* **90:**469-478.

Li, T., M.R. Stark, A.D. Johnson, and C. Wolberger. 1995. Crystal structure of the MATa1/MATα2 homeodomain heterodimer bound to DNA. *Science* **270:**262-269.

Lin, L., and W. McGinnis. 1992. Mapping functional specificity in the *Dfd* and *Ubx* homeo domains. *Genes & Dev.* **6:**1071-1081.

Lin, S.C., S. Li., D.W. Drolet, and M.G. Rosenfeld. 1994. Pituitary ontogeny of the Snell dwarf mouse reveals Pit-1-independent and Pit-1-dependent origins of the thyrotrope. *Development* **120:**515-522.

Lin, R., P. Beauparlant, C. Makris, S. Meloche, and J. Hiscott. 1996. Phosphorylation of IκBa in the C-terminal PEST domain by casein kinase II affects intrinsic protein stability. *Mol. Cell. Biol.* **16:**1401-1409.

Lindsley, D.L., and G.G. Zimm. 1992. *The genome of Drosophila melanogaster*. Academic Press, San Diego, USA. p37-38.

Liu, Z., R.L. Galindo, and S.A. Wasserman. 1997. A role for CKII phosphorylation of the Cactus PEST domain in dorsoventral patterning of the *Drosophila* embryo. *Genes & Dev.* **11:**3413-3422.

Lopez, A.J., and D.S. Hogness. 1991. Immunochemical dissection of the *Ultrabithorax* homeoprotein family in the *Drosophila melanogaster. Proc. Natl. Acad. Sci. USA* **88:**9924-9928.

Lu, Q., and M. Kamps. 1996. Structural determinants of Pbx1 mediating cooperative DNA-binding with pentapeptide-containing HOX proteins. *Mol. Cell Biol.* **16:**1632-1640.

MacDonald, P.M., and G. Struhl. 1986. A molecular gradient in early *Drosophila* embryos and its role in specifying the body pattern. *Nature* **324:**537-545.

Macias, A., S. Pelaz, and G. Morata. 1994. Genetic factors controlling the expression of the *abdominal-A* gene of *Drosophila* within its domain. *Mech. Dev.* **46:**15-25

Maier, D., A. Preiss, and J.R. Powell. 1990. Regulation of the segmentation gene *fushi tarazu* has been functionally conserved in *Drosophila. EMBO J.* **9:**3957-3966.

Maier, D., D. Sperlich, and J.R. Powell. 1993. Conservation and change of the developmentally crucial *fushi tarazu* gene in *Drosophila. J. Mol. Evol.* **36:**315-326.

Mak, A., and A.D. Johnson. 1993. The carboxy-terminal tail of the homeodomain protein alpha2 is required for function with a second homeodomain protein. *Genes & Dev.* **7:**1862-1870.

Malicki, J., K. Schughart, and W. McGinnis. 1990. Mouse Hox-2.2 specifies thoracic segmental identity in *Drosophila* embryos and larvae. *Cell* **63:**961-967.

Manak, J.R., and M.P. Scott. 1994. A class act: conservation of homeodomain protein functions. *Development* **(Suppl):**61-71.

Mangelsdorf, D.J., C Thummel, M. Beato, P. Herrlich, G. Schütz, K. Umesono, B. Blumberg, P. Kastner, M. Mark, P. Chamborn, and R.M. Evans. 1995. The nuclear receptor superfamily: the second decade. *Cell* **83:**835-839.

Mann, R.S. and D.S. Hogess. 1990. Functional dissection of *Ultrabithorax* proteins in *D. melanogaster*. *Cell* **60:**597-610.

Mann, R.S. 1995. The specificity of homeotic gene function. *BioEssays* **17:**855-863.

Mann, R.S., and S.K. Chan. 1996. Extra specificity from *extradenticle*: the partnership between HOX and PBX/EXD homeodomain proteins. *Trends Genet.* **12:**258-62

Mann, R.S., and M. Abu-Shaar. 1996. Nuclear import of the homeodomain protein Extradenticle in response to Decapentaplegic and Wingless signaling. *Nature* **383:**630-633.

Manoukian, A.S., and H.M. Krause. 1992. Concentration-dependent activities of the *even-skipped* protein in *Drosophila* embryos. *Genes & Dev.* **6:**1740-1751.

Martinez-Arias, A., and P.A. Lawrence. 1985. Parasegments and compartments in the *Drosophila* embryo. *Nature* **313:**639-642.

Martinez-Balbas, M.A., A.J. Bannister, K. Martin, P. Haus-Seuffert, M. Meisterernst, and T. Kouzarides. 1998. The acetyltransferase activity of CBP stimulates transcription. *EMBO. J.* **17:**2886-2893.

McCormick, A., H. Brady, L.E. Theill, and M. Karin. 1990. The pituitary specific homeobox gene GHF-1, is regulated by cell-autonomous and environmental cues. *Nature* **345:**829-832.

McGinnis, W., R.L. Garber, J. Wirz, A. Kuroiwa, and W.J. Gehring. 1984a. A homologous protein coding sequence in *Drosophila* homeotic genes and its conservation in other metazoans. *Cell* **38:**403-409.

McGinnis, W., M.S. Levine, E. Hafen, A. Kuroiwa, and W.J. Gehring. 1984b. A conserved DNA sequence in homeotic genes of the *Drosophila* Antennapedia and Bithorax complexes. *Nature* **308:**428-433.

McGinnis, N., M.A. Kuziora, and W. McGinnis. 1990. Human Hox-4.2 and *Drosophila Deformed* encode similar regulatory specificities in *Drosophila* embryos and larvae. *Cell* **63:**969-976.

McGinnis, W., and R. Krumlauf. 1992. Homeobox genes and axial patterning. *Cell* **68:**283-302.

McGinnis, W. 1994. A century of homeosis, a decade of homeoboxes. *Genetics* **137:**607-611.

Mercurio, F., H.Y. Zhu, B.W. Murray, A. Shevchenko, B.L. Bennett, J.W. Li, D.B. Young, M. Barbosa, and M. Mann. 1997. IKK-1 and IKK-2- cytokine-activated IκB kinases essential for NF-κB activation. *Science* **278:**860-866.

Mitchell, P.J., and R. Tjian. 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* **245:**371-378.

Mlodzik, M., and W.J. Gehring. 1987. Expression of the *caudal* gene in the germ line of *Drosophila*: formation of an RNA and protein gradient during early embryogenesis. *Cell* **48:**465-478.

Montminy, M. 1997. Transcriptional regulation by cyclic AMP. *Annu. Rev. Biochem.* **66:**807-822

Morisato, D., and K.V. Anderson. 1995. Signaling pathways that establish the dorsal-ventral pattern of the *Drosophila* embryo. *Annu. Rev. Genet.* **29:**371-399.

Müller, M., M. Affolter, W. Leupin, G. Otting, K. Wüthrich, and W.J. Gehring. 1988. Isolation and sequence-specific DNA binding of the *Antennapedia* homeodomain. *EMBO J.* **7**:4299-4304.

Müller, J., and M. Bienz. 1992. Sharp anterior boundary of homeotic gene expression conferred by the fushi tarazu protein. *EMBO J.* **11**:3653-3661.

Nambu, P.A., and J.R. Nambu. 1996. The *Drosophila fish-hook* gene encodes a HMG domain protein essential for segmentation and CNS development. *Development* **122**:3467-75

Napper, S., J.M. Anderson, F. Georges, J.W. Quail, L.T.J. Delbaere, and E.B. Waygood. 1996. Mutation of serine-46 to aspartate in the histidine-containing protein of *Escherichia coli* mimics the inactivation by phosphorylation of serine-46 in HPrs from gram-positive bacteria. *Biochemistry* **35**:11260-11267.

Ner, S.S., and A.A. Travers. 1994. HMG-D, the *Drosophila melanogaster* homologue of HMG 1 protein, is associated with early embryonic chromatin in the absence of histone H1. *EMBO J.* **13**:1817-22.

Nolan, G.P., S. Ghosh, H.C. Liou, P. Tempst, and D. Baltimore. 1991. DNA binding and IκB inhibition of the cloned p65 subunit of NF-κB, a *rel*-related polypeptide. *Cell* **64**:961-969.

Norris, J.L., and J.L. Manley. 1992. Selective nuclear transport of the *Drosophila* morphogen *Dorsal* can be established by a signaling pathway involving the transmembrane protein *Toll* and protein kinase A. *Genes & Dev.* **6**:1654-1667.

Nüsslein-Volhard, C., and E. Wieschaus. 1980. Mutations affecting segment number and polarity in *Drosophila. Nature* **287**:795-801.

Odenwald, W.F., J. Garbern, H. Arnheiter, E. Tournier-Lasserve, and R.A. Lazzarini. 1989. The Hox-1.3 homeo box protein is a sequence-specific DNA-binding phosphoprotein. *Genes & Dev.* **3**:158-172.

Ogryzko, V.V., R.L. Schiltz, V. Russanova, B.H. Howard, and Y. Nakatani. 1996. The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* **87**:953-959.

Ohkuma, Y., M. Horikoshi, R.G. Roeder, and C. Desplan. 1990. Binding site-dependent direct activation and repression of *in vitro* transcription by *Drosophila* homeodomain proteins. *Cell* **61**:475-484.

Okimura, Y., P.W. Howard, and R.A. Maurer. 1994. Pit-1 binding sites mediate transcriptional responses to cyclic adenosine 3',5'-monophosphate through a mechanism that does not require inducible phosphorylation of Pit-1. *Mol. Endocrinology* **8**:1559-1565.

Pai, C.Y., T.S. Kuo, T.J. Jaw, E. Kurant, C.T. Chen, D.A. Bessarab, A. Salzberg, and Y.H. Sun. 1998. The Homothorax homeoprotein activates the nuclear localization of another homeoprotein, Extradenticle, and suppresses eye development in *Drosophila*. *Genes & Dev.* **12**:435-446.

Palombella, V.J., O.J. Rando, A.L. Goldberg, and T. Maniatis. 1994. The ubiquitin-proteasome pathway is required for processing the NF-κB1 precursor protein and the activation of NF-κB. *Cell* **78**:773-785.

Parker, D., K. Ferreri, T. Nakajima, V.J. LaMorte, R. Evans, S.C. Koerber, C. Hoeger, and M.R. Montminy. 1996. Phosphorylation of CREB at Ser-133 includes complex formation with CREB-binding protein via a direct mechanism. *Mol. Cell. Biol.* **16**:694-703.

Patel, N.H., E. Martin-Blanco, K.G. Coleman, S.J. Poole, M.C. Ellis, T.B. Kornberg, and C.S. Goodman. 1989a. Expression of *engrailed* proteins in Arthropods, Annelids, and Chordates. *Cell* **58**:955-968.

Patel, N.H., B. Schafer, C.S. Goodman, and R. Holmgren. 1989b. The role of segment polarity genes during *Drosophila* neurogenesis. *Genes & Dev.* **3**:890-904.

Patel, N.H. 1994. Imaging neuronal subsets and other cell types in whole-mount *Drosophila* embryos and larvae using antibody probes. In *Drosophila melanogaster*: Practical uses in cell and molecular biology. ed. L.S.B. Goldstein, and E.A. Fyrberg. Academic Press, San Diego, USA. pp445-487.

Payet, D., and A. Travers. 1997. The acidic tail of the high mobility group protein HMG-D modulates the structural selectivity of DNA binding. *J. Mol. Biol.* **266**:66-75.

Peifer, M., and E. Wieschaus. 1990. Mutations in the *Drosophila* gene *extradenticle* affect the way specific homeo domain proteins regulate segmental identity. *Genes & Dev.* **4**:1209-1223.

Percival-Smith, A., M. Muller, M. Affolter, and W.J. Gehring. 1990. The interaction with DNA of wild-type and mutant *fushi tarazu* homeodomains. *EMBO J.* **9**:3967-3974.

Perler, F., A. Efstratiadis, P. Lomedico, W. Gilbert, R. Kolodner, and J. Dodgon. 1980. The evolution of genes: the chicken preproinsulin gene. *Cell* **20**:555-566.
Peterson, M.G., J. Inostroza, M.E. Maxon, O. Flores, A. Admon, D. Reinberg, and R. Tjian. 1991. Structure and functional properties of human general transcription factor IIE. *Nature* **354**:369-373.

Peverali, F.A., A. Isaksson, A.G. Papavassiliou, P. Plastina, L.M. Staszewski, M. Mlodzik, and D. Bohmann. 1996. Phosphorylation of *Drosophila* Jun by the MAP kinase *Rolled* regulates photoreceptor differentiation. *EMBO J.* **15**:3943-3950.

Pfaffle, R.W., G.E. DiMattia, J.S. Parks, M.R. Brown, J.M. Wit, M. Jansen, H. Van Der Nat, J.L. Van Den Brande, M.G. Rosenfeld, and H.A. Ingraham. 1992. Mutation of the POU-specific domain of Pit-1 and hypopituitarism without pituitary hypoplasia. *Science* **257**:1118-1121.

Phelan, M.L., I. Rambaldi, and M.S. Featherstone. 1995. Cooperative interactions between HOX and PBX proteins mediated by a conserved peptide motif. *Mol. Cell. Biol.* **15**:3989-3997.

Pick, L., A. Schier, M. Affolter, T. Schmidt-Glenewinkel, and W.J. Gehring. 1990. Analysis of the *ftz* upstream element: germ layer-specific enhancers are independently autoregulated. *Genes & Dev.* **4**:1224-1239.

Poleev, A., O. Okladnova, A.M. Musti, S. Schneider, B. Royer-Pokora, and D. Plachov. 1997. Determination of functional domains of the human transcription factor PAX8 responsible for its nuclear localization and transactivating potential. *European J. Biochem.* **247**:860-869.

Pöpperl, H., M. Bienz, M. Studer, S.K. Chan, S. Aparicio, S. Brenner, R. Mann, and R. Krumlauf. 1995. Segmental expression of *Hoxb-1* is controlled by a highly conserved autoregulatory loop dependent upon *exd/Pbx*. *Cell* **81**:1031-1042.

Ptashne, M., and A. Gann. 1997. Transcriptional activation by recruitment. *Nature* **386**:569-577.

Qian, Y.Q., M. Billeter, G. Otting, M. Muller, W.J. Gehring, and K. Wüthrich. 1989. The structure of the *Antennapedia* homeodomain determined by NMR spectroscopy in solution: comparison with prokaryotic repressors. *Cell* **59**:573-580.

Qian, Y.Q., K. Furukubo-Tokunaga, D. Resendez-Perez, M. Muller, W.J. Gehring, and K. Wüthrich. 1994. Nuclear magnetic resonance solution structure of the *fushi tarazu* homeodomain from *Drosophila* and comparison with the *Antennapedia* homeodomain. *J. Mol. Biol.* **238**:333-345.

Radhakrishnan, I., G.C. Perez-Alvarado, D. Parker, H.J. Dyson, M.R. Montminy, and P.E. Wright. 1997. Solution structure of the KIX domain of CBP bound to the transactivation domain of CREB: a model for activator:coactivator interactions. *Cell* **91**:741-752.

Radovick, S., M. Nations, Y. Du, L.A. Berg, B.D. Weintraub, and F.E. Wondisford. 1992. A mutation in the POU-homeodomain of Pit-1 responsible for combined pituitary hormone deficiency. *Science* **257**:1115-1118.

Rauskolb, C., M. Peifer, and E. Wieschaus. 1993. *extradenticle*, a regulator of homeotic gene activity, is a homolog of the homeobox-containing human proto-oncogene *pbx1*. *Cell* **74:**1101-1112.

Reach, M., R.L. Galindo, P. Towb, J.L. Allen, M. Karin, and S.A. Wasserman. 1996. A gradient of *cactus* protein degradation establishes dorsoventral polarity in the *Drosophila* embryo. *Dev. Biol.* **180:**353-64.

Read, D., and J.L. Manley. 1992. Alternatively spliced transcripts of the *Drosophila tramtrack* gene encode zinc finger proteins with distinct DNA binding specificities. *EMBO J.* **11:**1035-1044.

Rechsteiner, M., and S.W. Rogers. 1996. PEST sequences and regulation by proteolysis. *Trends in Biochem. Sci.* **21:**267-271.

Rieckhof, G.E., F. Casares, H.D. Ryoo, M. Abu-Shaar, and R.S. Mann. 1997. Nuclear translocation of Extradenticle requires *homothorax*, which encodes an Extradenticle-related homeodomain protein. *Cell* **91:**171-183.

Riedl, A., and M. Jacobs-Lorena. 1996. Determinants of *Drosophila fushi tarazu* mRNA instability. *Mol. Cell. Biol.* **16:**3047-3053.

Riley, G.R., E.M. Jorgensen, R.K. Baker, and R.L. Garber. 1991. Positive and negative control of the *Antennapedia* promoter P2. *Development* **1(suppl):**177-85.

Roberts, C.W., J.R. Shutter, S.J. Korsmeyer. 1994. Hox11 controls the genesis of the spleen. *Nature* **368:**747-749.

Roberts, D.B. 1986. *Drosophila* a practical approach. IRL Press Limited. Oxford, England.

Roberts, S.B., N. Segal, and N. Heintz. 1991. Differential phosphorylation of the transcription factor Oct1 during the cell cycle. *Science* **253:**1022-1026.

Robertson, H.M., C.R. Preston, R.W. Phillis, D.M. Johnson-Schlitz, W.K. Benz, and W.R. Engels. 1988. A stable genomic source of P element transposase in *Drosophila melanogaster. Genetics* **118:**461-470.

Rogers, S., R. Wells, and M. Rechsteiner. 1986. Amino acid sequences common to rapidly degraded proteins: The PEST hypothesis. *Science* **234:**364-368.

Ronchi, E., J. Treisman, N. Dostatni, G. Struhl, and C. Desplan. 1993. Down-regulation of the *Drosophila* morphogen bicoid by the *torso* receptor-mediated signal transduction cascade. *Cell* **74:**347-355.

Rosfjord, E., B. Scholtz, R. Lewis, and A. Rizzino. 1995. Phosphorylation and DNA binding of the octamer binding transcription factor Oct-3. *Biochem. Biophy. Res. Comm.* **212:**847-53.

Rosenberg, A.H., B.N. Lade, D. Chui, S. Lin, J.J. Dunn, and F.W. Studier. 1987. Vectors for selective expression of cloned DNAs by T7 RNA polymerase. *Gene* **56:**125-135.

Roth, S., D. Stein, and C. Nüsslein-Volhard. 1989. A gradient of nuclear localization of the *dorsal* protein determines dorsoventral pattern in the *Drosophila* embryo. *Cell* **59:**1189-1202.

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

Rubin, G.M., and A.C. Spradling. 1983. Vectors for P element-mediated gene transfer in *Drosophila. Nucl. Acids Res.* **11:**6341-6351.

Rushlow. C.A., K. Han, J.L. Manley, and M. Levine. 1989. The graded distribution of the *dorsal* morphogen is initiated by selective nuclear transport in *Drosophila. Cell* **59:**1165-1177.

Sauer, F., S.K. Hansen, and R. Tjian. 1995. Multiple $TAF_{II}s$ directing synergistic activation of transcription. *Science* **270:**1783-1788.

Scherer, D.C., J.A. Brockman, Z. Chen, T. Maniatis, and D.W. Ballard. 1995. Signal-induced degradation of IkBa requires site-specific ubiquination. *Proc. Natl. Acad. Sci. USA* **92:**11259-11263.

Schier, A., and W.J. Gehring. 1992. Direct homeodomain DNA interaction in the autoregulation of the *fushi tarazu* gene. *Nature* **356:**804-807.

Schier, A., and W.J. Gehring. 1993a. Functional specificity of the homeodomain protein *fushi tarazu*: The role of DNA-binding specificity *in vivo*. *Proc. Natl. Acad. Sci. USA* **90:**1450-1454.

Schier, A., and W.J. Gehring. 1993b. Analysis of a *fushi tarazu* autoregulatory element: multiple sequence elements contribute to enhancer activity. *EMBO J.* **12:**1111-1119.

Schneuwly, S., R. Klemenz, and W.J. Gehring. 1987. Redesigning the body plan of *Drosophila* by ectopic expression of the homeotic gene *Antennapedia*. *Nature* **325:**815-818.

Scott, M.P., and A.J. Weiner. 1984. Structural relationships among genes that control development: Sequence homology between the *Antennapedia*, *Ultrabithorax*, and *fushi tarazu* loci of *Drosophila*. *Proc. Natl. Acad. Sci. USA* **81:**4115-4119.

Scott, M.P., J.W. Tamkun, and G.W. Hartzell III. 1989. The structure and function of the homeodomain. *Bioch. Biophy. Acta* **989:**25-48.

Scott, M.P. 1992. Vertebrate homeobox gene nomenclature. *Cell* **71:**551-553.

Scott, M.P. 1993. A rational nomenclature for vertebrate homeobox (HOX) genes. *Nucleic Acids Res.* **21:**1687-1688.

Segil, N., S.B. Roberts, T. Heintz. 1991. Mitotic phosphorylation of the Oct-1 homeodomain and regulation of Oct-1 DNA binding activity. *Science* **254:**1814-186.

Sen, R., and D. Baltimore. 1986. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell* **46:**705-716.

Sharkey, M., Y. Graba, and M.P. Scott. 1997. Hox genes in evolution: protein surfaces and paralog groups. *Trends Genet.* **13:**145-151.

Shepherd, J.C.W., W. McGinnis, A.E. Carasco, E.M. De Robertis, and W.J. Gehring. 1984. Fly and frog homeo domains show homology with yeast mating-type regulatory proteins. *Nature* **310:**70-71.

Shikama, N., J. Lyon, and N.B. La Thangue. 1997. The p300/CBP family: integrating signals with transcription factors and chromatin. *Trends Cell Biol.* **7:**230-236.

Shore, P., A.D. Sharrocks. 1995. The MADS-box family of transcription factors. *Eur. J. Biochem.* **229:**1-13.

Siegfried, E., L.A. Perkins, T.M. Capaci, and N. Perrimon. 1990. Putative protein kinase product of the *Drosophila* segment polarity gene, *zeste-white 3*. *Nature* **345:**825-829.

Siegfried, E., L. Ambrosio, and N. Perrimon. 1990b. Serine/threonine protein kinases in *Drosophila*. *Trends Genet.* **6:**357-362.

Simmons, D.M., J.W. Voss, H.A. Ingraham, J.M. Holloway, R. S. Broide, M.G. Rosenfeld, and L.W. Swanson. 1990. Pituitary cell phenotypes involve cell-specific Pit-1 mRNA translation and synergistic interactions with other classes of transcription factors. *Genes & Dev.* **4:**695-711.

Smith, D.L., and A.D. Johnson. 1992. A molecular mechanism for combinatorial control in yeast: MCM1 protein sets the spacing and orientation of the homeodomains of an alpha 2 dimer. *Cell* **68:**133-142.

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

St. Johnston, D., and C. Nüsslein-Volhard. 1992. The origin of pattern and polarity in the *Drosophila* embryos. *Cell* **68:**201-219.

Steward, R., S.B. Zusman, L.H. Huang, and P. Schedl. 1988. The *dorsal* protein is distributed in a gradient in early *Drosophila* embryos. *Cell* **55:**487-495.

Steward, R. 1989. Relocalization of the *dorsal* protein from the cytoplasm to the nucleus correlates with its function. *Cell* **59:**1179-1188.

Stroumbakis, N.D., and P.T. Tolias. 1994. Localized maternal and zygotic expression of the gene encoding *Drosophila* HMG D. *Biochem. Biophy. Acta* **1218:**245-249.

Struhl, G. 1985. Near-reciprocal phenotypes caused by inactivation or indiscriminate expression of the *Drosophila* segmentation gene *ftz*. *Nature* **318:**677-680.

Swope, D.L., C.L. Mueller, and J.C. Chrivia. 1996. CREB-binding protein activates transcription through multiple domains. *J. Biol. Chem.* **271:**28138-28145.

Tanaka, M., and W. Herr. 1990. Differential transcriptional activation by Oct-1 and Oct-2: interdependent activation domains induce Oct-2 phosphorylation. *Cell* **60:**375-386.

TenHarmsel, A., and M.D. Biggin. 1995. Bending DNA can repress an eukaryotic basal promoter and inhibit TFIID binding. *Mol. Cell. Biol.* **15:**5492-5498.

Thanos, D., and T. Maniatis. 1995. NF-κB: a lesson in family values. *Cell* **80:**529-532.

Topol, J., C.R. Dearolf; K. Prakash, and C.S. Parker. 1991. Synthetic oligonucleotides recreate *Drosophila fushi tarazu* zebra-stripe expression. *Genes & Dev.* **5:**855-867.

Tower, J., G.H. Karpen, N. Craig, and A.C. Spradling. 1993. Preferential transposition of *Drosophila* P elements to nearby chromosomal sites. *Genetics* **133:**347-359.

Traenckner, E.B., H.L. Pahl, T. Henkel, K.N. Schmidt, S. Wilk, and P.A. Baeuerle. 1995. Phosphorylation of human IκBa on serines 32 and 36 controls IκBa proteolysis and NF-κB activation in response to diverse stimuli. *EMBO J.* **14:**2876-2883.

Treisman, J., P. Gönczy, M. Vashishtha, E. Harris, and C. Desplan. 1989. A single amino acid can determine the DNA binding specificity of homeodomain proteins. *Cell* **59:**553-562.

Tsai, C., and P. Gergen. 1995. Pair-rule expression of the *Drosophila fushi tarazu* gene: a nuclear receptor response element mediates the opposing regulatory effects of *runt* and *hairy*. *Development* **121:**453-462.

Ueda, H., S. Sonoda, J.L. Brown, M.P. Scott, and C. Wu. 1990. A sequence-specific DNA-binding protein that activates *fushi tarazu* segmentation gene expression. *Genes & Dev.* **4:**624-635.

Um M., Li C., and J.L. Manley. 1995. The transcriptional repressor *even-skipped* interacts directly with TATA-binding protein. *Mol. Cell. Biol.* **15:**5007-5016.

Utley, R.T., K. Ikeda, P.A. Grant, J. Côté, D.J. Steger, A. Eberharter, S. John, and J.L. Workman. 1998. Transcriptional activators direct histone acetyltransferase complexes to nucleosomes. *Nature* **394:**498-502.

Van Antwerp, D.J., and I.M. Verma. 1996. Signal-induced degradation of IκBa: Association with NF-κB and the PEST sequence in IκBa are not required. *Mol. Cell. Biol.* **16:**6037-6045.

Van Dijk, M.A., and C. Murre. 1994. *extradenticle* raises the DNA binding specificity of homeotic selector gene products. *Cell* **78:**617-624.

Van Renterghem, P., S. Dremier, G. Vassart, and D. Christophe. 1995. Study of TTF-1 gene expression in dog thyrocytes in primary culture. *Mol. Cell. Endocrinology* **11:**83-93.

Verma, I.M., J.K. Stevenson, E.M. Schwarz, D.V Antwerp, and S. Miyamoto. 1995. Rel/NF-κB/IκB family: intimate tales of association and dissociation. *Genes & Dev.* **9:**2723-2735.

Vershon, A.K., and A.D. Johnson. 1993. A short, disordered protein region mediates interactions between the homeodomain of the yeast α2 protein and the MCM1 protein. *Cell* **72:**1-20.

Vider, B.Z., A. Zimber, D. Hirsch, D. Estlein, E. Chastre, S. Prevot, C. Gespach, A. Yaniv, and A. Gazit. 1997. Human colorectal carcinogenesis is associated with deregulation of homeobox gene expression. *Biochem. Biophy. Res. Comm.* **232:**742-748.

Voelker, R.A., A.L. Greenleaf, H. Gyurkovics, G.B. Wisely, S. Huang, and L.L. Searles. 1984. Frequent imprecise excision among reversions of a P element-caused lethal mutation in *Drosophila. Genetics* **107:**279-294.

Wakimoto, B.T., and T.C. Kaufman. 1981. Analysis of larval segmentation in lethal genotypes associated with the *Antennapedia* gene complex in *Drosophila melanogaster. Dev. Biol.* **81:**51-64.

Wakimoto, B.T., F.R. Turner, and T.C. Kaufman. 1984. Defects in embryogenesis in mutants associated with the *Antennapedia* gene complex of *Drosophila melanogaster. Dev. Biol.* **102:**147-172.

Wall, N.A., M. Jones, B.L.M. Hogan, and C.V.E. Wright. 1992. Expression and modification of Hox 2.1 protein in mouse embryos. *Mech. Dev.* **37:**111-120.

Wasserman, S.A. 1993. A conserved signal transduction pathway regulating the activity of the rel-like proteins dorsal and NF-κB. *Mol. Biol. Cell* **4**:767-771.

Weiner, A.J., M.P. Scott, and T.C. Kaufman. 1984. A molecular analysis of *fushi tarazu*, a gene in *Drosophila melanogaster* that encodes a product affecting embryonic segment number and cell fate. *Cell* **37**:843-851.

Weir M.P., and T. Kornberg. 1985. Patterns of *engrailed* and *fushi tarazu* transcripts reveal novel intermediate stages in *Drosophila* segmentation. *Nature* **318**:433-439.

Winslow, G.M., S. Hayashi, M. Krasnow, D.S. Hogness, and M.P. Scott. 1989. Transcriptional activation by the *Antennapedia* and *fushi tarazu* proteins in cultured *Drosophila* cells. *Cell* **57**:1017-1030.

Wilson, D.S., et al. 1996. Conservation and diversification in homeodomain-DNA interactions: A comparative genetic analysis. *Proc. Natl. Acad. Sci. USA* **93**:6886-91

Wolberger, C., A.K. Vershon, B. Liu, A.D. Johnson, and C.O. Pabo. 1991. Crystal structure of a *MATa2* homeodomain-operator complex suggests a general model for homeodomain-DNA interactions. *Cell* **67**:517-528.

Wu, H.K., H.H.Q. Heng, D.P. Siderovski, W.F. Dong, Y. Okuno, X.M. Shi, L.C. Tsui, and M.D. Minden. 1996. Identification of a human LIM-Hox gene, *hLH-2*, aberrantly expressed in chronic myelogenous leukaemia and located on 9q33-34.1. *Oncogene* **12**:1205-1212

Xue, D., Y. Tu, and M. Chalfie. 1993. Cooperative interactions between the *Caenorhabditis elegans* homeoproteins UNC-86 and MEC-3. *Science* **261**:1324-1328.

Yan, C., and J.A. Whitsett. 1997. Protein kinase A activation of the surfactant protein B gene is mediated by phosphorylation of thyroid transcription factor 1. *J. Biol. Chem.* **272**:17327-17332.

Yu, Y., and L. Pick. 1995. Non-periodic cues generate seven *ftz* stripes in the *Drosophila* embryo. *Mech. Dev.* **50:**163-175.

Yu, Y., W. Li, K Su, M. Yussa, W. Han, N. Perrimon, and L. Pick. 1997. The nuclear hormone receptor Ftz-F1 is a cofactor for the *Drosophila* homeodomain protein Ftz. *Nature* **385:**552-548.

Yuan, W., G. Condorelli, M. Caruso, A. Felsani, and A. Giordano. 1996. Human p300 protein is a coactivator for the transcription factor MyoD. *J. Biol. Chem.* **271:** 9009-9013.

Zandi, E., D.W. Rothwarf, M. Delhase, M. Hayakawa, and M. Karin. 1997. The IκB kinase complex (IKK) contains two kinase subunits, IKK-α and IKK-β, necessary for IκB phosphorylation and NF-κB activation. *Cell* **91:**243-252.

Zannini, M., A. Acebron, M.D. Felice, M.I. Arnone. 1996. Mapping and functional role of phosphorylation sites in the thyroid transcription factor-1 (TTF-1). *J. Biol. Chem.* **271:**2249-2254.

Zappavigna, V., D. Sartori, and F. Mavilio. 1994. Specificity of HOX protein function depends on DNA-protein and protein- protein interactions, both mediated by the homeo domain. *Genes & Dev.* **8:**732-744.

Zappavigna, V., L. Falciola, M.H. Citterich, F. Mavilio, and M.E. Bianchi. 1996. HMG1 interacts with HOX proteins and enhances their DNA binding and transcriptional activation. *EMBO J.* **15:**4981-4991.

Zeng, W., J. Deborah, A. Laura, D. Mathies, M.A. Horner, and M.P. Scott. 1993. Ectopic expression and function of the *Antp* and *Scr* homeotic genes: the N terminus of the homeodomain is critical to functional specificity. *Development* **118:**339-352.

Zhang, H., K.M. Catron, and C. Abate-Shen. 1996. A role for the Msx-1 homeodomain in transcriptional regulation: residues in the N-terminal arm mediate TATA binding protein interaction and transcriptional repression. *Proc. Natl. Acad. Sci. U.S.A.* **93:**1764-1769.

Zhang, P., and A.C. Spradling. 1993. Efficient and Dispersed local P element transposition from *Drosophila* females. *Genetics* **133:**361-373.

Zhao, J.J., R.A. Lazzarini, and L. Pick. 1993. The mouse Hox-1.3 gene is functionally equivalent to the *Drosophila Sex combs reduced* gene. *Genes & Dev.* **7:**343-354.

Zhu, A., and A.K. Kuziora. 1996. Homeodomain interaction with the β subunit of the general transcription factor TFIIE. *J. Biol. Chem.* **271:**20993-20996

Zwilling, S., H. Konig and H. Wirth. 1995. High mobility group protein 2 functionally interacts with the POU domains of octamer transcription factors. *EMBO J.* **14:**198-208.

Zwilling, S., A. Dieckmann, P. Pfisterer, P. Angel, and T. Wirth. 1997. Inducible expression and phosphorylation of coactivator BOB.1/BOF.1 in T cells. *Science* **277:**221-225.

Pulp & Paper Centre
University of Toronto
200 College Street, Room 420
Toronto Ontario Canada
M5S 3E5

Telephone: (416) 978-3062
Fax: (416) 971-2106
E-mail: paper@chem-eng.utoronto.ca
Internet: http://www.chem-eng.toronto.edu/~pphome

Ph.D. Examinations Office
School of Graduate Studies
University of Toronto

CC: The Graduate Coordinator of the Department of Chemical Engineering and Applied Chemistry

To Whom It May Concern:

This is to certify that Esa Vilen has completed the minor corrections to his thesis as recommended by the Examination Committee.

Dec 11/98

Douglas W. Reeve
Professor
Dept. of Chem. Eng. And Applied Chemistry