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Functional analysis of homeobox genes in *Drosophila*

Zhao, Jiagang, Ph.D.

City University of New York, 1993

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**FUNCTIONAL ANALYSIS OF HOMEBOX GENES
IN DROSOPHILA**

by

JIAGANG ZHAO

A dissertation submitted to the Graduate Faculty in
Biomedical Sciences, in partial fulfillment of the
requirements for the degree of Doctor of Philosophy,
The City University of New York

1993

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This manuscript has been read and accepted for the Graduate Faculty in Biomedical Science in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT**FUNCTIONAL ANALYSIS OF HOMEBOX GENES
IN DROSOPHILA**

by

Jiagang Zhao

Advisors : Robert A. Lazzarini and Leslie Pick

The main goals of this work are: to characterize the function of the mouse *Hox-1.3* gene; to analyze its evolutionary conservation; and to provide a novel strategy for studying genes involved in the embryogenesis of *Drosophila melanogaster*. The developmental system of *Drosophila melanogaster* has several characteristics that make it an excellent experimental subject. This study reflects an effort to combine biochemistry, molecular biology, and classical genetics tools to achieve the goals. Accordingly, the specific aims of my research are: (1) to examine the evolutionarily conserved relationships between the mouse *Hox-1.3* gene and its fly counterpart, *Sex combs reduced*; (2) to define the functional motifs of the mouse *Hox-1.3* gene in cultured cells and in the transgenic flies system; (3) to study Hox-1.3 protein-DNA interactions *in vitro*; and (4) to utilize a targeted ribozyme as a potential tool for studying gene function in *Drosophila*.

ACKNOWLEDGMENTS

To Dr. Robert A. Lazzarini, I am very grateful for the great freedom he allowed me to reorient the direction of my thesis project, for his patience, advice, high standard criticisms, and continuous support during past three and a half years. I feel that I am gaining the ability to be creative and independent on research under his advice. For the successful completion of my thesis, I am indebted especially to Dr. Leslie Pick. Her lectures on *Drosophila* homeotic genes, in the "Advanced molecular Biology" course, led me viewing homeobox containing genes of different species from an evolutionary point and inspired me to propose the principle thought frames for this dissertation. She has generously provided me a nice laboratory space and the whole fly research supplies for last one and half year. I also wish to thank the members of the Lazzarini and Pick laboratories for creating a diversity, stimulating, and friendly atmosphere. Finally, I would like to give my deepest appreciation to my family, especially to my wife, Ying Wu. Without their comprehension, inspiration and constant support, my graduate student life in New York City would be pale and much longer.

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CHAPTER 1

Introduction I

A fundamental challenge to biologists is to understand how organisms develop a characteristic body pattern. As a result of profound progresses in recombinant DNA technology, molecular biologists have begun to understand the major issues of development, such as the determination of cell fate, the differentiation of tissues, and emergence of organs and organ systems, at the level of selective turning on and off regulatory genes. Until recently, the advanced knowledge of pattern formation in the developing embryo has been obtained from *Drosophila melanogaster*, with its sophisticated classical genetics and many mutations affecting pattern formation. One prominent example of how genetics contributes to our knowledge of development is that homeotic mutations cause one body segment to be transformed into another in *Drosophila*. The mutated genes are known as homeotic genes. Each of these genes is expressed in a certain region of the embryo and controls the cell identity in that part of the embryo during *Drosophila* development. Most of these homeobox genes are clustered in two complexes, the *Antennapedia*-complex (ANT-C), and the *bithorax*-complex (BX-C), on chromosome 3 (reviewed in Gehring et al. 1986).

In 1984, it was discovered by cross-hybridization that a 180 bp coding sequence, known as the homeobox, was shared among three *Drosophila* genes *ftz*, *Antp* and *Ubx* (McGinnis et al. 1984a; Scott et al. 1984). The sequence has a coding capacity for a 60 amino-acid residue domain, known as the homeodomain (McGinnis et al. 1984b). The existence of a well conserved sequence in genes controlling embryonic development of *Drosophila* suggested that similar sequences may play a key role in morphogenesis in other species (McGinnis et al. 1984b). In fact, the homeobox motif is spread widely through three kingdoms: fungal, plant, and animal. The common ancestor might have arisen about one billion years ago (Vollbrecht et al. 1991). In unicellular yeast, the genes determining cell mating types, *MATa1* and *MAT α 2*, are homeobox-containing

genes (Shepherd et al. 1984; reviewed in Scott et al. 1989). In multicellular organisms, homeobox genes may have acquired new developmental features to control spatial patterning in certain time periods during embryogenesis. It has been postulated that homeobox genes are integrated into a regulatory hierarchy. In the hierarchy, they are expressed in defined regions of embryos under the control of other regulatory genes (that may or may not be homeobox genes). Their products, then, regulate the expression of downstream regulatory genes which could act on the local morphogenesis control genes. The most complete analysis has been done in *Drosophila*, because of its sophisticated classical genetics and many mutations in homeobox-containing genes that affect pattern formation.

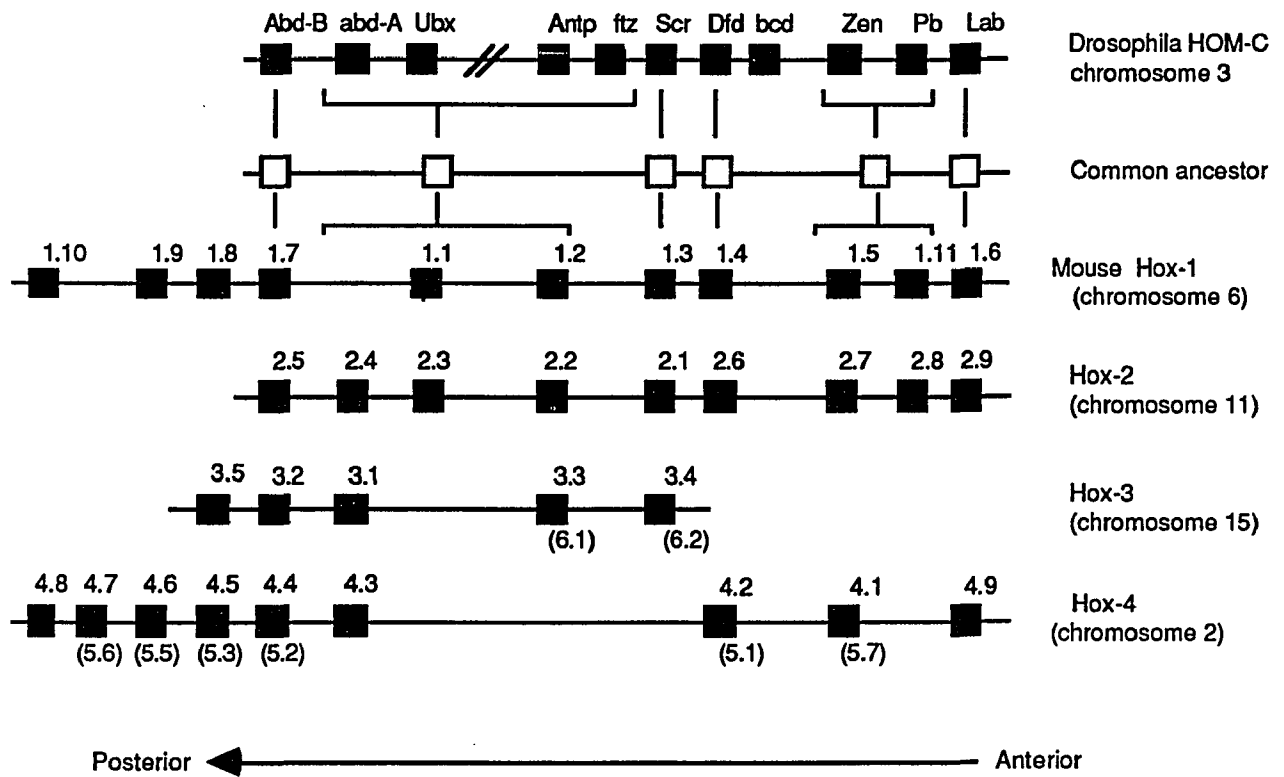
Mouse Hox genes and clusters

Since homeotic-like mutants are not found naturally in mice, the murine homeobox-containing genes have been cloned solely by homology searches using *Drosophila* homeobox sequences. To date, about 50 genes have been found in the murine genome. Based on sequence similarity, they fall into several classes such as *Antennapedia*-like *Hox* genes, *paired*-like *Pax* genes, and *engrailed*-like *En* genes. Another divergent family of homeodomain proteins initially characterized as including transcription factors later proved to contain a homeodomain as well as a 75 amino-acid residue POU (Pit/Oct/Unc) specific domain. Both domains form the POU-domain shared by the members of family (reviewed in Rosenfeld 1991).

Virtually all known murine *Hox* genes are tightly compacted in four gene clusters (*HOX*-1, -2, -3, and -4 clusters) on chromosome 6, 11, 15, and 2 respectively. Notably, conservation of this organization is extended to humans. Based on sequence similarity, the members of the four clusters can be classified into cognate subfamilies. Since the corresponding positions of members in a cognate subfamily are also relatively conserved, the clusters can be aligned in parallel (depicted in Figure 1-1). The exact

size of these clusters is not known yet, but most genes in one cluster are compacted in a relatively short genomic region. For instance, the *Hox-1* gene cluster (*Hox-1.1*, *-1.2*, *-1.3*, *-1.4*, *-1.5*, *-1.11* and *Hox-1.6*) spans only about a 70 kbp region of chromosome 6. Interestingly, Krumlauf and Duboule independently observed that the *Drosophila* HOM-C locus (including the homeobox gene clusters ANT-C and BX-C) can be aligned with murine *HOX* cognate groups on the basis of the homeobox sequences (Duboule et al. 1989; Graham et al. 1989). Similar homeobox cluster structures are also found in the nematode *Caenorhabditis elegans* (Bürglin et al. 1991) and the beetle *Tribolium*, which diverged from *Drosophila* 300 million years ago or more (Beeman 1987; Stuart et al. 1991). It is thought that the cluster structures were formed by two duplication events during evolution. First, an ancestral homeobox gene was duplicated successively to form the cluster on one chromosome of an early metazoan. Then, at some time during the evolution of vertebrates, duplications of the cluster occurred, resulting in four clusters located on different chromosomes. For instance, the human *Hox-4.2* shows about 36% identity in sequence of the whole protein and 55/61 amino acid identity for the homeodomain with *Drosophila Deformed (Dfd)* gene (McGinnis et al. 1990). Thus, the protein similarity between the two species must have been maintained for about 600 million years. Besides the homeodomain, some regions of the proteins, such as the amino end sequence as well as the residues adjacent to the homeodomain, are also conserved. One controversy is that the *Drosophila* genes are compacted into two complexes which are separated by a large chromatin fragment on chromosome three. However, the red flour beetle *Tribolium castaneum*, a more primitive insect than *Drosophila*, and the nematode *Caenorhabditis elegans* have only a single homeotic cluster HOM-C which contain the ANT-C and BX-C equivalent genes (Beeman 1987; Bürglin et al. 1991; Stuart et al. 1991). Therefore, a split HOM-C of certain insects may reflect a more recent evolutionary event than the divergence between vertebrates and insects.

Figure 1-1. Putative evolutionary relationships between mouse *Hox* clusters and the *Drosophila* HOM complex. Based on figures from Graham et al. (1989), Kessel and Gruss (1990), and Hunt et al. (1991).



Expression patterns of Hox genes during mouse embryogenesis

Based mainly on expression patterns of *Hox* genes detected by *in situ* hybridization, it has been postulated that homeobox genes may define regional specificities in mouse development (Holland et al. 1988; Kessel et al. 1990). Like *Drosophila* homeobox genes, murine *Hox* genes also display region specific expression patterns along the anterior-posterior axis. The order of genes within a cluster is correlated with the anterior boundaries of their expression region. The messenger RNA has been detected as early as in pre-somitic embryos. The highest level of expression is usually observed during day 9 to day 12 of embryogenesis. Later, *Hox* RNA levels go down, but remain relatively abundant in the central nervous system. In adult mice, many organs still contain detectable amounts of *Hox* mRNAs. Since the RNA level does not always directly correlate to the protein level, there still remain questions about when and where the *Hox* genes function. One way to localize *Hox* proteins is immunohistochemistry using antibodies against the most divergent peptides in the protein (Odenwald et al. 1987). Nevertheless, cross-reactivity is a major setback for using this approach.

Interactions between the homeodomain and DNA

The first evidence of homeodomain containing DNA binding activity came from studying the *Drosophila engrailed* protein (Desplan et al. 1985). Since then, several groups have reported similar DNAase I protection results for different homeodomain-containing proteins from different organisms (Fainsod et al. 1987; Beachy et al. 1988; Hoey et al. 1988; Desplan et al. 1988; Müller et al. 1988; Odenwald et al. 1989).

The homeodomain consists of three alpha helices and a flexible N-terminal arm. The most convincing structural data comes from the NMR spectroscopy study of *Antennapedia* homeodomain-DNA complex and the crystallographic analysis of *engrailed* homeodomain-DNA complex (Qian et al. 1989; Kissinger et al. 1990; Otting et al. 1990). The DNA fragments used in both cases contain the critical sequence 5'-A-T-

T-A-3' which exists in virtually all homeodomain protein binding sites (Odenwald et al. 1989). In the protein-DNA complex, helices 1 (10 to 21 residue) and 2 (28 to 38 residue) are anti-parallel to each other but do not contact the DNA backbone. The major role of these two helices is to stabilize the folded structure of the homeodomain in the complex with DNA. Helix 3 (42 to 58 residue) contacts the DNA major groove (according to NMR analysis, this long stretch region has two helices). A more flexible helix 4 (residues 53-59) bends slightly from helix 3 (Qian et al. 1989). It may provide additional stability to the DNA-protein complex. The four invariant residues, Trp48, Phe 49, Asn51 and Arg53 in the middle of the helix 3, are key residues for fitting in the major groove. Trp48 and Phe49, which are in the key part of the hydrophobic core, control the relative conformation of helices 1 and 3, thus stabilizing the folded structure for DNA fitting. Asn51 builds up strong hydrogen bonds with one of the adenine bases of the T-A-A-T sequence, and Arg53 forms hydrogen bonds with two phosphate groups on the opposite strand. In addition, in the *engrailed* model, Ile47 makes a hydrophobic interaction to the methyl group of the T base on the T-A-A-T subsequence, while van der Waals' forces attract the Gln50 side chain and the methyl group of the T base just to the 3' side of the A-T-T-A subsequence. These two residues are conserved within subsets of the homeodomain proteins. The model is consistent with the genetic analysis on the DNA recognition of the *bicoid* homeodomain (Hanes et al. 1989). Their work showed that the residue Lys9 (equal to Gln50 of *engrailed* homeodomain) of the third helix is critical for the sequence-specificity recognition by the *bicoid* protein and must contact the neighboring base of the A-T-T-A motif. The another interesting aspect of the homeodomain-DNA complex is that the N-terminal arm (9 residues) reaches into the minor groove. This arm is flexible in the absence of DNA. The residue, Arg5, which is conserved in most of homeodomain proteins, directly contacts a thymidine base within the consensus motif by hydrogen bonds. But in the case of *Antp* homeodomain, it touches a base adjacent to T-A-A-T. This difference may also reflect binding

specificities of different classes of homeodomain proteins. Since most of these contacts are located in the major and minor grooves around the T-A-A-T consensus sequence, it is understandable why this motif is so common in many homeodomain binding sites.

Sequence specific DNA-binding and transcriptional activities of homeodomain proteins

So far, all homeodomain proteins have been localized to the nucleus. The genetic analyses of their regulatory functions and *in vitro* biochemical studies on their DNA-binding capability suggested that these proteins could act as transcriptional factors. A number of experiments have been done to test this hypothesis. Transcription factors frequently contain two functional protein domains: one for sequence-specific binding activity, the other for stimulating or preventing RNA polymerase from transcribing adjacent genes.

The *in vitro* DNA-binding studies of homeodomain proteins have demonstrated the conclusively that the homeodomain is involved in the sequence-specific DNA-binding function of these proteins (Hall et al. 1987; Müller et al. 1988). The genetic analysis by Porter and Smith also indicated that the homeodomain is necessary for the function of the *MAT α 2* protein *in vivo* (Porter et al. 1986). They found mutations which affected the repressor function of the *MAT α 2* protein corresponded to amino acid residues conserved between *MAT α 2* and *Antp* homeodomains. Nevertheless, homeodomain proteins show an apparently loose sequence-specific recognition capability and low affinity DNA binding *in vitro*.

The analyses of binding specificity by different *Drosophila* homeodomain proteins indicated that many proteins bind similar sequences (Desplan et al. 1988; Hoey et al. 1988). As pointed out above, an ATTA core sequence exists in virtually all of the binding sites with various flanking sequences. The estimated binding sites could be a vast number in the whole genome. In fact, however, these proteins function as very specific

regulators *in vivo* and only a few genes are their targets. The abnormal phenotypes of the homeobox gene mutations have been only observed in restricted regions, organs, segments, and at certain developmental pathways. How could the specificity of binding be achieved *in vivo*?

First, nucleosomes of chromatin may not allow occupation by homeodomain proteins at both major and minor grooves of specific binding sites. This physical obstruction may limit the number of suitable binding sites (Kissinger et al. 1990). Second, additional factors may increase the binding affinity by interacting with homeodomain proteins at particular sites. The dimerizations of some proteins may also increase the binding affinity to specific sites in certain circumstances (Ingraham et al. 1990). Finally, the residues outside of the homeodomain may modulate binding specificity (Gibson et al. 1990).

There is evidence that the sequence of the homeodomain is important for the DNA binding specificity. Hoey et al. (1988) swapped the *eve* and *en* homeodomains and showed that the new binding specificity of the chimeric proteins more closely resembled the binding specificity of the homeodomain donor protein. Treisman et al. (1989) have shown that it is possible to change binding specificity of the *prd* protein by changing one amino acid at position 9 in helix 3. The wild-type *prd* has a serine residue at this position and does not bind to the *en* consensus binding site. When glutamine was substituted for serine, the mutated *prd* protein bound well to the *en* consensus binding site. They have also shown that substitution of serine for lysine changes the binding specificity of the *prd* protein to that of *bicoid* protein. A recent genetic analysis clearly demonstrated that residues in the N-terminals of homeodomains of *Drosophila* *Dfd* and *Ubx* proteins are also important for their targeting specificities (Lin et al. 1992). These experiments indicate that different homeodomains have preferences for different binding sequences and suggest that this may partly provide the regulatory specificity in the embryo.

The evidence for the trans-acting activity of the homeodomain was first obtained from analyses of three mammalian proteins Pit-1, Oct-1, and Oct-2. They all share a conserved POU domain which contains a divergent homeodomain and a POU-specific domain (Herr et al. 1988). These proteins were initially identified as transcription factors. Oct-1 and Oct-2 recognize the same octamer motif, but Oct-1 is a ubiquitous protein implicated in regulation of a variety of cellular genes and Oct-2 is a lymphoid-specific regulator for transcription of immunoglobulin genes. Pit-1 is a pituitary-specific factor that regulates transcription of the growth hormone and prolactin genes. The specific promoters were activated *in vitro* by purified proteins in reconstituted transcription systems (Scheidereit et al. 1987; Nelson et al. 1988; Fletcher et al. 1989).

Besides POU proteins, *Drosophila* homeodomain proteins were also shown to regulate transcription by co-transfection experiments in *Drosophila* cultured cells. The results are consistent with regulatory models deduced by earlier genetic analyses. It has been known that autoregulation and cross-regulation are two apparent roles of *Drosophila Antennapedia* class homeodomain proteins. For instances, *Antp* and *Ubx* proteins each can activate their own promoter in cultured cells, suggesting that both genes are auto-activated (Krasnow et al. 1989; Winslow et al. 1989). Nevertheless, *Ubx* protein is a dual functional transcription factor and can also repress an *Antp* promoter (P1) in co-transfection experiments. Since the embryo is a totally different environment from cultured cells, there is no guarantee that interactions seen in cultured cells are also present in the embryo. The *even-skipped* (*eve*) protein is a possible activator of the engrailed gene in embryo (Harding et al. 1986). Han et al., however, found that the *engrailed* promoter containing the putative *eve* target sites was repressed by *eve* protein (Han et al. 1989).

A disadvantage of the co-transfection technique is that it is unable to distinguish whether a homeodomain protein acts on its putative target promoter directly or indirectly.

The more convincing data has been drawn from studies of transcription in a cell free system. The purified *Drosophila eve* protein repressed transcription of the *Ubx* promoter in *in vitro* assays (Biggin et al. 1989).

The functions of mouse Hox genes

The mammalian *Hox* gene products are suspected to be important regulators of embryogenesis because they are homeodomain proteins, but direct supporting evidence is only beginning to emerge. The lack of progress stems from the poor knowledge of mammalian regulatory hierarchies in development. Unlike *Drosophila* genetics, which provides a firm biological basis for studying gene interactions, mammalian genetics has generated few insights into the black box of the regulatory hierarchy.

Since no *Hox* locus mutant has been found directly in nature, the functions of the *Hox* genes can only be inferred. Although profound differences exist in the embryogenesis of the mouse and the fruit fly, the anterior boundaries of the expression domains of *Hox* genes coincide with boundaries in the hindbrain of central nervous system, according to a rather regular pattern (Wilkinson et al. 1989). The hindbrain consists of clear repetitive segmental structures known as rhombomeres at a certain stage of embryonic development. This striking parallels of expression patterns between the vertebrate *Hox* and insect homeotic gene clusters indicate that *Hox* genes may be also important for pattern formation in mouse embryogenesis as their counterparts are in *Drosophila*. The experiment of swapping genes between mouse and *Drosophila* clearly supported this notion (Malicki et al. 1990; McGinnis et al. 1990; Awgulewitsch et al. 1992; Malicki et al. 1992). The results also suggest that similarities between the two species are not only at the protein structural level but also extend to the interaction with the general transcriptional apparatus and other regulators. Most recently, recessive mutations in murine *Hox-1.5*, *Hox-1.6*, and *Hox-3.1* loci were generated by homologous recombinations in embryonic stem cells which were, then, used to produce homozygous

Hox-1.5, *Hox-3.1*, and *Hox-1.6* null mutant mice (Chisaka et al. 1991; Lufkin et al. 1991; Mouellic et al. 1992). The results demonstrated that *Hox* genes do specify regional information of the mouse embryo and in one case, the *Hox-3.1* null mutant mice display obvious anterior transformations in skeletal segments. However, knocking out *Hox-1.5* and *Hox-1.6* genes did not cause significant anterior transformations in mice as those seen in the recessive loss-of-function mutants of *Drosophila*. Some organs and tissues, where *Hox-1.5* and *Hox-1.6* mRNAs have been detected in wild-type mice, were dramatically affected in the homozygous mutant mice, but some were still normal. This suggests that there may be functional redundancy among the *Hox* cognates. The functional overlap may be restricted only along the antero-posterior axis. One report showed that *Hox* genes from different clusters seem to have different dorsoventral patterns of expression (Graham et al. 1991).

The patterns of expression and phenotypes of a limited number of mutant mice, however, do not address how the regulatory network of *Hox* genes works. To have a real molecular understanding of the roles of *Hox* genes, important questions such as whether the *Hox* proteins are real transcription factors, how to define the target genes of *Hox* proteins, and how different patterns of gene expression are established during development, have to be solved.

Previous studies on *Hox-1.3* gene

The *Hox-1.3* gene consists of two exons and one intron (Odenwald et al. 1987; Fibi et al. 1988). It belongs to the HOX-1 cluster on chromosome 6. The 270 amino-acid residue *Hox-1.3* protein is rich in proline, serine, and glycine and undergoes phosphorylation (Odenwald et al. 1989). The homeodomain is located between residues 195 and 254. Its N-terminal sequence MSSYFVN and the IYPWM pentapeptide are conserved among many vertebrate *Hox* genes and *Drosophila* genes.

The distribution of *Hox-1.3* is progressively restricted during early mouse embryogenesis. In very early embryos, it is expressed in nearly all cells (Tani et al. 1989). In midgestation embryos, *Hox-1.3* mRNA is expressed at high levels in the mesoderm and neuroectoderm with an anterior boundary near the junction of the hindbrain and spinal cord (Dony et al. 1987; Fibi et al. 1988). In adults, the mRNA and protein have also been detected in some tissues including liver, lung, kidney, and central nervous system (Odenwald et al. 1987; Garbern et al. 1989). The protein expression has been also found in the cultured cells through immunostaining (Odenwald et al. 1987).

It has been demonstrated that *Hox-1.3* containing nuclear extract from a baculovirus expression system has a sequence-specific binding activity. The footprint assay identified an 80 bp region in its own proximal promoter. It was speculated that the *Hox-1.3* gene may be autoregulated, since a number of homeobox genes have been shown to be under the control of autoregulation in the fruitfly. These include *ftz* (Hiromi et al. 1987), *Dfd* (Kuziora et al. 1988), and *Ubx* (Bienz et al. 1988). The consensus DNA binding motif that includes an ATTA core sequence is present in the *Hox-1.3* gene promoter, as well as in its own intron, in other *Hox* gene promoters, and in some viral cis-regulatory elements identified by the gel-retardation assays (Odenwald et al. 1989; Tournier-Lasserre et al. 1989). It is reasonable to postulate that a gene (not necessarily a *Hox* gene) transiently turns on the *Hox-1.3* gene and the *Hox-1.3* product maintains its own expression and regulates other cellular genes. However, to date there has been no direct evidence that the *Hox-1.3* protein modulates gene expression *in vivo* via interacting with this motif.

Another interesting aspect of the *Hox-1.3* gene is that its product accumulates in the nucleus of non-confluent cultured cells but remains in the cytoplasm of confluent culture cells. Immunostaining of adult murine CNS tissue with anti-*Hox-1.3* specific

antibody was also observed in the nucleus and cytoplasm of neuron cells (Odenwald et al. 1987). A similar cytoplasmic retention phenomenon has been found for the *Drosophila dorsal* protein and a mammalian transcription factor NF- κ B. The *dorsal* protein is thought to be retained in the cytoplasm of cleavage stage embryos by the product of the *cactus* gene, but is redistributed as a nuclear dorso-ventral gradient at the cellular blastoderm stage (Rushlow et al. 1989). NF- κ B was first identified as a tissue-specific transcription factor in nuclei of B cells; then, its inactive form was found in non-B cells. NF- κ B is inactivated and retained in the cytosol by an inhibitor protein, I κ B (reviewed in Lenardo et al. 1989).

The specific activity of the *Hox-1.3* promoter has been studied in transgenic mice (Zakany et al. 1988; Tuggle et al. 1990). It was demonstrated that a 912 bp of 5' flanking sequence of the *Hox-1.3* gene could direct *lacZ* reporter gene expression specifically in the spinal cord, while the 308 bp proximal sequence is inactive. The remaining 604 bp fragment, interestingly, could function in either orientation and direct the expression of a mouse hsp68 promoter-*LacZ* fusion gene to the spinal cord. The result indicates that enhancer elements located between -234 to -894 bp of the *Hox-1.3* promoter are sufficient to confer specific expression in the spinal cord of mouse embryos.

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CHAPTER 2

The Mouse Hox-1.3 Gene Is Functionally Equivalent To The Drosophila Sex Combs Reduced Gene

(accepted with minor revisions for publication
in Genes & Development)

Abstract

To test whether the mouse *Hox-1.3* gene is a cognate of *Drosophila Sex combs reduced* (*Scr*) gene, we inserted a *hsp70-Hox-1.3* fusion gene into the *Drosophila* genome. Transgenic flies displayed *Scr*-like homeotic transformations after ectopic expression of *Hox-1.3* induced by heat shock. In larvae, the thoracic segments T2 and T3 are transformed towards T1. In adults, head structures are dramatically disrupted, including transformation of the antenna towards leg. Transformations are not due to ectopic activation of the endogenous *Scr* gene. Rather, *Hox-1.3* appears to directly regulate *Scr* target genes, as demonstrated by the ectopic activation of *forkhead* by *Hox-1.3*. The results suggest that mouse *Hox-1.3* can not only functionally substitute for *Drosophila Scr* in the determination of external structures but also participate in the regulatory hierarchy of insect organogenesis.

Introduction

The conserved homeobox sequences, first found in *Drosophila* regulatory genes, encode a 60 amino acid residue domain, known as the homeodomain (McGinnis et al. 1984a; McGinnis et al. 1984b; Scott et al. 1984). Substantial genetic evidence demonstrates that mutations in homeobox-containing genes can dramatically affect the development of *Drosophila*. Since homeotic mutations have not been found naturally in mice, the mouse homeobox-containing (*Hox*) genes were isolated solely on the basis of homology to *Drosophila* homeobox sequences. Most *Hox* genes are tightly linked in four gene clusters (*Hox-1*, *-2*, *-3*, and *-4* clusters) on chromosomes 6, 11, 15, and 2. Based upon

homeobox sequence similarity, two groups independently observed that the members of the four mouse *Hox* clusters can be aligned with the *Drosophila Antennapedia*-complex and *bithorax*-complex (collectively known as HOM-C) and classified into cognate subfamilies (Duboule et al., 1989; Graham et al., 1989; reviewed in McGinnis et al., 1992). Interestingly, the physical order of homeotic genes in *Drosophila* HOM-C corresponds to the order along the antero-posterior axis of the segments affected by the genes (reviewed in Akam, 1987). The correlation between regions of gene expression along the body axis and physical order along the chromosome is also conserved in mouse (Duboule et al. 1989; Graham et al. 1989).

In vitro and *in vivo* studies of *Drosophila* and mammalian homeodomain proteins indicate that they are transcription factors and that the homeodomain is required for nuclear targeting and sequence specific DNA-binding activity (Desplan et al. 1985; Fainsod et al. 1987; Hall et al. 1987; Desplan et al. 1988; Hoey et al. 1988; Müller et al. 1988; Kuziora et al. 1989; Odenwald et al. 1989; Treisman et al. 1989; Kissinger et al. 1990). These findings, along with the observation that mammalian *Hox* genes are expressed in a tissue-specific fashion during embryonic development, suggest that *Hox* genes may play important roles in controlling embryogenesis in vertebrates as do *Drosophila* homeobox-containing genes. This idea is strongly supported by recent results using homologous recombination to disrupt endogenous *Hox* genes in mice (Chisaka et al. 1991; Lufkin et al. 1991; Le Mouellic et al. 1992).

The *Hox-1.3* gene was initially identified by screening a mouse genomic library with an *Antp* homeobox probe under low stringency (Colberg-Poley et al. 1985). The gene was then characterized by Odenwald et al. (1987) and Fibi et al. (1988). It consists of two exons and one intron. The 270 amino-acid residue *Hox-1.3* ORF is rich in proline, serine, and glycine and is phosphorylated at multiple sites (Odenwald et al. 1989). The homeodomain is located between residues 195 and 254. The protein also contains an

octapeptide (MSSYFVNS) and a pentapeptide (IYPWM) that are conserved among many vertebrate and *Drosophila* homeobox genes (Odenwald et al. 1987). The distribution of *Hox-1.3* is progressively restricted during early mouse embryogenesis. In very early embryos, it is expressed in nearly all cells (Tani et al. 1989). In midgestation embryos, *Hox-1.3* mRNA is expressed at high levels in the mesoderm and neuroectoderm with an anterior boundary near the junction of the hindbrain and spinal cord (Dony et al. 1987; Fibi et al. 1988). In adults, the mRNA and protein have also been detected in some tissues including liver, lung, kidney, and central nervous system (Odenwald et al. 1987; Garbern et al. 1989). DNA-binding assays showed that *Hox-1.3* high-affinity binding sites contain an ATTA core motif (Odenwald et al. 1989) which is also the core of *Drosophila Antp* class high-affinity binding sites (reviewed in Scott et al., 1989). Nevertheless, the biological function of *Hox-1.3* is unknown.

If homeobox-containing genes play a basic role in embryonic development that is conserved throughout the animal kingdom, they would be under selective pressure to maintain certain functional specificities during evolution. One way to test this notion is to assay the ability of a given homeobox-containing gene from one organism to functionally substitute for a putative cognate gene in another organism. Two previous studies have shown that the mouse *Hox-2.2* and human *Hox-4.2* genes can carry out some of the functions of their *Drosophila* cognates when introduced into flies (Malicki et al. 1990; McGinnis et al. 1990).

We have chosen to use the mouse *Hox-1.3/Drosophila Scr* putative cognate pair as a model to examine the functional conservation of *Hox* genes at both morphological and regulatory levels. We have inserted the mouse *Hox-1.3* ORF into the *Drosophila* genome under the control of a *Drosophila* heat shock promoter (*hsp 70*) which gives rise to ubiquitous expression after heat treatment. Abnormalities induced by the ectopic expression of *Hox-1.3* protein during both embryonic and larval phases of development

were *Scr*-like. These results strongly suggest that the mouse *Hox-1.3* is the *bona fide* cognate of *Scr* but not the closely related and neighboring *Antennapedia* (*Antp*) gene. We further show that the mouse *Hox-1.3* gene can participate in *Drosophila* regulatory networks by activating the *forkhead* (*fkh*) gene, a downstream target of *Scr*. Thus, the functional properties of this cognate pair appear to have been conserved over 600 million years since the divergence of vertebrates and arthropods.

Results

Expression of the mouse *Hox-1.3* gene in transgenic flies

The mouse *Hox-1.3* and three *Drosophila* homeodomain proteins (*Scr*, *Antp*, and *Dfd*) are highly homologous in the homeodomain (Figure 2-1A). In addition, two other short regions (Odenwald et al. 1987), one at the amino terminus of the proteins (the octapeptide) and the other just adjacent to the homeodomain (the pentapeptide), are also highly conserved among these proteins. However, the overall similarity of the 270 amino acid *Hox-1.3* protein to the three is low: 28% (75 aa) to *Scr*; 26% (70 aa) to *Antp*; and 24% (66 aa) to *Dfd*. Because of the slightly higher degree of sequence similarity and the position of *Hox-1.3* in the *Hox-1* cluster, the mouse *Hox-1.3* was proposed to be a cognate of the *Scr* gene (Duboule et al. 1989; Graham et al. 1989).

We examined the potential *Scr*-like function of *Hox-1.3* protein in transgenic flies by placing the *Hox-1.3* gene under the control of a heat inducible promoter-*hsp70* (Figure 2-1B). The full-length *Hox-1.3* open reading frame (Odenwald et al. 1987) was inserted between the *Drosophila hsp70* promoter and trailer sequences in the P element vector CaSpeR-hs (Pirotta 1988) to create the fusion gene *hs-Hox-1.3*. The construct was inserted into the *Drosophila* genome by P element-mediated transformation (Rubin et al. 1982; Spradling et al. 1982). Ten *hs-Hox-1.3* independent transformant lines were generated and all described phenotypes were observed in at least three independent lines.

Figure 2-1. *Hsp70-Hox-1.3* transgene constructs and amino acid sequences comparison

(A) Alignment of mouse *Hox-1.3* amino acid sequences with three *Drosophila* homeotic gene products *Scr*, *Antp*, and *Dfd* (*Scr*, LeMotte et al., 1989; *Antp*, Schneuwly et al., 1986; *Dfd*, Regulski et al., 1987). Within the conserved domains, identical residues are marked with a dashed line. Homeodomain residues are in bold type. The asterisks indicate missing positions in terms of alignment. The dots represent large non-conserved regions (not to scale).

(B) Schematic representation of pCaSpeR-*hs-Hox-1.3* transgenic plasmids. The vector contains the mini white (w^+) gene flanked by the ends of the P-transposon (Pirodda 1988). The multiple cloning site separates the *hsp70* promoter (-245 to +207) from the *hsp70* trailer sequences (a 525 bp fragment containing polyadenylation site). The full length *Hox-1.3* cDNA was inserted into the polycloning sites (see Experimental Procedure). The orientations of *Hox-1.3* and *white* ORFs are indicated by arrows. Besides the homeodomain (indicated as HD), two other short conserved regions are indicated by "O" (the octapeptide) and "P" (the pentapeptide).

A

<i>Hox-1.3</i>	MSSY*FVNS		PQIYPWMRKLHI		GPE	GKRARTAYTRYQTLELE
<i>Scr</i>	----Q----		-----KRV-L		-*-	T--Q--S-----
<i>Antp</i>	-T--*-T--		-*L-----*****		N*-	R--G-QT-----
<i>Dfd</i>	---F*****		RI-----K-I-V		-MD	P--Q-----H-I----

<i>Hox-1.3</i>	KEFHFNRYLTRRRRIEIAHALCLSERQIKIWFQNRRMKWKKDN	KLKSM	..
<i>Scr</i>	-----T-----	EH	-MA-- ..
<i>Antp</i>	-----T-----	E-	-T-GE ..
<i>Dfd</i>	----Y-----	T-V-	--PNT ..

B

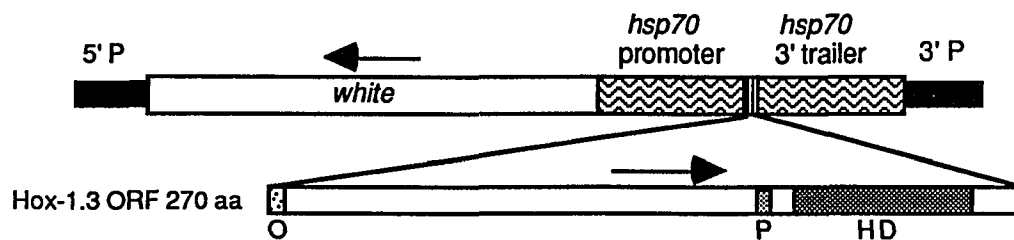


Figure 2-2. Induction of mouse *Hox-1.3* protein in transgenic embryos.

(A) No *Hox-1.3* protein is detected in the non-heat-shocked transgenic embryos at the germ band extension stage.

(B) Heat induced expression of *Hox-1.3* in *hs-Hox-1.3* bearing embryos. *Hox-1.3* protein was detected by immunostaining with anti-*Hox-1.3* antibodies after one hour recovery at 25°C following a one hour heat shock at 6 hr $\pm$ 30 min AEL. Note that the signal is located in the nuclei of all embryonic cells.

(C) Embryos were heat treated as in panel B, but immunostaining was carried out after 3 hr of recovery at 25°C.

**A****B****C**

Under normal growth condition (25°C), transformant embryos carrying *hs-Hox-1.3* do not express detectable levels of *Hox-1.3* proteins (Figure 2-2A). However, after a one hour heat shock, *Hox-1.3* protein products are distributed in most nuclei of the embryo (Figure 2-2B). The expressed *Hox-1.3* protein products persist in the embryos and are still abundant in embryos even after 3 hr of recovery at 25°C (Figure 2-2C). In order to rule out general effects of heat shock and protein ectopic expression, we also inserted a *Hox-1.3* gene fragment lacking the coding sequence for homeodomain and part of carboxyl terminus into the *Drosophila* genome. The protein products of the truncated form are detected in the cytoplasm of cell (shown in Figure 3-11A).

Homeotic transformations Induced by the ectopic expression of *Drosophila* *Scr*

In order to compare the morphological consequences induced by ectopic expression of the mouse *Hox-1.3* and *Drosophila* *Scr* genes in transgenic flies, we have re-examined the defects produced in *hs-Scr* flies. Here we expand the description of the induced phenotypes.

Larval transformations were induced optimally by a one hour heat shock (37°C) of *hs-Scr* bearing embryos at 6 hr $\pm$ 30 min after egg laying (AEL). The cuticular phenotypes were essentially the same as previously reported (Gibson et al. 1990). Briefly, more than 80% of heat-shocked *hs-Scr* cuticles display thoracic transformation, although to varying degrees. Compared to a wild type larva (Figure 2-3A), the transformation is marked by the unique T1 ventral beards appearing ectopically in the T2 and T3 segments (Figure 2-3B). After a one hour heat shock, most resulting *hs-Scr* larvae have the T2 towards T1 transformation, and 70% of them had T1 epidermal identity in both T2 and T3. Shorter heat shocks (e.g. 20 minutes) stimulate ectopic beards only in T2 (data not shown). Additional effects induced by ectopic expression of *Scr* are found in the head segments of resulting larvae. Head involution is disrupted and

the cephalopharyngeal skeleton is dramatically reduced. The extent of malformation in various head structures is variable and depends on the time and length of heat shock. Even though there is a strong transformation of T3, no transformation of abdominal segments was observed in these *hs-Scr* cuticles.

In the adult, morphological changes were induced by treating *hs-Scr* bearing larvae with multiple heat shocks starting at late second instar or early third instar larval stage, as described in Experimental Procedures. The result is largely consistent with a previous report (Gibson et al., 1990). Compared to a normal head (Figure 2-3C), the aberrant expression of *Scr* leads to a dramatic reduction of the compound eyes (observed in 42 of 45 flies), induces arista to tarsal transformation (or thickened arista, observed in 40 of 45 flies; Figure 2-3D), and causes disfigured maxillary palps, labial palps (observed in 24 of 45 flies, Figure 2-3E), and swollen occiputs with many thickened bristles in the hindhead (observed in 30 of 45 flies, Figure 2-3F). The occiput transformation is similar to a phenotype induced by ectopic expression of *Antp* (Schneuwly et al. 1987; Gibson et al. 1988) and a natural dominant mutation *Cephalothorax (Ctx)* in the *Antennapedia* complex (Duncan et al. 1982). The hindhead seems to be a sensitive and plastic region which can be modified to thoracic identity by both *Antp* and *Scr* genes.

Homeotic transformations induced by the ectopic expression of mouse Hox-1.3

To compare the phenotypic consequences of ectopically expressed mouse *Hox-1.3* to those of *Scr*, ~5.5-6.5 hr old *hs-Hox-1.3* bearing embryos were subjected to heat treatment at 37°C for one hour, as was done for *hs-Scr* bearing embryos. As shown in Figure 2-4B, the larvae display *Scr*-like transformations (compare to Figure 2-3B). Characteristically, mouse *Hox-1.3* induces the transformation of thoracic segments T2 and T3 towards T1. The ventral beard, a unique feature of T1, clearly appears in the T2 and T3 thoracic segments. The thoracic segment transformation induced in *hs-Hox-1.3*

animals is *Scr*-like, but not *Antp*-like since the ectopic expression of *Antp* causes the loss of the T1 ventral beard, an antagonistic transformation to that induced by *Scr* (Gibson et al., 1988; Gibson et al., 1990). Other obvious abnormalities caused by misexpression of *Hox-1.3* are the failure of head involution and the absence or misplacement of many cuticular head structures (e.g. epistomal sclerite, ectostomal and hypostomal sclerites, the pigmented H-piece, the mouth hooks, and sensory organs). Transformation of cuticular pattern is not detectable in abdominal segments. Embryos carrying the *Hox-1.3* fragment lacking homeodomain coding sequence are still able to hatch after heat shock and the larval cuticles are indistinguishable from that of wild-type larvae (shown in Figure 3-12E), as are those of non-heat shocked *hs-Hox-1.3* bearing embryos (Figure 2-4A).

To test if the ectopic expression of *Hox-1.3* could induce *Scr*-like homeotic transformations in adult animals, *hs-Hox-1.3* bearing larvae were subjected to one or more heat treatments at varying stages of development. Ectopic expression of *Hox-1.3* reproduced all *Scr*-like transformations in the external head structures of transgenic flies (Figure 2-5A, compare to Figure 2-3D). Although some flies eclosed after heat treatment, many of the phenotypes were observed in animals that were dissected from pupal cases. The abnormalities include reduced and irregular compound eyes (observed in 157 of 203 flies), malformation of mouth parts (observed in 91 of 203 flies), and thickening of the arista (observed in 102 of 203 flies). We have often observed a *Ctx*-like transformation in the hindhead (155 of 203 flies). Intriguingly, the antenna is transformed towards a relatively well formed leg (observed in 42 of 203 flies, Figure 2-5B). For *hs-Hox-1.3* animals, this antenna towards leg transformation could be induced by even a single one hour heat shock between 63 and 93 hr AEL (observed in 10 of 65 flies). Occasionally, structures strongly resembling sex comb teeth were seen on the well-transformed antennal legs (Figure 2-5C), suggesting that the ectopic legs have T1 leg identity. In contrast to this, ectopic expression of *Antp* (*hs-Antp*) induces transformation of antenna towards T2 leg, as indicated by the presence of an apical

bristle on the ectopic leg (Schneuwly et al. 1987; Gibson et al. 1988) . We have never observed an apical bristle on the *hs-Hox-1.3* induced ectopic legs. In some *hs-Hox-1.3* adult heads, the maxillary palps were severely affected and split into two segments (Figure 2-5D). This morphological consequence is similar to the transformation previously described for *hs-Dfd* and *hs-Hox4.2* transgenic flies (McGinnis et al. 1990). As for the embryonic phenotype, no transformation was observed in embryos carrying the *Hox-1.3* fragment lacking the homeodomain (data not shown). Thus these transformations are not due to non-specific effects of heat shock. In addition, the homeodomain is required for all transformations observed.

Hox-1.3 action is not mediated by *Drosophila* homeotic genes

Since *Hox-1.3* can cause *Scr*-like homeotic transformations of posterior thoracic segments towards T1 in embryos and *Scr*- and *Dfd*-like defects in the adult head, we investigated whether the induced defects might result from activation of the endogenous *Scr* and/or *Dfd* genes. For these purposes, we compared the localizations of *Scr* and *Dfd* proteins in treated *hs-Hox-1.3* embryos with wild-type embryos by immunostaining. In wild-type embryos, *Scr* protein is present in the anterior compartment of T1, in the labial segment, and in a small group of cells in the posterior region of the maxillary segment at the germ-band-extension stage (Riley et al. 1987; LeMotte et al. 1989). *Dfd* protein is localized to the maxillary and mandibular segments (Jack et al. 1988).

In heat shocked *hs-Hox-1.3* bearing embryos, *Scr* and *Dfd* expression appears normal. No expansion of expression of *Scr* or *Dfd* in either posterior or anterior direction has been observed (Figure 2-6A and 2-6B), although there are abundant *Hox-1.3* protein products in the whole embryo (see Figure 2-2C). We conclude that the endogenous *Scr* and *Dfd* genes are not activated by *Hox-1.3* in heat treated embryos. Furthermore, *Antp* gene expression was normal in heat shocked *hs-Hox-1.3* bearing embryos (data not shown). Similar experiments were carried out to test the role of endogenous *Scr* in

Figure 2-3. Transformations resulting from ectopic expression of *Drosophila Scr*.

(A) Wild type first instar larva cuticular structures. The arrow indicates the ventral beard which marks the first thoracic segment (T1).

(B) Morphological consequences induced by ectopic expression of *Scr* in *hs-Scr* bearing embryos. Embryos were subjected to a one hour heat shock at 6 hr $\pm$ 30 min AEL and allowed to develop to larvae. Authentic and ectopic T1 beards of a first instar larva are indicated by the arrows.

(C) Wild type adult head.

(D) An example of phenotypes induced in the adult head by misexpression of *Scr* during larval development. See text for details.

(E) The malformed mouthparts of a heat-shocked *hs-Scr* bearing animal. Note that the maxillary palps are severely disfigured (arrows).

(F) The dorsal head of a heat-shocked *hs-Scr* bearing animal. The arrows indicate the Cephalothorax-like transformation.

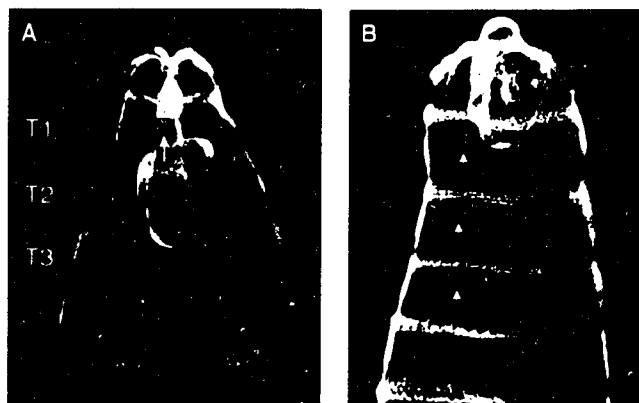


Figure 2-4. Ectopic expression of mouse *Hox-1.3* induces segmental transformations in *Drosophila* larvae

(A) Cuticular pattern of a non-heat-shocked *hs-Hox-1.3* bearing first instar larva. There is no transformation detected (compare to the wild type larva in Figure 3A). The T1 beard is indicated by an arrow.

(B) Embryos carrying the *hs-Hox-1.3* fusion gene were subjected to a one hour heat shock at 6 hr $\pm$ 30 min AEL at 37°C and allowed to develop to larvae. A typical first instar larva is shown. Authentic and ectopic T1 beards are indicated by the arrows.

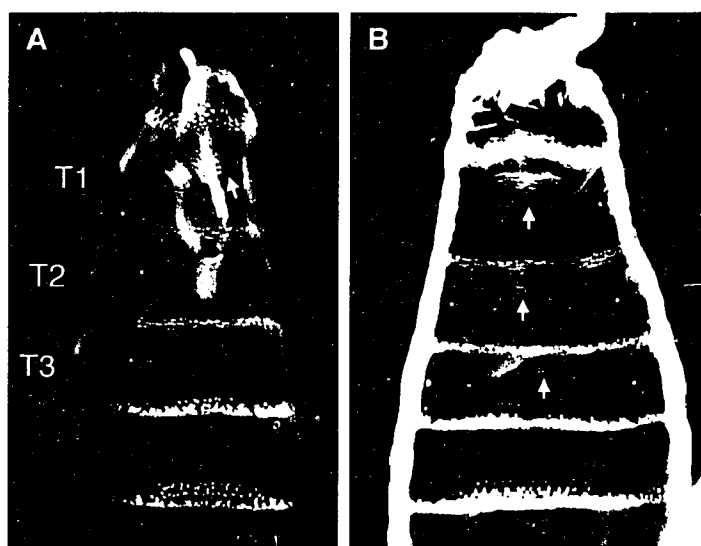


Figure 2-5. Adult phenotypes induced by ectopic expression of mouse *Hox-1.3*.

(A) Adult head phenotypes induced by misexpression of *hs-Hox-1.3*. Larva carrying the *hs-Hox-1.3* fusion gene were subjected to multiple heat shocks (see Experimental Procedure). Typical transformations shown include the thickening of the arista, small compound eyes, irregular shape of maxillary palps, and *Cephalothorax*-like transformation on the dorsal head.

(B) One example of transformation of antenna towards leg in a *hs-Hox-1.3* fly. Arrows indicate the ectopic legs.

(C) High-power showing a sex comb tooth (indicated by the arrow) on an ectopic leg.

(D) Malformations of mouth parts induced by improper expression of *Hox-1.3*. Note the maxillary palps are split into two segments (pointed out by arrows).

A



B



D

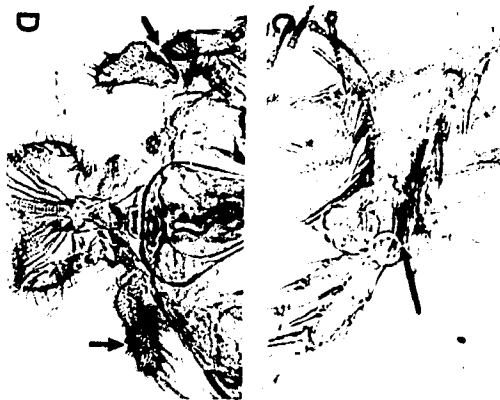


Figure 2-6. The expression patterns of *Drosophila Scr* and *Dfd* are not affected by *Hox-1.3*. Embryos carrying the *hs-Hox-1.3* fusion gene were subjected to a one hour heat shock at 6 hr $\pm$ 30 min AEL at 37°C and allowed to recover at 25°C for 3 hr (for *Scr* immunostaining) and 2 hr (for *Dfd* immunostaining). Expression patterns of *Scr* (A) and *Dfd* (B) proteins were detected by immunostaining. No difference from wild type patterns was detected. For panels C and D, larvae were subjected to two one-hour heat shocks at the third instar larval stage (see Materials and methods). No ectopic expression of *Scr* was detected in the eye-antenna imaginal disc (C), while expression in T1 leg discs was normal (D). Thus, ectopic expression of *Hox-1.3* did not induce the endogenous *Scr* gene.

A



B



C



D



inducing the observed adult phenotypes. Larvae were subjected to two heat shocks, as described above. Although strong transformations were observed in adults in these experiments, no ectopic expression of *Scr* was observed in eye-antennal discs (Figure 2-6C). In addition, we have observed *Hox-1.3* induced embryonic transformations in *Scr* animals (data not shown). These results are consistent with the lack of genetic evidence that the endogenous *Scr* gene is autoregulated and strongly suggest that the *Hox-1.3* is acting directly to regulate the downstream targets of *Scr*.

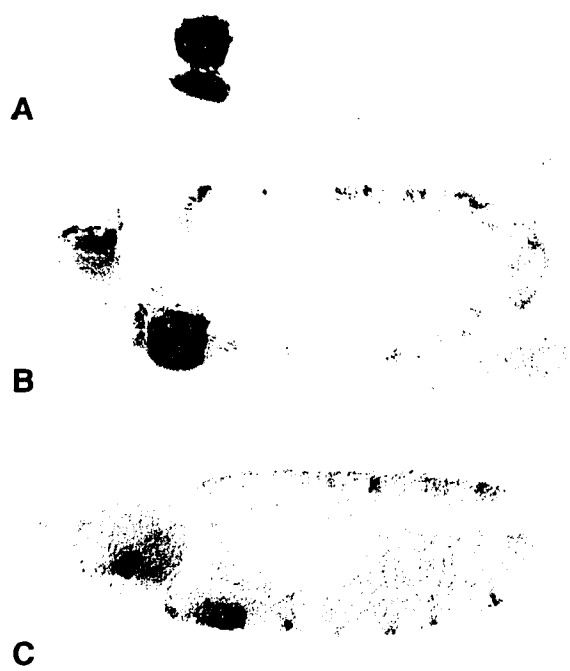
Hox-1.3* activates forkhead (*fkh*), a target gene of *Scr

Drosophila Scr is expressed in the primordia of the salivary gland and is required for its development (Panzer et al. 1992). One of the *Scr* target genes involved in this process is *fkh*, a transcription factor participating in the development of the salivary gland and gut (Weigel et al. 1990). In *Scr* null mutant embryos, no *fkh* expression and no salivary placode are detected, while misexpression of *Scr* induces ectopic salivary placodes in the procephalon, in parasegments 1 and 3, and in A8 (Panzer et al. 1992). A *fkh-lacZ* fusion gene containing a *fkh* salivary gland specific enhancer element directs *lacZ* expression specifically in salivary placodes of early embryos (strain p[ry⁺, HNK01], Panzer et al., 1992; and Figure 2-7A). This fusion gene responds to *Scr* overexpression as does the endogenous *fkh* gene (see Figure 2-7B and Panzer et al., 1992). To test the ability of mouse *Hox-1.3* to participate in the salivary gland regulatory pathway of *Drosophila*, we crossed the *hs-Hox-1.3* strain with p[ry⁺, HNK01]. After a 30 min heat shock of 1-4 hr old hybrid embryos and recovery at 25°C for 6 hours, ectopic expression of the *fkh-lacZ* fusion gene is observed. β -galactosidase is detected ectopically in the procephalon, and small patches in ventrolateral parts of almost all other parasegments (Figure 2-7C). This expression pattern is very similar to that induced by ectopic *Scr* expression (compare to Figure 2-7B). Thus, the mouse *Hox-1.3* gene mimics the effects of *Scr* in regulating the expression of the *fkh* gene.

Figure 2-7. Ectopic expression of a *fkh-lacZ* fusion gene induced by misexpression of mouse *Hox1.3*.

(A) The expression of the *fkh-lacZ* fusion gene in HNK01 transformant embryos was detected with anti- β -galactosidase antibodies. Note the prominent band of expression in the salivary gland primordia.

(B, C) Hybrid embryos carrying either *hs-Scr* and *fkh-lacZ* (B) or *hs-Hox-1.3* and *fkh-lacZ* (C) were subjected to a 30 min heat shock at 1-4 hr AEL at 37°C and allowed to develop at 25°C for another 6 hr. *fkh-lacZ* expression is detected in patches that extend to the posterior segments of the embryo in each case. Embryos at the germ-band extension stage are shown (lateral view).



Discussion

The ectopic expression of mouse *Hox-1.3* mimics *Scr* homeotic transformations

In this report, we show that the mouse *Hox-1.3* can induce specific homeotic transformations when ubiquitously expressed in *Drosophila*. These transformations are similar to those morphological consequences induced by ectopic *Scr* gene expression.

After a 60 min heat shock to germ-band extended embryos, the first instar larvae bearing either *hs-Scr* or *hs-Hox-1.3* display a prominent abnormality in the thoracic region. The two posterior segments (T2 and T3) gain T1 epidermal identity evidenced by the presence of ectopic ventral beards. This transformation must result from the presence of *Scr/Hox-1.3* proteins in the whole thoracic region which reprograms the fate of ventral beard precursor cells, and possibly additional thoracic precursors, by overriding the effects of *Antp* and *Ubx* activities in this region. These results suggest that there must be strict selective pressure to maintain the *Scr* expression pattern within sharp boundaries. Other morphological abnormalities are displayed mainly in head segments including the failure of head involution and the absence or misplacement of head structures. However, these alterations may not reflect the functional specificity of *Scr* and *Hox-1.3*, since ectopic expressions of other *Drosophila* homeotic selector genes and a mouse cognate of *Antp* (*Hox-2.2*) also generated similar abnormalities (Gibson et al. 1988; Kuziora et al. 1988; Malicki et al. 1990; Mann et al. 1990).

More dramatic homeotic transformations are generated in adult animals after heat treatment of the larvae, the most striking being a transformation of antenna towards leg. These legs appear to correspond to T1 legs since sex comb teeth were displayed in a few *hs-Hox-1.3* male pupae. This result strongly indicates that the transformation is *Scr* specific since one of the functions of *Scr* is to produce sex combs on T1 legs of male flies (Pattatucci et al. 1991). The transformation of antenna towards leg by *Hox-1.3* is

stronger than that induced by *Scr* and is detected with a higher frequency (observed in 42 of 203 flies examined). This rather surprising observation may be explained by the increased viability of *hs-Hox-1.3* flies as compared to *hs-Scr* flies following heat shock. Under equivalent conditions, we found that more *hs-Hox-1.3* flies develop to adult than do *hs-Scr* flies. This most likely results from the mouse *Hox-1.3* having lost functions present in the *Drosophila Scr* that prevent development during larval and pupal stages. Thus the potential for stronger transformation of antenna towards leg is uncovered in animals which can develop to adult.

In addition to antennal transformations, both *Scr* and *Hox-1.3* induce a *Cephalothorax*-like transformation in adult flies (swollen occiputs with many dark ectopic bristles in the dorsal aspect of hind head; Duncan et al., 1982; Schneuwly et al., 1987; Gibson et al., 1988). At present time, we are unable to determine the segmental identity of this transformation and thus cannot distinguish between *Antp* and *Scr* induced effects on these structures. Unlike dominant *Scr* mutant males that have ectopic sex comb teeth on both T2 and T3 legs (Pattatucci et al., 1991), *hs-Scr* and *hs-Hox-1.3* adult animals do not exhibit a posterior leg towards foreleg transformation. Perhaps the timing or persistence of heat induced expression differs from that of the *Scr* protein produced in dominant gain of function mutants. It is possible that in these mutants a rearranged cis-acting regulatory element leads to ectopic expression of *Scr* exclusively in the primordial cells of the T2 and T3 leg imaginal discs that have the potential to generate sex combs.

The anteriorized transformation in heat shocked larvae containing *Scr/Hox-1.3*

As shown in Figure 2-2, the ectopic expression of *Scr* causes transformation of T2 and T3 towards T1. The anteriorisation is probably due to the ectopic presence of *Scr* protein products in T2 and T3, since it is similar to the phenotype of *Antp* recessive mutants where *Scr* proteins can be found in T2 and T3 (Riley et al. 1987). This observation is consistent with the model that posteriorly acting homeotic genes normally

down-regulate anteriorly acting genes in the thoracic region. For instance, *Antp* can repress *Scr* (Riley et al. 1987) and *Ubx* can repress *Antp* (Hafen et al. 1984; Harding et al. 1985). Thus, the improper expression of *Scr* can override *Antp* and *Ubx* in two other thoracic segments (T2 and T3) and cause them to gain the epidermal identity of T1 segment which is normally determined by *Scr*. Ectopic expression of *Antp* leads to a transformation of T1 to T2 identity (Gibson et al. 1988). T3 is not transformed, presumably because its identity normally is determined by both *Antp* and *Ubx*. A transient excess of *Antp* protein cannot override *Ubx* activity and alter the developmental program of T3. Ectopic expression of *Ubx*, which normally determines T3 (together with *Antp*) and A1 (alone) segment identities, causes a transformation of all thoracic segments towards A1 (González-Reyes et al., 1990a; Mann et al., 1990).

However, why doesn't the anteriorized transformation occur in all abdominal segments of embryos in which anteriorly acting genes are ectopically expressed? One explanation is that BX-C gene products present in abdominal segments are functionally dominant to ANT-C gene products such that ectopic expression of ANT-C genes cannot override their effects in abdominal segments even when expressed at high levels, a phenomenon termed phenotypic suppression (González-Reyes et al., 1990a; González-Reyes et al., 1990b; Mann et al., 1990). Thus far, almost all reported homeotic transformations induced by misexpression of HOM-C genes fit within this model. Nevertheless, our results and a previous report (Panzer et al. 1992) demonstrate that this model may not be suitable for explaining other sorts of transformations. In fact *fkh*, a downstream gene of *Scr*, can be activated ectopically in the ventrolateral epithelial cells of most posterior segments by overexpression of either *Scr* or *Hox-1.3* (see Figure 2-5). Apparently, the functional dominance of BX-C genes is not applicable to this subset of cells in posterior segments. Consistent with this idea, ectopic expression of ANT-C genes also altered the developmental program of the peripheral nervous system in the abdominal segments (Heuer et al. 1992). Thus, when markers for individual cell identity

are utilized, transformation can be observed at a cellular level along the entire anterior-posterior body axis. These transformations may not be manifested at the level of morphological change in cuticular pattern because of the large number of cells and/or sets of genes in each individual cell that must be simultaneously reprogrammed to produce such a dramatic phenotypic change as segmental transformation.

A primordial gene for *Hox-1.3* and *Scr*

Several investigators proposed that a common ancestor for vertebrates and arthropods contained five homeotic genes in a primordial cluster (Duboule et al. 1989; Graham et al. 1989; McGinnis et al. 1992). These genes were arrayed in an order corresponding to *lab*, *pb*, *Dfd*, one of *Scr*/*Antp*, and *Abd-B* of the HOM-C of *Drosophila*. Akam (1988) suggested that *Scr*, *Ubx* and *Abd-A* emerged after the divergence of vertebrates and arthropods by duplication of a single *Antp*-like primordial gene. As shown in Figure 2-1A, *Hox-1.3* shares 55 of 61 amino acids within the homeodomain of both *Antp* and *Scr*, leaving the assignment of primordial gene identity ambiguous. Our observations demonstrate that despite this sequence similarity, *Hox-1.3* is functionally equivalent to *Scr* and not to *Antp*. In addition, *Hox-1.3* shares more homology to *Scr* than to *Antp* because of the conserved octapeptide and pentapeptide regions (see Figure 2-1A). This homology suggests that *Hox-1.3* and *Scr* arose from a single primordial gene, although we cannot rule out convergent evolution of *Scr* and *Hox-1.3*.

The results presented here reflect the strongest conservation of functional specificity among the three cognate pairs of *Hox* genes that have been examined. In the *Dfd/Hox-4.2* case, the resulting transformation is probably the consequence of activation of endogenous *Dfd* by ectopic expression of a human cognate *Hox-4.2* (McGinnis et al. 1990). In embryos carrying the mouse *Hox-2.2* transgene, an *Antp*-like transformation was not dependent upon the activation of endogenous *Antp* gene (Malicki et al. 1990). However, there is a difference between the cuticular phenotypes induced by *Antp* and

Hox-2.2. *Hox-2.2* has little effect on the morphology of T1, while the ectopic expression of *Antp* resulted in T1 to T2 transformation. This may be attributed to a partial loss of functional specificity during evolution, or alternatively, to a convergent evolution of *Antp* and its vertebrate cognates. In this study, we have shown that phenotypes induced by ectopic expression of *Hox-1.3* are virtually identical to (if not stronger than) those of *hs-Scr*. Thus, we suggest that an independent primordial gene for *Scr* and *Hox-1.3* existed in a primordial cluster of a common ancestor. Possibly this gene was retained under more stringent selective pressure because it plays a more important role than an *Antp*-like gene for pattern formation in vertebrate development.

How does a cognate maintain its regulatory specificity?

The present study supports previous finding that mammalian *Hox* genes can functionally substitute for cognate *Drosophila* homeotic selector genes in fruit flies (Malicki et al. 1990; McGinnis et al. 1990). For each one of the three examined cognate pairs (*Antp*/mouse *Hox-2.2*, Malicki et al., 1990; *Dfd*/human *Hox-4.2*, McGinnis et al., 1990; and *Scr*/mouse *Hox-1.3*, this paper), comparison of primary amino acid sequence reveals only small regions of conservation, the strongest homology being in the homeodomain (57/61, 54/60, and 56/61 respectively). In addition, similarities in the octapeptide and pentapeptide regions between *Scr* and *Hox-1.3* suggest that these regions may contribute to functional specificity as well. However, these regions of similarity are extremely short.

Thus, the remarkable specificities of these proteins seems to be based only on a few individual amino acids in conserved regions, relatively independent of the rest of the protein sequence. At the same time, however, functional specificity of different homeobox-containing genes is accomplished despite the striking similarities of their homeodomains (*Antp* vs. *Scr*, 56/61; *Antp* vs. *Dfd*, 49/60; and *Scr* vs. *Dfd*, 50/60). These analyses suggest that specificities of these proteins may be accomplished by

secondary or tertiary protein structures whose existence is not revealed by primary amino acid sequence analysis and whose function is to interact with auxiliary factors (Hayashi et al. 1990). Further, the combined results of the mouse/fly cognate experiments suggest that position within a *Hox* cluster may be more predictive of function than is analysis of primary amino acid sequence:

Although the different members of mouse *Hox* complexes are expressed in patterns having unique anterior boundaries, there are large regions of the body in which several members of each complex are expressed. For example, the expression domains of *Hox-1.2*, *Hox-1.3*, and *Hox-1.4* partially overlap (Gaunt et al. 1988; Dressler et al. 1989; Gaunt et al. 1989; Gaunt et al. 1990). In flies, the *fkf* gene is activated by ectopic expression of *Scr* but not of *Antp* (Panzer et al. 1992). The observation that the *fkf* gene is activated by *Hox-1.3* in flies suggests that it might have the ability to regulate specific genes that are not regulated by other members of the *Hox-1* cluster e.g. *Hox-1.2*, the *Antp* cognate. A further speculation is that each *Hox* gene may thus regulate different sets of target genes. The overlapping expression of different combinations of *Hox* genes in the mouse embryo would thereby provide a combinatorial code that would give each cell in the developing embryo a unique identity.

Hox-1.3 activates a downstream gene of *Scr*

The ectopic activation of *fkf* following the induction of mouse *Hox-1.3* in fly embryos provides the first molecular evidence to support the notion that mammalian *Hox* gene products can activate a target gene of an insect homeotic selector gene and participate in the genetic circuitry of *Drosophila*. The transformation and the activation of the *fkf-lacZ* fusion gene observed in *hs-Hox-1.3* bearing animals do not appear to be attributable to the activation of the endogenous cognate *Scr* gene since ectopic expression of *Scr* is not observed after induction. Panzer et al. (1992) pointed out that the action of *Scr* on *fkf* expression is restricted to the early phase of embryogenesis

- (within 4 hr AEL). After that, *Scr* is unable to induce *fkh* ectopically. The response time phase of *fkh* to *Scr/Hox-1.3* induction further supports the hypothesis that *Hox-1.3* directly activates *fkh* transcription. In *hs-Hox-1.3/fkh-lacZ* bearing hybrid embryos, the ectopic presence of β -galactosidase is indistinguishable from that of *hs-Scr/fkh-lacZ* embryos (see figure 5). It is unlikely to be mediated by activation of the endogenous *Scr* gene after heat induced expression of *Hox-1.3*, because the *Scr* gene has an approximate 25 kb long transcription unit (LeMotte et al. 1989) and would take approximately 3 hr to produce protein products ectopically. Thus, enough *Scr* protein products would only accumulate after the sensitive period for *fkh* induction in *hs-Hox-1.3* embryos.

It is possible that the extent of conservation in developmental programs between vertebrates and invertebrates is far more than expected. Sequence comparison reveals that the mammalian HNF-3 gene family, whose products were originally purified from rat liver nuclear extracts, is the counterpart to *Drosophila fkh* (Lai et al. 1991). Like *fkh* which is specifically expressed in gut and salivary gland tissues, the HNF-3 family is also expressed at different levels in the organs or tissues (such as liver, lung, and intestine et al.) derived from the embryonic gut tube. Lai et al. (1991) suggested that the HNF-3 family may maintain certain *fkh* functions in forming gut-related terminal embryonic structures during evolution (Lai et al. 1991). Interestingly, *Hox-1.3* transcripts also have been found in liver and lung (Odenwald et al. 1987; Garbern et al. 1989). The co-localization of *Hox-1.3* and HNF-3 gene products in the same tissues may not be a coincidence. Rather, the mammalian *Hox-1.3* may participate in organogenesis by activating HNF-3 in a particular group of cells, as *Scr* and *fkh* dictate the ontogenesis of the *Drosophila* salivary gland. The HNF-3 products, a new class of transcription factors, may then act on downstream targets to direct the formation of terminal organ structures.

Materials and Methods

DNA construction

The P element vector CaSpeR-hs (Pirota 1988) was cleaved within the polycloning sites with restriction enzymes HpaI and XbaI. A full length *Hox-1.3* coding sequence from plasmid pCDM81.35+ (obtained from W. Odenwald) was cleaved with XhoI. Protruding ends were filled in with Klenow enzyme, and the linearized DNA was digested with XbaI. The fragment produced by this manipulation contains the *Hox-1.3* open reading frame flanked by the 5' end TCGAGCAACAAGGAA linker and the 3' end GCATCTGAGC GGCCAAAGTACTGTCTGACTCTAGA untranslated sequence. The fragment and the vector DNA were ligated together to generate *hs-Hox-1.3*. Standard methods were used for all procedures (Sambrook et al. 1989)

Transgenic flies

Flies were grown at 25°C on standard medium containing cornmeal, sugar, yeast, and agar. P element transformation of *Df(1)w* embryos was carried out using standard techniques with the helper plasmid p π 25.1wc (Rubin et al. 1982; Karess et al. 1984). Stocks were maintained as homozygotes or with the balancer chromosomes *yw/CyO* or *yw/TM6b*. All experiments were repeated with three or more of the more than five independent transformant lines established to rule out positional effects of insertion.

Three independent *hs-Scr* lines were kindly provided by D. Andrew, W-L. Zeng, and M. Scott. p[ry⁺, HNK01] carrying a *fkh* enhancer-*lacZ* fusion gene was a gift from B. Zhou and S. Beckendorf.

Heat treatment and phenotypic analysis

Heat shock treatments were performed as described by Gibson and Gehring (1988, and Gibson, personal communication) except for some modification. For examining the cuticular consequences of heat shock, embryos are pre-collected on yeasted apple juice

plates for one hour and collected on fresh yeasted apple juice plates for 30 min. They are allowed to mature at 25°C for ~5.5-6 hr until heat shock treatment. Embryos are immersed in still water of a 37°C waterbath for 60 min. After heat shock treatment, the embryos are allowed to further mature for another 20 hr on plain apple juice plates. They are dechorinated in 3% sodium hypochlorite (supplied by VWR) for 2 min and vitelline membranes are removed by vigorously shaking in heptane/methanol (1:1) solution. After washing twice with methanol, embryos are incubated in acetic acid/glycerol (4:1) at 45°C for two days and mounted in Hoyer's medium:lactic acid (1:1) on a glass slide (Van der Meer, 1977). They are placed at 45°C for one week. The cuticular structures are analysed and photographed using dark-field and phase-contrast microscopy. By delivering a 20 min heat shock at different times to *hs-Hox-1.3* embryos, we found that the most sensitive stage to ubiquitous *Hox-1.3* expression was at 5-6 hr AEL; 70% of the treated embryos were not able to hatch to first instar larvae.

For larval heat shock, embryos are collected over one hour, washed and allowed to age on plain apple juice plates at 25°C for 24 hr. In order to test in which period *hs-Hox-1.3* larvae would be most severely affected by heat shock, hatched larvae were picked and inoculated into several yeasted cornmeal vials, in batches of 20. Animals in each vial grew at 25°C to a predetermined stage and were heat shocked at 36.8°C for 45 min (heat shock is given to individual vials at 3 hr intervals starting at 48 hr and ending at 120 hr AEL). To get stronger transformations, heat shock is given multiple times usually from 68 to 92 hr AEL at 36.8°C for 45 min with minimal 4 hr intervals. Heat is delivered to animals by simply placing the vials in a 36.8°C waterbath. It was found that the temperature of waterbath is crucial, and a 0.3°C higher temperature could kill all larvae before they reach the pupal stage. After heat shock, usually, 70-80% of the larvae die prior to eclosion (for *hs-Scr* flies, more than 90% die). Adult flies and unhatched pupae (which were first dissected out of sacs) are treated as described by McGinnis et al. (1990).

Immunohistochemical staining

Embryos are allowed to age 6 hr and heat shocked for the indicated time. For *Hox-1.3* antibody staining, the heat treatment is for one hour and embryos recovered at 25°C for 1 hr. For *Scr* and *Dfd* antibody staining, embryos are heat shocked for 1 hr and allowed to recover at 25°C for 3 and 2 hr, respectively. Embryo collection and heat treatment for the *fkf* enhancer-*lac Z* construct is according to the previous description (Panzer et al. 1992). Immunostaining is carried out by modifications of standard methods (T. Gutjahr and M. Noll, personal communication) using a preformed ABC (Avidin and Biotinylated horseradish peroxidase macromolecular Complex) reagent (Vectastain Elite ABC Kit). The *Hox-1.3* epitope is located in a non-conserved region from amino acid residue 53 to 72 and the preparation of *Hox-1.3* antiserum was described previously (Odenwald et al. 1987). The *Scr* and *Dfd* monoclonal antisera are gifts from D. Bowery and W. McGinnis. The anti- β -galactosidase antibody is from Cappel. Imaginal disc staining was carried out as described by Pattatucci et al. (1991).

Photography

The immunostaining embryos were photographed with Nomarski optics and the larval cuticles were photographed in darkfield on a Zeiss Axiophot microscope. The adults were photographed on an Olympus BH-2 microscope.

Note: The new nomenclature for *Hox-1.3* is *HoxA5* (Scott 1992).

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CHAPTER 3

Functional Dissection of The Mouse Hox-1.3 Gene

(manuscript in preparation)

Abstract

This study presents an effort to combine biochemistry, molecular biology, and genetic tools for a systematic functional analysis of the mouse *Hox-1.3* gene. The interactions between *Hox-1.3* protein and its target DNA sequence within the promoter have been characterized. The potential autoregulation of the *Hox-1.3* gene has been studied by using a transient expression assay. The transcription activities of a series of truncated forms of the *Hox-1.3* protein were first examined in cultured mammalian cells by the cotransfection assay. Then their biological roles were assayed in transgenic flies by heat shock-induced ectopic expression. The results demonstrated that the homeodomain is essential for the function of the protein because of its roles in DNA binding and nuclear targeting; the pentapeptide region is required for biological specificity even though it does not seem to contribute to transcription activation potential; the octapeptide region contributes significantly to transcription activity in cultured cells but appears to play no role in determination of functional specificity of the *Hox-1.3* protein in flies.

Introduction

The development of an organism is an amazingly heritable process. In terms of genetics, the one-dimensional regulatory genes hierarchically instruct a four-dimensional animal (a three-dimensional body plus aging). One group of these regulatory genes is the family of the homeobox genes. They were first identified in *Drosophila* (McGinnis et al. 1984; Scott et al. 1984) and later, similar sequences were found in many other

species. In *Drosophila*, the homeobox genes have been demonstrated to function as transcription factors and play crucial roles during embryogenesis. In mouse, similar roles of *Hox* genes have been suggested from analyses of loss-of-function phenotypes resulting from homologous recombination (Chisaka et al. 1991; Lufkin et al. 1991; Mouellic et al. 1992). Based on the similarities of homeobox sequences, position within a cluster, and expression patterns between the mouse *Hox* cluster and *Drosophila* HOM-C (including ANT-C and BX-C) homeobox genes, an evolutionary conservation hypothesis that both vertebrate and insect homeobox gene complexes diverged from a common ancestor has been proposed (Duboule et al. 1989; Graham et al. 1989; McGinnis et al. 1992; Zhao et al. submitted). Gene swap experiments performed in both mouse and fly solidify this idea (Malicki et al. 1990; McGinnis et al. 1990; Awgulewitsch et al. 1992; Malicki et al. 1992). By putting the mouse *Hox-1.3* under the control of heat-inducible promoter in transgenic flies, I have also reached a similar conclusion that the mouse *Hox-1.3* gene is functionally equivalent to the *Drosophila Sex combs reduced* gene (see Chapter 2). These assays suggest that vertebrate *Hox* and insect homeotic genes retain certain degrees of common function. Thus, the transgenic fly technology may also provide a simple, easy and low-cost biological system for preliminary functional studies of mammalian genes in the context of *Drosophila* embryogenesis.

In vertebrates, the functions of *Hox* genes have not been well characterized. The mouse *Hox-1.3* gene is one of several *Hox* genes which has been studied at a molecular level (Odenwald et al. 1987; Fibi et al. 1988). The gene is located in the middle of the HOX-1 cluster on chromosome 6. It consists of two exons and one intron. One open reading frame encodes a 270 amino acid residue long protein which is rich in proline, serine, and glycine residues. The homeodomain is encoded by the second exon and located between residues 195 and 254. Two other small regions (the N-terminal octapeptide region and the pentapeptide region) are found to be highly conserved in most *Hox* genes and in different species. Both of them are of unknown function. Two

groups have reported that proline-rich and serine-rich domains are transcription activation domains in CTF/NF-1 (CCAAT-binding transcription factor; Mermod et al. 1989) and Pit-1 (a pituitary POU domain protein; Ingraham et al. 1990) respectively. In fact, a number of proteins involved in the control of gene expression including *ftz* and *Krüppel* proteins in *Drosophila* (Laughon et al. 1984; Rosenberg et al. 1986), the receptors for the steroids, and the enhancer binding factor AP-2 also contain proline-rich sequences in regions of the molecules that participate in their transcription activation (Groneneyer et al. 1987; Kumar et al. 1987; Williams et al. 1991). Thus, the ability of the pro-, ser-rich regions, and the two short conserved sequences of the *Hox-1.3* protein to affect its transcription activity is an open question. Odenwald et al. (Odenwald et al. 1989) previously demonstrated that the *Hox-1.3* protein can bind its own promoter in a sequence-specific manner *in vitro*. This suggests that the *Hox-1.3* gene may be autoregulated. Here I presents results to show that *Hox-1.3* is able to regulate its own expression in cultured cells as expected. Then, I dissected out the contributions of different portions of the *Hox-1.3* protein to its transcription activation ability in cultured cells.

Since the cultured cell is a totally different environment from the embryo in the sense of development, it is extremely important to develop a biological assay to measure protein functions in the organism. A direct way is to make transgenic mice and to examine the effects of ectopic expression of the transgene, or alternatively, to study the phenotypes of a null mutation made by homologous recombination in embryonic stem cells. However, these approaches are costly and time-consuming. It is not affordable for researchers in a small group. An attractive alternative is the fly system, since I have shown that *Hox-1.3* retains functional specificity in this organism. In this Chapter, I report the utilization of the transgenic fly system as a biological assay to further analyze the functional domains of *Hox-1.3* protein and compare this study with the results from a transient expression assay in tissue cultured cells.

Results

The Hox-1.3 DNA binding site analysis

Previously, Odenwald et al. (1989) identified a 30-nucleotide-long DNA fragment, which is derived from the putative *Hox-1.3* promoter (-165 to -135 bp), that can specifically interact with *Hox-1.3* protein. A search was carried out to identify a shorter DNA fragment which has a binding constant comparable to the 30-mer used in earlier binding studies of *Hox-1.3*. The shorter version probe would better define the sequence and could reduce the non-specific binding in gel retardation assays. The partially overlapping oligonucleotide probes: d(CCCCCATTAGTGACGAG) "18-mer", d(CAACTCCCCATTAGTG) "17-mer", and their complementary-strands were chemically synthesized. After annealing two complementary strands, the binding activities of *Hox-1.3* proteins to the 18-mer, 17-mer and the 30-mer were compared. The deletion of the 5' TGC and the downstream 3' sequence, CACGAGACCT, did not significantly alter the binding affinity with respect to that of the 30-mer. In contrast, further deletion at the 5' end, between the 18-mer and 17-mer, apparently lead to dramatically reduced binding affinity (Figure 3-1). This result is consistent with the conclusion drawn from studies of *Antp* binding sites (Otting et al. 1990). Therefore the sequence CAACTCCCCATTAGTG, containing a core ATTA sequence, is sufficient for the *Hox-1.3* protein binding and has been used in further gel retardation assays.

Changing degenerate codons dramatically increases the expression level of mouse Hox-1.3 protein in bacteria

Although bacteria are useful systems to generate great amounts of recombinant proteins, many eukaryotic proteins are not expressed at high levels in *E. coli*. One notion is that since these eukaryotic proteins are exogenous products, they could be

toxic for bacteria. Alternatively, one of the most inhibitory factors for bacteria expressing eukaryotic proteins may be that higher eukaryotic organisms use different sets of codons for some amino acids.

Initial efforts to express the *Hox-1.3* protein at a high level in *E. coli* failed using its natural codons. Even under very strong induction conditions using the T7 expression system, the full-length *Hox-1.3* protein and the shortest truncated form were undetectable in coomassie blue stained SDS-PAGE gels, and hard to detect even on Western blots. Therefore, the coding sequence for the *Hox-1.3* homeodomain (HD) was reconstructed (Figure 3-2) by using synthetic oligonucleotides containing codons frequently utilized by *E. coli* (Abate et al. 1990). The synthetic sequence has been confirmed by sequencing and subcloned into a BamH I cloning site of the *E. coli* expression vector pET3a (Studier et al. 1990) (kindly provided by Dr. Studier). One surprising result of using this synthetic template is that the expression level of the homeodomain was dramatically elevated in *E. coli* after induction by isopropyl β -D-thiogalactopyranoside (IPTG) for 2 hours (compare lane 1 and 2 in Figure 3-3). The *Hox-1.3* HD constituted approximately 75% of the total protein in the bacterial lysate (Figure 3-3, lane 2) and had an apparent molecular weight of 8 KDa on the coomassie blue stained SDS/polyacrylamide gel. Newly synthesized *Hox-1.3* HD peptides did not need to be solubilized in the presence of a high concentration of guanidine, since they were partially soluble in the cell-lysis buffer (10 mM Tris-HCl, 25 mM EDTA, pH 8.0) (Figure 3-3, lane 4 and 6). In this case, the remaining insolubility may be due to the presence of huge amounts of homeodomain protein inside the cell body and is not due to the different protein-folding pathway in bacteria as suggested by others. This soluble fraction contains a high DNA-binding activity and was used in the gel retardation assays and DNAase I protection analyses.

Figure 3-1. The binding activities of three oligonucleotide probes.

		<u>binding activity</u>
30 mer	5'-TGCCAACTCCCCCATTAGTGCACGAGACCT-3'	strong
18 mer	5'-CCCCCATTAGTGCACGAG-3'	weak
17 mer	5'-CAACTCCCCCATTAGTG-3'	strong

Figure 3-2. The natural and synthetic nucleotide sequences and predicted amino acid sequence of the *Hox-1.3* homeodomain. The changed nucleotides are underlined.

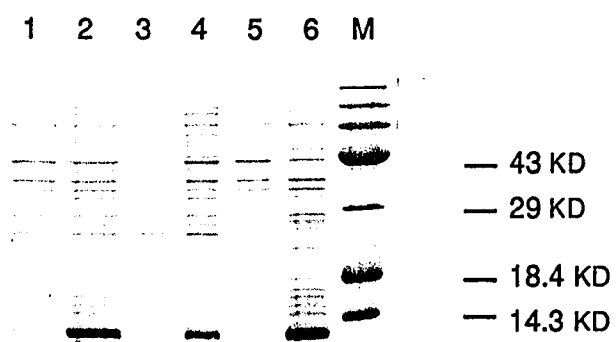
Original	GGC	AAA	AGG	GCC	CGG	ACG	GCC	TAC	ACT	CGC	TAC	CAG	ACC	CTG
Synthetic	<u>GGT</u>	AAA	<u>CGT</u>	<u>GCG</u>	<u>CGT</u>	<u>ACC</u>	<u>GCG</u>	<u>TAT</u>	<u>ACC</u>	<u>CGT</u>	TAC	CAG	ACC	CTG
Protein	Gly	Lys	Arg	Ala	Arg	Thr	Ala	Tyr	Thr	Arg	Tyr	Gln	Thr	Leu

GAG	CTG	GAG	AAA	GAA	TTC	CAC	TTC	AAC	CGC	TAC	CTG	ACC	CGC	CGA	AGA
<u>GAA</u>	CTG	GAG	AAA	GAA	TTC	CAC	TTC	AAC	<u>CGT</u>	<u>TAT</u>	CTG	ACC	CGC	<u>CGT</u>	<u>CGC</u>
Glu	Leu	Glu	Lys	Glu	Phe	His	Phe	Asn	Arg	Tyr	Leu	Thr	Arg	Arg	Arg

AGG	ATC	GAA	ATA	GCT	CAT	GCC	CTT	TGC	CTC	TCC	GAG	AGA	CAA	ATT	AAA
<u>CGT</u>	ATC	GAA	<u>ATC</u>	<u>GCG</u>	<u>CAC</u>	<u>GCG</u>	<u>CTG</u>	TGC	<u>CTG</u>	TCC	<u>GAA</u>	<u>CGT</u>	CAG	<u>ATC</u>	AAA
Arg	Ile	Glu	Ile	Ala	His	Ala	Leu	Cys	Leu	Ser	Glu	Arg	Gln	Ile	Lys

ATC	TGG	TTC	CAA	AAC	AGG	AGG	ATG	AAG	TGG	AAA	AAA	GAT	AAT	
ATC	TGG	TTC	CAG	AAC	<u>CGT</u>	<u>CGC</u>	ATG	<u>AAA</u>	TGG	AAA	AAA	<u>GAC</u>	<u>AAC</u>	TAA
Ile	Trp	Phe	Gln	Asn	Arg	Arg	Met	Lys	Trp	Lys	Lys	Asp	Asn	End

Figure 3-3. Coomassie blue-stained gel of *E. coli* expressed recombinant *Hox-1.3* homeodomain proteins. Lane 1 and 2 are whole cell lysates. Lane 3 and 4 are soluble fractions. Lane 5 and 6 are insoluble fractions. MW, protein molecular weight markers (supplied by BRL). Samples in odd number lanes are cell lysates before IPTG induction. Samples in even number lanes are cell lysates after IPTG induction for 2 hr.



Comparison of footprinting patterns using recombinant proteins expressed in insect cells and bacteria

The initial effort to demonstrate that *Hox-1.3* protein can bind to the proximal promoter region of its own gene was performed by Odenwald et al. (Odenwald et al. 1989). The protected regions, a and b (-160 to -137 bp) containing the consensus core motif ATTA, were easily detected in DNase I protection assays even using small amounts of insect nuclear extract containing *Hox-1.3* protein (0.4 to 1.2 µg of total protein); the adjacent sequences, c and d regions (-128 to -75bp), were protected after adding large amounts of extract (9.6 µg), but were not observed when using the extracts without *Hox-1.3* protein (Figure 3-4A). The c and d regions do not have homologous sequences with each other or with the a-b box. The authors also demonstrated that a 30-bp fragment derived from the c region was unable to compete away *Hox-1.3* protein specific binding to the a-b derived 30 mer in a gel retardation assay. My observation is consistent with this result since there was no detectable binding activity for a probe containing the whole c box sequence in gel retardation assays using *Hox-1.3* nuclear extract. The appearance of c and d regions footprinting was specific for the presence of high amount baculovirus expressed *Hox-1.3* protein nuclear extract and the c box itself did not have any detectable binding activity. This suggested that either *Hox-1.3* proteins bind to these regions cooperatively via first binding to the a-b region, or alternatively, that endogenous insect factors interact with the *Hox-1.3* protein-DNA complex. The assumed factors could not be DNA-binding proteins that bind independently. They could bind but only cooperate with *Hox-1.3* since no sequence was protected in the footprinting assay with extracts from wild type insect cells. To investigate the existence of such an insect cofactor, I compared footprinting patterns in the region (-156 to -75 bp) of the *Hox-1.3* promoter with *E. coli* produced recombinant *Hox-1.3* proteins and the nuclear extract used in previous studies. Rationally, bacteria expressed DNA-binding proteins are better

for defining specific binding sequences than crude nuclear extracts, because bacterial factors are unlikely to interact with transcriptional factors from higher eukaryotes.

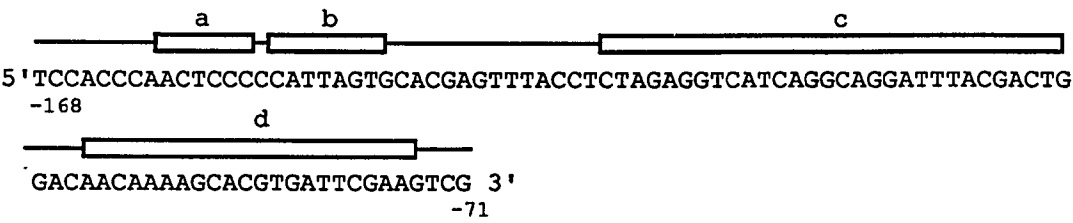
To analyze this complex footprinting pattern in the *Hox-1.3* promoter, a 256 bp (from -182 to +74 bp) fragment was used in DNAase I protection assays. The preliminary result shown in Figure 3-4B was obtained by employing *E. coli* overexpressed *Hox-1.3* HD and *Hox-1.3* containing nuclear extracts from baculovirus expression system. Interestingly, the recombinant *Hox-1.3* HD only protected the putative a-b region with a base shorter at 5' end of a-b box (compare lane 4, 5 and lane 7, 8 in Figure 3-4B). This difference may be a result that the *Hox-1.3* HD has smaller size and permits DNAase I digestion closer to the edge of protection sequence than the full-length *Hox-1.3* protein does. The c box was protected from DNAase I digestion after increasing amounts of *Hox-1.3* nuclear extract in the reaction (from 5.8 to 14.5 μ g). However, the footprinting did not extend to the d region under this condition (Figure 3-4B, lane 4 and 5). Significantly, the c-d region was not protected even by elevating amounts of *E. coli* produced the *Hox-1.3* HD (Figure 3-4B, lane 7 and 8). The lack of protection by the *Hox-1.3* HD indicates that the N-terminal sequence of *Hox-1.3* protein is important for the protein-protein interactions.

Figure 3-4. The footprinting pattern of the *Hox-1.3* proximal promoter region.

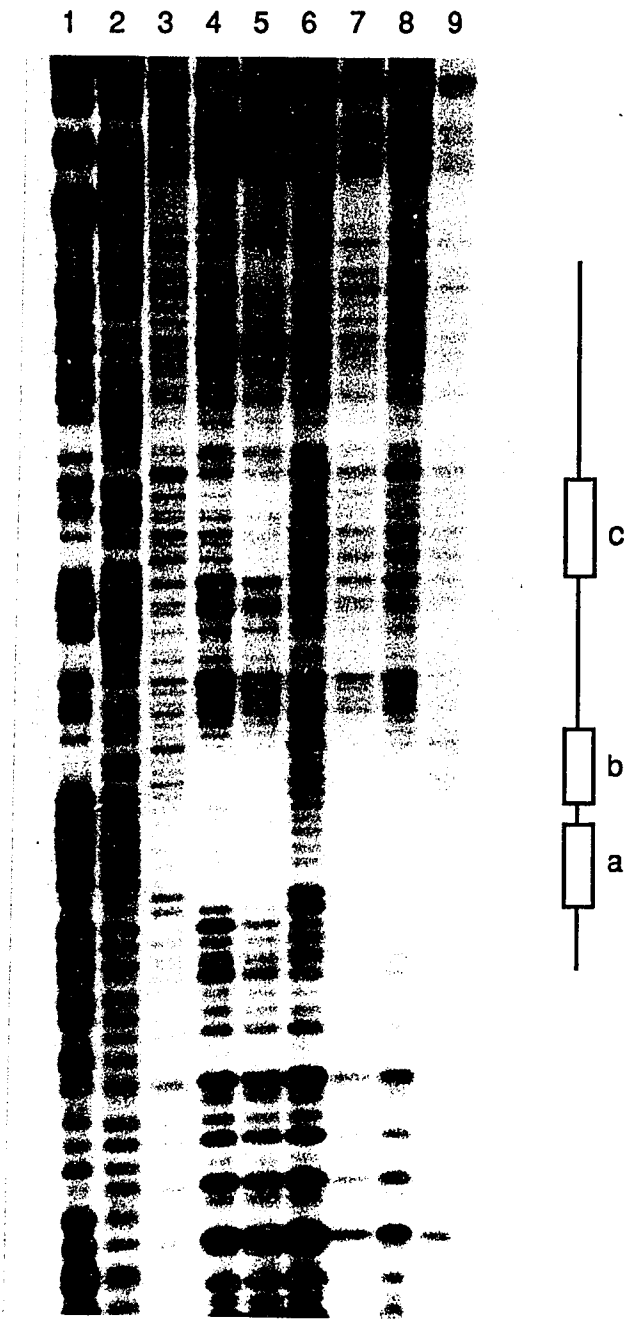
(A) The diagram of footprinting regions in the *Hox-1.3* promoter [adapted from Odenwald et al. (1989)].

(B) Characterization of footprinting patterns in the *Hox-1.3* promoter. A 256 bp fragment from -182 to +74 bp of the *Hox-1.3* promoter was employed. Lane 1 and 2 correspond to naked DNA cleaved by the chemical sequencing method either specific for G (lane 1) and A+G (lane 2). The DNA cleaved by DNAase I in the absence of proteins (lane 3). DNAase I treatments after probes were preincubated with different amounts of *Hox-1.3* containing nuclear extract (5.4 μ g and 14.5 μ g, lane 4 and 5, respectively); with 9.2 μ g uninfected nuclear extract (lane 6); with different amounts of the *Hox-1.3* HD containing bacterial lysates (2.0 and 4.0 μ g, lane 7 and 8 respectively); and with 4.1 μ g normal *E. coli* lysate (lane 9).

A



B



Evidence for positive autoregulation of the *Hox-1.3* gene

To test the ability of *Hox-1.3* protein to regulate its own gene expression *in vivo*, a transient expression assay in cultured COS7 cells was utilized. For these experiments, Cells are co-transfected with an expression plasmid encoding the full-length *Hox-1.3* protein together with a plasmid containing the putative target *Hox-1.3* promoter fused to the sensitively assayable human growth hormone (GH) gene. The results are presented in Figure 3-5A. Several conclusions could be drawn from these studies. First, a 2.2 kb putative intact *Hox1.3* promoter fragment (EcoRI to SacI) was able to raise growth hormone level up to eight fold over the basal level when the COS7 cells were co-transfected with a plasmid producing the *Hox-1.3* protein. This indicated that *Hox-1.3* protein indeed positively stimulates its own promoter sequence, at least, in tissue culture cells. Second, the 201 bp proximal promoter sequence which lacks any putative *Hox-1.3* binding site could not stimulate expression above the basal level. Third, the inclusion of one single *Hox-1.3* protein-binding site, even in the context of its natural promoter, is not sufficient for the full activity of *Hox-1.3* promoter, since the 307 bp promoter construct only gave a mild enhancement of expression.

Interestingly, the strongest expression was observed by using a 913 bp promoter construct. Sequence analysis of this region identified two additional putative *Hox-1.3* binding consensus sequences (ATTA) in the upstream region between -312 and -333 (Figure 3-5B). Two novel sites are close to each other with a 7 bp intervening sequence. The presence of this element indicates that it could serve as an enhancer element for *Hox-1.3* gene regulation.

The results from these transient expression assays are consistent with data obtained from transgenic mice carrying *Hox-1.3* promoter sequences (Zakany et al. 1988; Tuggle et al. 1990). In the transgenic mice, the 308 bp sequence (equal to p307/GH) is not sufficient to activate *lacZ* reporter gene expression, while the 912 bp

fragment (equal to p913/GH) contains enhancer elements to confer expression in the spinal cord.

Characterization of functional motifs of Hox-1.3 protein in cultured cells

Since the *Hox-1.3* protein can function as a transcriptional activator, the next logical step is to study the contributions of different portions of the *Hox-1.3* protein to its transcriptional activation ability. A series of DNA constructs which encode truncated proteins were made by digesting the DNA with restriction enzymes and using linkers for in frame ligations (Figure 3-6). The full length *Hox-1.3* cDNA encodes a protein with a long (194 residues) amino-terminal segment which contains serine-rich and proline-rich regions, followed by the 60 residue homeodomain and a short (16 residues) carboxyl-terminal tail. As mentioned above, a number of proteins involved in control of gene expression have proline-rich and serine-rich domains that function as transcriptional activation domains. In addition, the *Hox-1.3* protein also has two conserved peptide sequences, one at the N terminal of the protein and the other in front of homeodomain. Both of them are shared by many *Drosophila* and mouse homeodomain proteins. To investigate whether these domains in *Hox-1.3* protein could function as potential trans-acting domains, I constructed five *Hox1.3* cDNA deletion clones which delete amino acids from 1 to 39, 2 to 68, 69 to 192, 177 to 192, and 4 to 192. Each of these constructs as well as the full length cDNA was subcloned into a modified transcription and translation vector pT7 β -G Δ 6Sal which contains the T7 promoter and the β -globin leader sequence. Some of these constructs were transcribed *in vitro* by T7 RNA polymerase to produce mRNA which could serve as templates for *in vitro* translation in wheat germ extracts (Figure 3-7A). Gel retardation assays carried out using the full-length *Hox-1.3* protein and three truncated forms (Δ 2-68, Δ 68-192, and Δ 4-192) produced *in vitro* with wheat germ extract demonstrated that deletions in the amino terminal portion of *Hox-1.3* protein do not abolish DNA-binding activity (Figure 3-7B). However, DNA-binding affinities of these proteins are quite different despite the fact that

the apparent efficiency of *in vitro* translation is similar for these constructs (Figure 3-7A). Since there are several regions having abundant serine residues and proline residues, it is possible that deletion of these regions may affect the protein folding. The *Hox-1.3* protein is a multiply phosphorylated protein (Odenwald et al. 1989). Serine residues are potential sites for phosphorylation. The richness of proline residues in the overall N-terminal domain makes this portion of protein in a relatively rigid structure. These observations suggest that the homeodomain of *Hox-1.3* protein is necessary for DNA binding, though other protein domains may be also important for adjusting the binding affinity through different protein folding conformations.

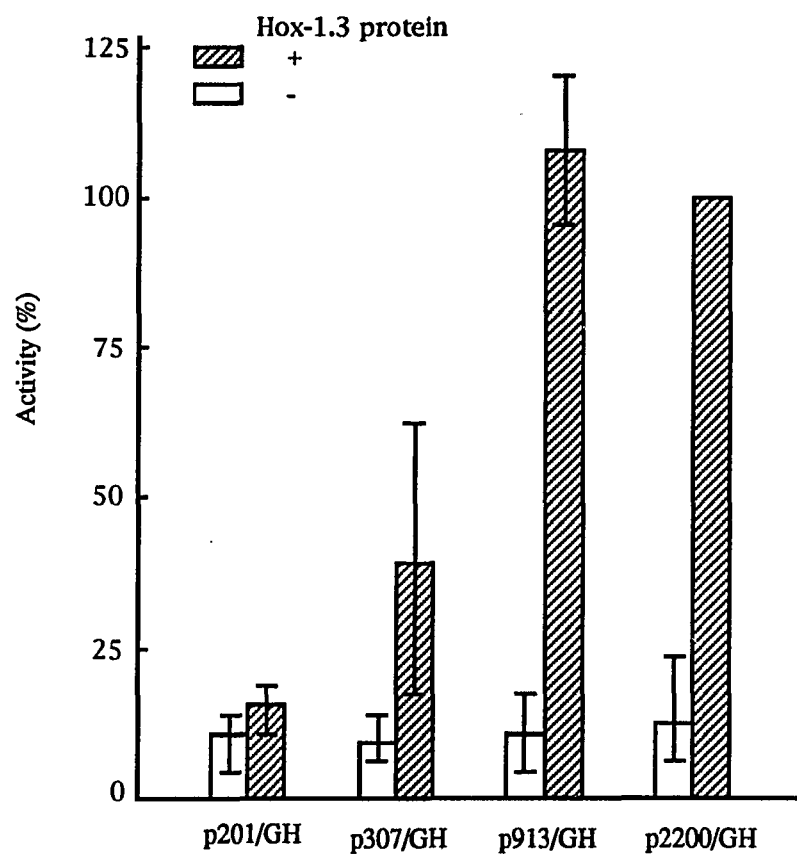
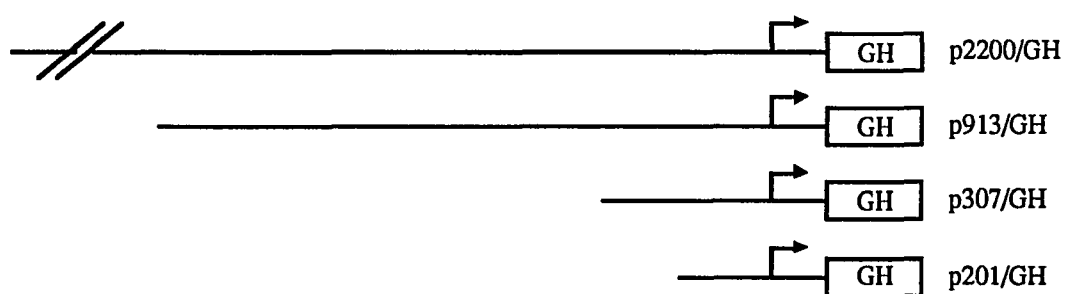
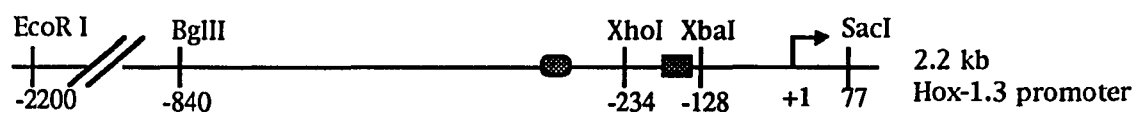
Results of the co-transfection experiments are shown in Table 3-1. The data indicated that the amino-terminal 39 residues are important for transcription activation. This domain contains a conserved amino-terminal MSSYFVNS octapeptide and a serine rich region. An internal deletion of 16 amino acids containing the highly conserved IYPWMR pentapeptide just upstream of the homeodomain, does not affect the trans-activation activity. The homeodomain and carboxyl tail alone are insufficient to activate transcription.

To confirm that loss of trans-acting activity is not due to the lack of nuclear targeting, the transfected culture cells were immunostained by anti-TAG antibody which recognizes a twelve peptide bacterial sequence inserted at the carboxyl end (see details in Materials and Methods). All of these constructs are targeted to the nucleus (Figure 3-8). Therefore, I concluded that a DNA-binding domain and a trans-acting domain are independent and separable in the murine *Hox-1.3* protein. The nuclear localization signal is likely to be in the homeodomain since it is rich in basic amino acid residues.

Figure 3-5. Diagram of the *Hox-1.3* promoter structure and auto-regulatory activity.

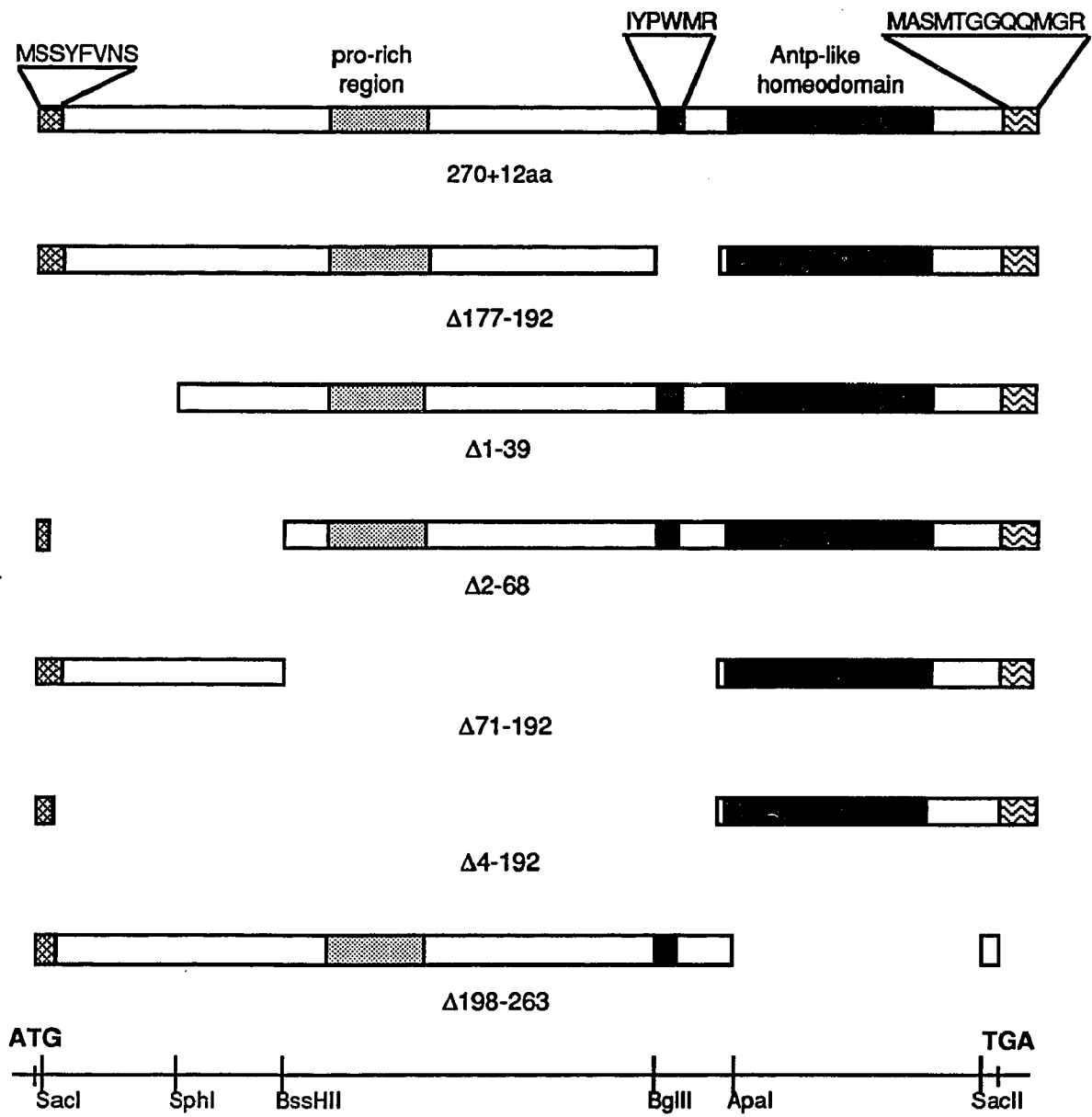
(A) The transcriptional activities of different length *Hox-1.3* promoter sequences in the presence of the *Hox-1.3* protein.

(B) The novel putative *Hox-1.3* protein binding sites. The sequence is from -333 to -312 bp of the *Hox-1.3* promoter. Two core motifs are shown in bold.

A**B**

5'--GCAATAATTTTGCCATAATGGG--3'
3'--CGTTATTAAACGGTATTACCC--5'

Figure 3-6. Structures of the recombinant *Hox-1.3* protein and its truncated forms. MSSYFVNS is a conserved octapeptide at the N-end of protein. IYPWMR is another conserved sequence in the front of homeodomain. The carboxyl terminal 12 amino acid peptide derived from bacteriophage T7 gene 10 coding region is indicated. The filled black box is the homeodomain. Constructions were made as described in the Materials and Methods.



The Hox-1.3 open reading frame

Figure 3-7. Translated products of *Hox-1.3* deletion constructs and their DNA-binding activities.

(A) *In vitro* translation of the full-length *Hox-1.3* protein and truncated forms in wheat germ extract. The protein products were labeled with ^{35}S -methionine. Lane 1, the full-length protein; lane 2, $\Delta 2-68$; lane 3, $\Delta 71-192$; and lane 4, $\Delta 4-192$.

(B) The DNA-binding activities were measured in a gel retardation assay. Naked probe only (lane 1); adding *in vitro* translation products of the full-length protein, $\Delta 2-68$, $\Delta 71-192$, and $\Delta 4-192$ polypeptides, and *E. coli* expressed the *Hox-1.3* HD (lane 2, 3, 4, 5 and 6, respectively).

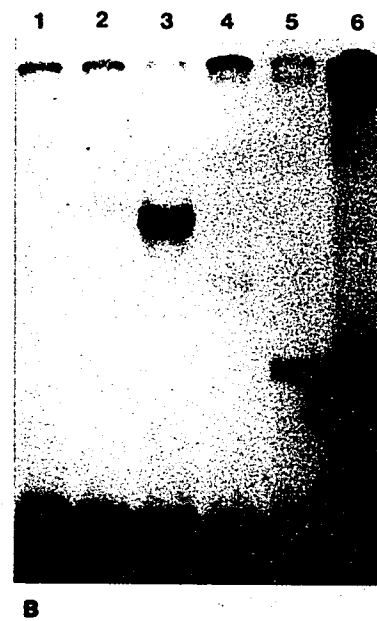
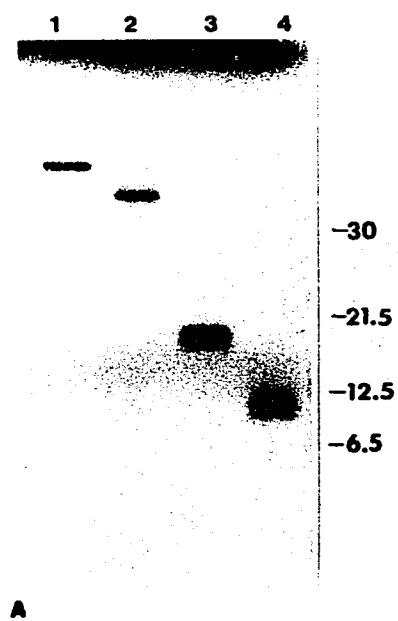


Figure 3-8. Subcellular distribution of the full-length and truncated forms of *Hox-1.3* polypeptides. The anti-TAG antibody was used for staining the COS7 cells in A, C, E, G, I, K, and M. Nuclei stained with DAPI are shown in B, D, F, H, J, L, and N.

(A) and (B): the cells expressed the full-length *Hox-1.3* protein;

(C) and (D): the cells expressed the $\Delta 177-192$ polypeptide;

(E) and (F): the cells expressed the $\Delta 1-39$ polypeptide;

(G) and (H): the cells expressed the $\Delta 2-68$ polypeptide;

(I) and (J): the cells expressed $\Delta 71-192$ polypeptide;

(K) and (L): the cells expressed $\Delta 4-192$ polypeptide;

(M) and (N): the cells expressed $\Delta 198-263$ polypeptide, which lacks the homeodomain, as a negative control.

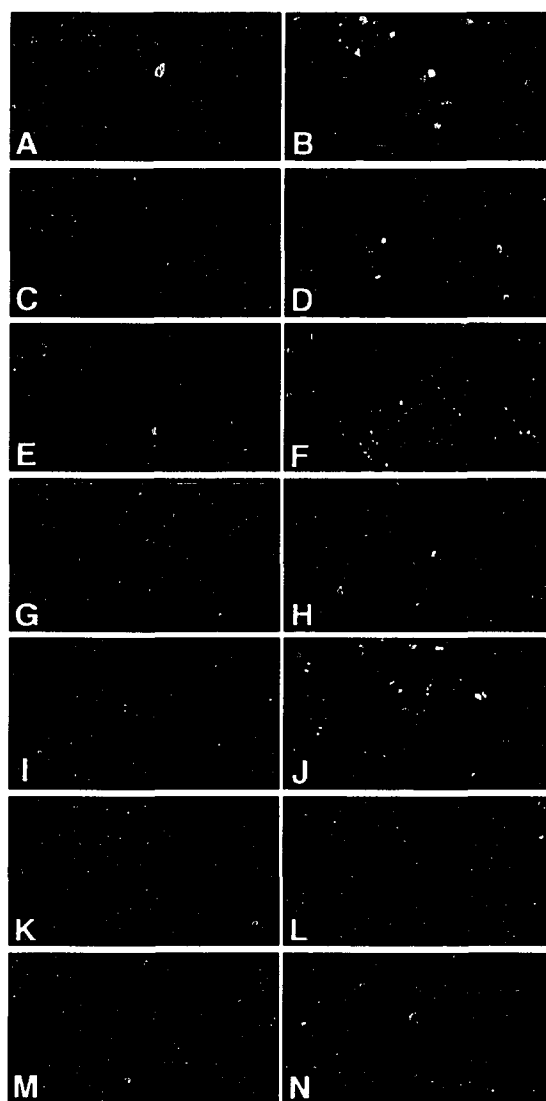


Table 3-1. Summary of Hox-1.3 trans-acting domains studies

Construct	Activity (%)	Subcellular localization
Hox-1.3 protein (full-length)	100.0	nuclei
Δ 177-192	98.0 ± 3.0	nuclei
Δ 1-39	20.0 ± 7.3	nuclei
Δ 2-68	9.1 ± 1.4	nuclei
Δ 71-192	22.8 ± 8.5	nuclei
Δ 4-192	9.5 ± 3.3	nuclei
non-expression vector	9.2 ± 2.0	

Ectopic expression of Hox-1.3 truncated-form proteins in *Drosophila*

The biological assay I have used to study *Hox-1.3* function *in vivo* depends on its ability to generate *Scr*-like homeotic transformations when ectopically expressed (see Chapter 2). To take advantage of this transgenic system, the truncated forms of *Hox-1.3* cDNA corresponding to $\Delta 1-39$, $\Delta 177-192$, $\Delta 4-192$, and $\Delta 198-263$ were each inserted into the pCaSpeR-hs vector (Pirrotta 1988). Each construct was introduced into the germline of flies as was done for the full-length *Hox-1.3* construct (see Chapter 2). Multiple independent transformant lines for each construct were obtained. All of them are viable under normal growth conditions.

Except for the hs-*Hox1.3* $\Delta 4-192$, which is missing the whole N-terminal region (containing the epitope for the anti-*Hox-1.3* antibody), the ectopic expression of these truncated forms can be detected after a one hour heat shock induction by using a *Hox-1.3* antiserum in whole mount embryo immunostaining (Figure 3-9). The truncated forms $\Delta 1-39$ and $\Delta 177-192$ are still nuclear proteins (Figure 3-9B and C), however, the protein products of the *Hox-1.3* $\Delta 198-263$, which lacks the coding sequence for the homeodomain and part of carboxyl terminus, are detected in the cytoplasm of cell (Figure 3-9A).

Effects of the different motifs of Hox-1.3 protein on induction of homeotic transformation in *Drosophila*

Previously I have demonstrated that transgenic flies bearing the full-length mouse *Hox-1.3* ORF can generate *Scr*-like homeotic transformations after heat-inducible ectopic expression. Thus, it provides an assay to test the contributions of different portions of *Hox-1.3* protein to its activity in the context of *Drosophila* development. All tested conditions for these deletion constructs are as same as those applied for the full-length hs-*Hox-1.3* construct.

Figure 3-9. Ectopic expression of different truncated forms of mouse *Hox-1.3* protein in transgenic embryos.

(A) Heat induced expression of hs-*Hox-1.3*Δ198-263.

(B) Heat induced expression of hs-*Hox-1.3*Δ1-39.

(C) Heat induced expression of hs-*Hox-1.3*Δ176-192.

A



B



C



The results are shown in Figure 3-10 for cuticular patterns and in Figure 3-11 for phenotypes of adult heads. As expected, the transgenic flies bearing *hs-Hox-1.3Δ198-263* which expresses a truncated protein lacking most of the homeodomain and 9 of 16 carboxyl terminus amino acid residues and targeted to the cytoplasm are indistinguishable from wild-type flies after heat induction (Figure 3-10E and Figure 3-11I and J). Therefore, this construct actually serves two purposes: first, it proves that observed transformations must not be attributed to the possible effects of heat shock and general protein ectopic expression; second, it also provides direct evidence that a mouse *Hox* homeodomain is necessary for its biological functions and harbors the nuclear targeting signal.

In heat-shocked *hs-Hox-1.3Δ1-39* embryos, the resulting larval transformations closely resemble those of the *hs-Scr* and/or *hs-Hox-1.3*. As shown in Figure 3-10B, the T2 and T3 thoracic segments are clearly transformed towards T1 as indicated by the appearance of T1 ventral beards. The ectopic expression of *hs-Hox-1.3Δ1-39* also blocks head involution and generates abnormalities in the head segments. The adult phenotype resulting from the ectopic expression of *hs-Hox-1.3Δ1-39* displays homeotic transformations in the head that are similar to the full-length *hs-Hox-1.3* (Figure 3-11). However, closer comparisons to the full-length *Hox-1.3* protein indicate that the transforming activity of this truncated protein may be slightly different in different tissue types. Both of them can dramatically affect the eye size and shape and induce the obvious *Cephalothorax*-like transformation in the hindhead. However, the antenna to leg transformation is observed more completely and frequently in animals bearing the *hs-Hox-1.3* than in animals bearing the *hs-Hox-1.3Δ1-39* (compare Figure 3-11C to Figure 2-5B).

Figure 3-10. Cuticular patterns in transgenic larvae expressing the mouse *Hox-1.3* truncated proteins. The transgenic embryos were subjected to a 60 min heat shock at 5.5~6.5 hr AEL in a 37°C waterbath and then allowed to develop to larvae.

(A) *hs-Hox-1.3*; (B) *hs-Hox-1.3Δ1-39*; (C) *hs-Hox-1.3Δ176-192*; (D) *hs-Hox-1.3Δ4-192*; (E) *hs-Hox-1.3Δ198-263*.

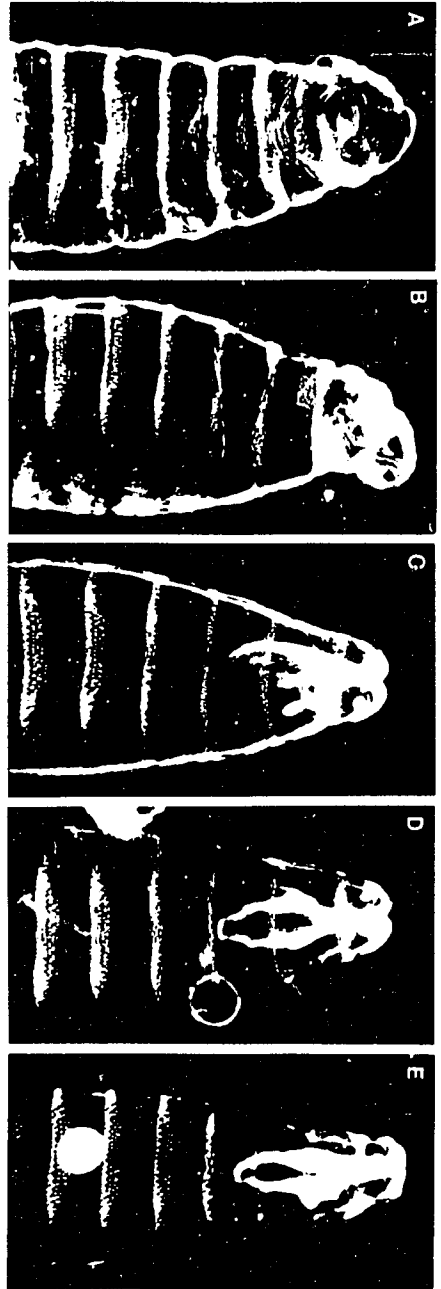


Figure 3-11. Adult phenotypes in transgenic flies expressing the mouse *Hox-1.3* truncated proteins. Larvae bearing the individual truncated transgene were subjected to multiple heat shocks. The larvae bearing the full-length *Hox-1.3* coding region, shown in A and B, was heat shocked only once. Cephalic views of adult heads are shown in A, C, E, G, and I; caudal views of adult heads are shown in B, D, F, H, and J.

(A), (B) *hs-Hox-1.3* ; (C), (D) *hs-Hox-1.3Δ1-39*; (E), (F) *hs-Hox-1.3Δ176-192*; (G), (H) *hs-Hox-1.3Δ4-192*; (I), (J) *hs-Hox-1.3Δ198-263*.

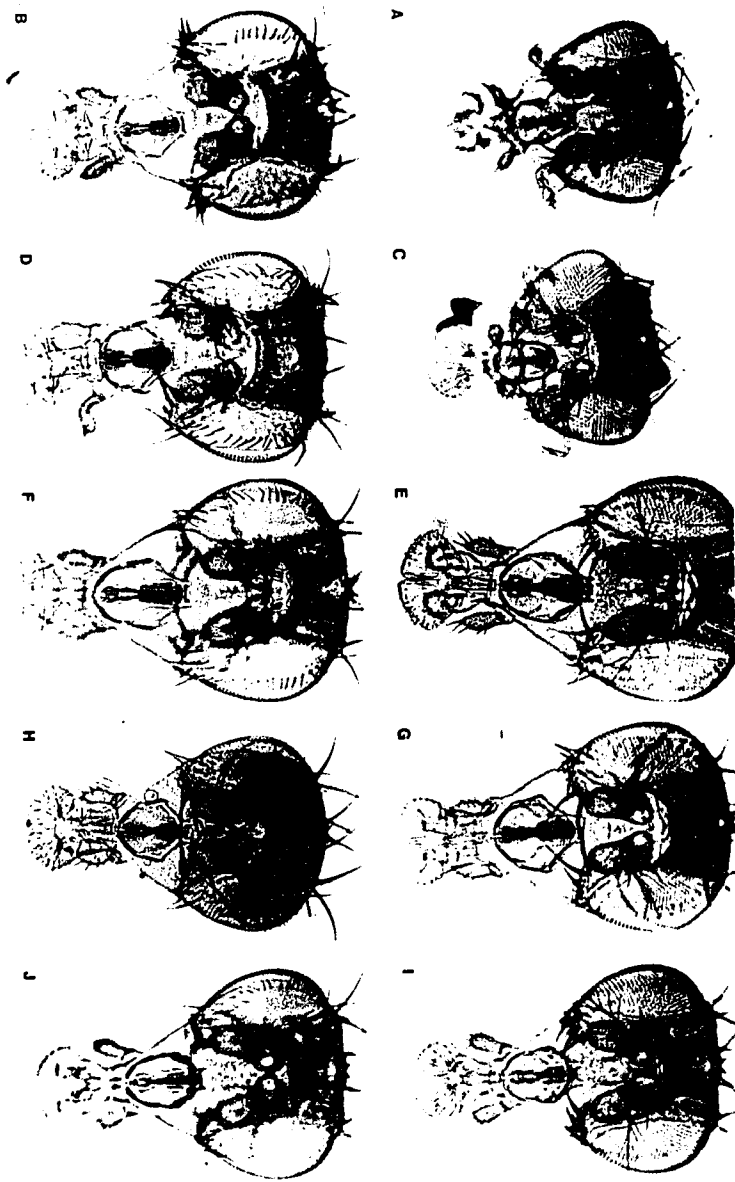


Figure 3-12. Ectopic expression of a *fkh-lacZ* fusion gene in transgenic flies expressing the mouse *Hox-1.3* truncated proteins. Hybrid embryos carrying *fkh-lacZ* and (A) *hs-Hox-1.3*, or (B) *hs-Hox-1.3Δ1-39*, or (C) *hs-Hox-1.3Δ176-192*, or (D) *hs-Hox-1.3Δ4-192*, or (E) *hs-Hox-1.3Δ198-263* were subjected to a 30 min heat shock at 1-4 hr AEL at 37°C and recovered at 25°C for 6 hr. The *fkh-lacZ* expression was monitored by immunostaining with an anti-β-gal antibody. The ectopic expression of *fkh-lacZ* was indicated by an arrow in panel B.

**A****B****C****D****E**

The ectopic expression of *hs-Hox-1.3* Δ 4-192 does not cause any detectable homeotic transformation in either larval cuticles or adults (see Figure 3-10D, and 3-11G and H). Surprisingly, animals bearing the *hs-Hox-1.3* Δ 176-192 (the construct lacking the pentapeptide region) also showed no morphological changes after heat shock treatment (Figure 3-10C, and 3-11E and F). This result suggests that the pentapeptide motif is important for the functional specificity of *Hox-1.3* even though it may not be responsible for the potency of trans-acting activity as assayed in co-transfection system (see above).

Deletions affect the ability of Hox-1.3 to activate *fkh*, a downstream target gene in *Drosophila*

As demonstrated in Chapter 2, the overexpression of the full-length *Hox-1.3* protein can induce the ectopic expression of one *Scr* downstream gene, *fkh* (Figure 2-7). To test whether the deletions in the *Hox-1.3* protein affect this regulatory function or not, the p[ry+, HNK01] line, which carries a *fkh-lacZ* fusion gene, was crossed with *hs-Hox-1.3* Δ 1-39, Δ 4-192, Δ 176-192, and Δ 198-263 transgenic strains separately. After the hybrid embryos were shifted to a 37°C waterbath for 30 min, and allowed to recover at normal temperature (25°C) for 6 hours, the expression of the *fkh-lacZ* fusion was examined by whole mount immunostaining. The ectopic expression of the *fkh-lacZ* transgene was detected only in hybrid embryos bearing *hs-Hox-1.3* Δ 1-39 but not in others (Figure 3-12). The activation ability of *Hox-1.3* Δ 1-39 on *fkh-lacZ* was considerably lower than that of the full-length *Hox-1.3*. The ectopic β -galactosidase was limited to the procephalon region but not in other parasegments (Figure 3-12B). This is consistent with the ability of this truncated protein to provide weak homeotic transformations in adult flies. Notably, the *Hox-1.3* Δ 176-192, which lacks the pentapeptide and apparently has full trans-acting activity in cultured cells (see above), was still inactive in terms of induction of expression of the *fkh-lacZ* reporter gene. This

observation, however, is consistent with the protein's inability to produce homeotic transformations in adults.

Discussion

On the basis of the results presented above, several functional properties of the mouse *Hox-1.3* gene have been uncovered. First, the data from the DNA-binding analysis using bacteria expressed *Hox-1.3* homeodomain instead of *Hox-1.3* containing nuclear extract from the baculovirus expression system further confirmed that the previously identified a-b region within the *Hox-1.3* proximal promoter (Odenwald et al. 1989) is a stable *Hox-1.3* binding site *in vitro*. However, the extended protection region (c-d, downstream of a-b consensus binding site) is only observed in the presence of large amounts of *Hox-1.3* nuclear extract. One speculation is that these downstream sequences have low affinities for the *Hox-1.3* protein. Despite attempts to optimize binding reactions, the double-strand probe derived from this region has not been able to form the complex with the *Hox-1.3* protein as examined by the gel retardation assay. However, the *Hox-1.3* protein could bind single strand probes from this region even in the presence of 100-fold excess of non-specific single-strand DNA in a preliminary study (data not shown). This suggests that *Hox-1.3* protein initially interacts with a high affinity binding site in a specific manner, then alters the adjacent downstream sequence to a single strand conformation (Straney et al. 1987) and enables additional transcription factors (*Hox-1.3* protein or others) to load. The formed DNA -protein complex could either function as a landmark for RNA polymerase II to find the correct transcriptional initiation site in the TATA-lacking *Hox-1.3* promoter or *Hox-1.3* bound to DNA could interact directly with the basal transcriptional machinery.

Second, the co-transfection experiments performed with the *Hox-1.3* gene coding region and the different lengths of the *Hox-1.3* promoter further support the notion that the expression of *Hox-1.3* may be autoregulated. The data suggests that the *Hox-1.3*

consensus binding site functions as a mild enhancer since the 307 bp proximal promoter which includes the footprinting a-b region were activated about two to three fold over the promoter without this cis element (p201) by the *Hox-1.3* protein. The 913 bp long promoter which contains two more putative binding sites can be upregulated most strongly by the transient expression of *Hox-1.3* in cultured mammalian cells. It has been previously demonstrated that, in transgenic mice, the 308 bp *Hox-1.3* proximal promoter region was silent and the 913 promoter region was able to direct a reporter gene (*lacZ*) expression in the spinal cord (Zakany et al. 1988; Tuggle et al. 1990) . Therefore, it is possible that the synergistic effect of three *Hox-1.3* binding sites (or the proximal *Hox-1.3* enhancer with other unidentified enhancer sites) is required for the activity and tissue specific expression of *Hox-1.3*.

Third, I have demonstrated that the bias usage of codons is the main cause of poor expression of mouse *Hox-1.3* protein in bacteria. Although in principle, a bacterial expression system is fast for engineering of exogenous gene products, selective, high-yield, and easy isolation of expressed proteins, a familiar experience for many researchers is that many engineered eukaryotic proteins are expressed at extremely low levels in bacteria. One possible explanation for this common problem is that some eukaryotic proteins are toxic to bacteria and the hosts expressing these proteins are not viable. An alternative explanation is that bacteria halt the synthesis of engineered proteins simply because they cannot produce enough of some kinds of tRNA which are rare in bacteria but are used frequently in higher eukaryotes. Although no systematic study has been done on this problem, the high yield of *Hox-1.3* homeodomain in bacteria after changing degenerate codons strongly supports the latter explanation.

Finally, and most importantly, the deletion analysis of the *Hox-1.3* protein further revealed the functional significance of at least three separable domains within the protein. The homeodomain most likely harbors the nuclear targeting signal and the full

DNA-binding ability. It is a necessary but not a sufficient domain for the *Hox-1.3* regulatory functions, since the *Hox-1.3* homeodomain itself (see the results with the *Hox-1.3* Δ 4-192 construct) is unable to function in cultured cells and fails to produce any visible homeotic transformation in transgenic animals.

The second defined motif is the pentapeptide conserved region, N-terminal to the homeodomain. While the deletion of this motif does not affect transcription activity of *Hox-1.3* in cultured cells, it significantly affects the function of *Hox-1.3* in transgenic flies. The *Hox-1.3* Δ 177-192 is no longer able to induce any homeotic transformation even though it has been expressed abundantly in most nuclei of embryo. Given the extended conservation of the pentapeptide motif between mouse *Hox-1.3* and *Drosophila Scr* (Figure 2-1), it could participate in protein-protein interactions to fine tune the regulatory specificity. This speculation is consistent with the biochemical studies that have shown the members of homeodomain families all bind to similar target sequences *in vitro*. Thus, the remarkable specificities of homeodomain proteins could be accomplished with the aid of auxiliary factors besides the contributions of some particular amino acids in the homeodomain (Lin et al. 1992). Whether the pentapeptide motif is involved in the formation of homodimers or heterodimers is currently unknown. To further investigate the role that the pentapeptide plays in determining the functional specificity of *Hox-1.3*, swap experiments should be performed. Pentapeptide motifs from different classes of homeodomain proteins (e.g. *Antp* or *Dfd*) would be substituted for the *Hox-1.3* pentapeptide and assayed both in cultured cells and transgenic flies.

The third defined motif is the N-terminal 1 to 39 amino acid residues of *Hox-1.3* including the conserved octapeptide motif and a serine rich region. Lacking this motif, the transcriptional activity of *Hox-1.3* protein dropped down ~80% in the transient expression assay system. The deletion effects on the trans-acting activity has been also observed in transgenic animals. Furthermore, a recent experiment performed by Dr. J.

Licht in this Department supports the notion that this motif is a separable trans-activation domain. They fused the 1-39 amino acids motif of *Hox-1.3* protein to a yeast protein *Gal4* DNA-binding domain (1-147 amino acids). When this fusion construct was cotransfected with a reporter gene (*CAT*) under the control of a promoter containing five *Gal4* binding sites, the level of *CAT* activity clearly increased around 15 fold comparing to co-transfected with *Gal4* DNA-binding domain alone (personal communication). However so far, no evidence has been presented to show that the octapeptide motif could participate in the determination of target specificity of any homeodomain protein. An open question that still remains is why it is so highly conserved? We cannot exclude the possibility that *Hox-1.3* may need the octapeptide motif in a different type of tissue or cell to adjust its target specificity, other than that examined in the transgenic fly experiments.

The studies presented here involved three different experimental systems: the *in vitro* biochemical assay, the transient expression assay in cultured cells, and ectopic expression assay in transgenic flies. In general, these different assays are complimentary to each other. The *in vitro* DNA-binding experiments provide physical evidences to show how the protein and its putative DNA target interact. One possible application of this assay is to address the question of homodimer or heterodimer formation by using bacterially expressed full-length *Hox-1.3* in the footprinting assay. If the footprinting pattern using the full-length form still include the a-b and the c-d regions, the homodimer model is more likely since a bacterial factor is unlikely to be involved in protein-protein interactions with a transcription factor from a mammal. Moreover, if the footprinting pattern given by using *Hox-1.3* Δ 177-192 protein is similar to the pattern given by the homeodomain, it could provide direct evidence that the pentapeptide motif is involved in the protein-protein interaction. The transient expression assay could provide useful information before attempting to transfer genes into an animal. The nuclear localization signal of *Hox-1.3* protein and cis-regulatory elements in the *Hox-1.3*

promoter could be further finely dissected out by small deletions or site-directed mutagenesis. However, the regulatory specificity of *Hox-1.3* is difficult to verify at this level, since it is known that different homeodomain proteins are capable of using the same binding site in cotransfection assays. Therefore, the animal system is ultimately required for understanding how a homeodomain protein specifically acts on its *bona fide* targets *in vivo*. *Drosophila melanogaster* has several merits to make it an excellent candidate for this purpose. Its genetics is well defined and it has a short life cycle. In addition, the significant functional conservation between the mouse *Hox* genes and *Drosophila* homeotic genes suggests that both organisms may use similar regulatory strategies during development.

While the co-transfection assay system was unable to elucidate the contributions of the octapeptide and pentapeptide motifs to the target specificity of the homeodomain proteins, they are clearly discernible by using this transgenic fly approach. A further application of this approach is to confirm the autoregulation specificity of *Hox-1.3* gene by crossing the *hs-Hox-1.3* transgenic strain with the *Hox-1.3* promoter-*lacZ* fusion gene transgenic strain. This genetic approach will open a novel way to elaborate specific DNA-regulatory interactions of mammalian genes. The data from this sort of experiment will provide more convincing information about the natural situation of gene regulation than those from the tissue culture experiment.

Therefore, before going to the transgenic mouse system, the basic regulatory functions of *Hox* genes can be elucidated in transgenic flies and the results could help to design new experiments to understand them in mouse. One surprising outcome of using this strategy is finding out that *Hox-1.3* can act on *fkf*, one of downstream gene of its cognate *Scr* in *Drosophila*. Interestingly, there are also mammalian *fkf* counterparts, known as the HNF-3 gene family (Lai et al. 1991). If the regulatory hierarchies of fly and mouse are conserved to a certain extent, one prediction would be that a HNF-3 family

gene may be a downstream target of *Hox-1.3*. Based on the above studies on *Hox-1.3* in the transgenic fly system, one possible experiment could be designed for transgenic mouse system. Fusing to a *Hox-1.6* promoter, the *Hox-1.3* proteins (the full-length and truncated forms) would be expressed in the *Hox-1.6* specific region of the embryo which is more anterior than *Hox-1.3* in mice. In the transgenic mice bearing these fusion genes, we can examine the resulting transformations, the effects of deletions of the octapeptide and pentapeptide motifs, and expression patterns of members of HNF-3 family.

Materials and Methods

Plasmids for In vitro translation

Translation vectors were based on pT7 β G Δ 6Sal, a derivative of pGEM2 in which the β -globin sequence is inserted between the polylinker HindIII PstI sites. Transcripts synthesized from this plasmid by T7 RNA polymerase begin with the nucleotides GGAGACCGGAAGCUUGCUU followed by the β -globin leader sequence.

pT7 β *Hox-1.3* contains the 1 kb SacI-SalI *Hox-1.3* cDNA fragment from pUC-Xho-rhomeo (obtained from W. F. Odenwald, originally constructed by E. Linney) inserted into NcoI-SalI cleaved pT7 β G Δ 6Sal. An adapter oligonucleotide was used to allow the in frame ligation of *Hox-1.3* cDNA to the β -globin ATG codon.

pT7 β *Hox-1.3* Δ 1-39: pUC-Xho-rhomeo was cut with Sph I and blunt ended with T4 DNA polymerase, then, a 0.88 kb DNA fragment was cut out using Sal I and subcloned into pT7 β G Δ 6Sal digested first with Nco I, blunt ended, and second with Sal I.

pT7 β *Hox-1.3* Δ 2-68: a 201 bp SacI-BssHII fragment was deleted from pT7 β *Hox*. After two incompatible stick ends were blunt-ended with T4 DNA polymerase, the linerized plasmid was religated.

pT7 β *Hox-1.3* Δ 71-192: a 387 bp coding sequence was deleted out of pT7*Hox* with BssH II and Apa I. An annealed synthetic sequence

CGCGCCGATATCCCAGAAGGCCAAAAGGGCC
GGCTATAGGGTCTTCCGTTTTC

was inserted into the deletion site to give a in frame ligation, reconstitute BssH II and ApaI sites, and engineer an EcoR V site between them.

pT7 β *Hox-1.3* Δ 4-192: the SacI-ApaI cleaved pT7 β *Hox* was recirculized with an annealed linker

CTGATATCCCAGAAGGCCAAAAGGGCC
TCGAGACTATAGGGTCTTCCGTTTTC

which reconstituted SacI and ApaI sites and introduced an EcoRV site .

pT7 β *Hox-1.3* Δ 198-263: the homeobox sequence was deleted from pT7 β *Hox* with restriction enzymes ApaI and SacII. After blunt ending with T4 DNA polymerase, both sites are lost and the plasmid was religated.

Plasmids for the transient expression assay

The *Hox-1.3* promoter sequence was derived from a plasmid pJG4 (a gift from J. Garbern) carrying a 3.8 kb EcoR I genomic fragment which starts in the *Hox-1.2* homeobox and extends to the *Hox-1.3* homeobox. From this plasmid, a 2.2 kb Sac I-EcoR I fragment containing the whole 5' untranslated sequence of *Hox-1.3* gene (see diagram in Figure 3-5) was subcloned into pUC19 to generate pJZH1. Since this fragment keeps the initial codon ATG, a primer GGGAGCTCGGATCCTTGTTTTTTGATATGTGTGCTTGA, which matches the sequence from +82 to +51 bp, was designed to get ride of the ATG. This introduces a novel BamH I site at this position through polymerase chain reaction (PCR) (Saiki et al. 1985). The second primer used in this PCR reaction was GGCTCGAGTCCGACTGAACGGCGGCAACGGGT derived from upstream sequence -235 to -205 bases. The 320 bp PCR product was cut with BamH I

and XbaI, then, the 201 bp subfragment was cloned into pØGH (Selden et al. 1986), to give p201/GH.

p2200/GH: a 2.0 kb Hind III-Xba I fragment from pJZH 1 was cloned into p201/GH linerized with Hind III and Xba I.

p913/GH: a 913 bp Bgl II-BamH I fragment from p2200/GH and pØGH digested with BamH I were ligated together.

p307/GH: a 1.9 kb Hind III-XhoI fragment was deleted from p2200/GH. The linerized plasmid was blunted by Klenow filling in and self-religated.

Constructions for the full-length and truncated *Hox-1.3* cDNA plasmids (pJZHox series) have been made by simply moving HindIII-BamHI fragments from constructs for *in vitro* translation (described above) into the expression vector pCMV5 (Anderson et al. 1989). The heterologous 12 amino acid "tag" was introduced into these expression plasmids, except pJZHoxΔ198-263 which lost the SacII site already, by placing the synthetic sequence (Figure 3-13) between SacII and BamHI sites.

Plasmids for bacterial expression

pET3d-*Hox-1.3*: An 804 bp XhoI-SacII fragment (the XhoI end was blunted by Klenow filling in) from pCDM81.35+ (a gift from Dr. Odenwald) containing the *Hox-1.3* first 262 amino acids and TCGAGCAACAAGGAA linker sequence at the upstream of ATG, the synthetic "tagged" *Hox-1.3* carboxy terminal (Figure 3-15), and the NcoI (blunted)-BamHI cleaved expression vector pET3d, were ligated together.

Figure 3-13. The nucleotide sequence and corresponded amino acid sequence of "tagged" *Hox-1.3* carboxyl terminal. The shadowed box indicates the sequence derived from first 11 amino acids of the T7 gene 10 protein (the major capsid protein of T7 phage) (Rosenberg et al. 1987) and an Arg residue. Codons for Thr, Gly and Gln have been substituted with synonymous codons frequently used in eukaryotes.

G	GCA	GGC	GGC	GCT	TTC	CGC	CCC	ATG	GCT	AGC	ATG	ACC	GGC	GGC
CGC	CGT	CCG	CCG	CGA	AAG	GCG	GGG	TAC	CGA	TCG	TAC	TGG	CCG	CCG
Ala	Ala	Gly	Gly	Ala	Phe	Arg	Pro	Met	Ala	Ser	Met	Thr	Gly	Gly
263							270							

CAG	CAG	ATG	GGC	CGC	TGA	TAG
GTC	GTC	TAC	CCG	GCG	ACT	ATC CTAG
Gln	Gln	Met	Gly	Arg	End	End

pET3d-*Hox-1.3* Δ 1-192: a 276 bp fragment containing the *Hox-1.3* homeodomain and carboxyl terminal was cut out of pJZ*Hox* Δ 4-192 with EcoRV and BamHI, and inserted into the NcoI (blunted)-BamHI cleaved expression vector pET3d.

pET3a-HD: The homeodomain of *Hox-1.3* (amino acids 195-254) was reconstructed using a total of 8 oligonucleotides containing the most frequently utilized codons of *E. coli* (Abate et al. 1990). Four sense strand oligonucleotides are:

HD-1P	GATCCGGTAAACGTGCGCGTACCGGTATACCGTTACCAGACCCTGGAA
HD-2P	CTGGAGAAAGAATTCCACTTCAACCGTTATCTGACCCGCCGTCGC
HD-3P	CGTATCGAAATCGCGCACGCGCTGTGCCTGTCCGAACGTCAGATCAA
HD-4P	AATCTGGTTCCAGAACCGTCGCATGAAATGGAAAAAGACAACCTAAG

and four complementary oligonucleotides respectively are:

HD-1M	TTCTCCAGTTCCAGGGTCTGGTAACGGGTATACGCGGTACGCGCACGTTTACCG
HD-2M	TCGATACGGCGACGGCGGGTCAGATAACGGTTGAAGTGAATTCT
HD-3M	ACCAGATTTTGATCTGACGTTCCGGACAGGCACAGCGCGTGCGCGATT
HD-4M	GATCCTTAGTTGTCTTTTTTCCATTTTCATGCGACGGTTCTGGA

The reconstructed fragment was confirmed by automatic sequencing, and cloned into the BamHI site of expression vector pET3a. This vector fused 12 amino acids (same as the sequence in the shadow box of Figure 16) to the N terminal of homeodomain.

Plasmids for transgenic flies

phs-*Hox-1.3* Δ 4-192: phs-*Hox-1.3* (see Materials and methods in Chapter 2 for details of construction) was digested with BglII and XbaI and filled in with Klenow enzyme. Two blunt ends were ligated.

phs-*Hox-1.3* Δ 1-39: The vector pCaSpeR-hs was cut with HpaI and BglII, then, a HindIII (blunt ended)--BamHI fragment from pJZ*Hox* Δ 1-39 was inserted.

pTF*Hox-1.3* Δ 176-192: A HindIII (blunt ended)--BamHI fragment from pJZ*Hox-1.3* Δ 176-192 was ligated into the vector pCaSpeR-hs.

pTF*Hox-1.3* Δ 4-192: The 330 bp HindIII (blunted)--BamHI fragment from pJZ*Hox-1.3* Δ 4-192 was subcloned into the HpaI-BglII sites of pCaSpeR-hs.

Expression of Hox-1.3 proteins in *E. coli*

Host strain *BL21(DE3)/plys* was employed for expression (Studier et al. 1990). Bacteria were grown in LB media containing 0.4% glucose, 150 μ g/ml ampicillin and 34 μ g/ml chloramphenicol at 37°C. At an A₆₀₀ between 0.4 and 0.6, IPTG was added to a final concentration of 1 mM and the cultures incubated a further 2 hr at 37°C. Cells were collected by centrifugation at 4000 X g for 10 min. For *BL21(DE3)/plys/pET3a-HD*, cells were resuspended in 1/20 of the original culture volume in 10 mM Tris-HCl, 25 mM EDTA, pH 8.0. and sonicated three times for 15 seconds on ice to break the cells. After centrifugation at 12,000 X g for 15 min at 4°C, the supernant was stored at -70°C. The pellet was dissolved in equal volume of 10 mM Tris-HCl, 25 mM EDTA, pH 8.0. 0.5% SDS, followed by centrifugation at 12,000 X g for 5 min at room temperature. The supernant was stored at -70°C. For *BL21(DE3)/plys/pET3d-Hox-1.3* and *BL21(DE3)/plys/pET3d-Hox* Δ 1-192, the cell pellets were resuspended in 1/200 of the original culture volume in Buffer Z (100 mM KCl, 25 mM HEPES pH 7.8, 12.5 mM MgCl₂, 1mM DTT, 0.1% Triton X-100, 20% glycerol, 1 mM PMSF, 2 mM benzamidine, 5 μ g/ml leupeptin) containing 4M guanidine-HCl and incubated on ice for 30 min. After sonication four times for 15 seconds followed centrifugation, the supernatants were subjected to sequential dialysis against buffer Z containing 1M guanidine-HCl first once and then buffer Z twice. The proteins were stored at -70°C. Protein concentrations were determined by Bradford assays.

In vitro transcription and translation

Plasmids were completely linearized with two restriction enzymes BamH I and Sal I, since supercoiled plasmids containing a bacteriophage promoter are very efficiently transcribed, yielding large, often multimeric transcripts. The linear DNA templates (100 µg/ml) were transcribed *in vitro* using T7 RNA polymerase (100 U/ml, supplied by Promega) in a buffer containing 40 mM Tris-HCl, pH 7.5, 6 mM MgCl₂, 2 mM Spermidine-HCl, 10 mM Dithiothreitol, 500 µM Each ATP, CTP, GTP, and UTP, and 1 U/µl RNasin (supplied by Promega). Reactions were allowed to proceed for 1 hr at 37°C. After the reaction was completed, DNA templates were digested by the addition of RNase-free DNase to a final concentration of 20 µg/ml for 10 min at 37°C. The mRNAs were extracted with an equal volume of phenol-chloroform, precipitated with 0.7 M ammonium acetate and 2.5 volumes of ethanol, and resuspended in 20 µl DEPC water. RNA products were checked by 1% agarose gel electrophoresis in 1X TBE buffer. Following electrophoresis, gels were stained for 10 min with 1 µg/ml ethidium bromide. Quantities of transcripts were roughly estimated from the gel by comparing to a total cellular RNA preparation of known concentration.

In vitro translation was performed using a wheat germ extract (Promega). A single reaction mixture consisted of 12.5 µl of wheat germ extract, 2 µl of 1 mM amino acid mixture (minus methionine), 1.5 µl of 1 M potassium acetate, 1.5 µl of DEPC H₂O, and 1.5 µl of translation grade ³⁵S-methionine (1186 Ci/mmol, New England Nuclear). After adding 3 µl of transcribed RNA, the reaction was run for 60 minutes at 25°C. The translation products were resolved on a 15% SDS-polyacrylamide gel along with ¹⁴C-labeled low molecular weight markers (New England Nuclear). The gels were fixed in 30% methanol and 10% acetic acid, treated with autoradiography enhancer (Amersham), dried, and autoradiographed at -70°C.

Gel retardation and DNAase I protection analyses

Gel retardation assays are based on previously described procedures (reviewed in Revzin 1989). Synthetic oligo probes were either labeled with T4 polynucleotide kinase and γ - ^{32}P -ATP (6000 Ci/mM) or by filling in recessed 3' ends with the Klenow fragment of DNA polymerase I and two α - ^{32}P labeled dNTPs. DNA-protein binding reactions usually contained 10 mM Tris-HCl pH7.9, 50 mM NaCl, 5 mM MgCl_2 , 0.5 mM EDTA, 1 mM DTT (dithiothreitol), 10% glycerol, 2.5 μg poly (dl-dC) or 1 μg sonicated salmon sperm DNA, various amounts of proteins and 15 fmol of labeled probe (~ 25,000 cpm).

DNAase I protection assays used here were originally developed by Galas and Schmitz (Galas et al. 1978) and modified by Amemiya et al. (Amemiya et al. 1989). The standard reaction contained, in 50 μl , ~15,000 cpm of probe and were of the same composition as for gel retardations. The binding reactions were carried out on ice for 30 min. After incubation for another 2 min at room temperature, 50 μl of a diluent solution containing 5 mM CaCl_2 and 10 mM MgCl_2 was added, followed by an empirically determined amount of DNAase I. After 2 min, digestions were terminated with 100 μl of stop buffer (200 mM NaCl, 20 mM EDTA, 1% SDS and 50 $\mu\text{g}/\text{ml}$ tRNA). Following extraction with phenol/chloroform, nucleic acids were precipitated with 2.5 volumes of ethanol on ice for 15 min. Pellets were washed with 80% ethanol, dried, and then electrophoresed through 6% polyacrylamide gels containing 7M urea. Reference ladders were generated by the chemical sequencing method (Maxam et al. 1977). Gels were dried and autoradiographed with an intensifying screen at -70°C .

Co-transfection and transient expression assays

Monkey kidney COS7 cells were grown on 100 mm tissue culture dishes or T75 flasks in Dulbecco's Modified Eagles Medium (D-MEM) supplemented with 10% FBS. Calcium phosphate transfection was performed as described in Auebel et al. (1987). Equimolar amounts of test plasmids (10 μg to 20 μg) plus 5 μg of pSV2CAT (Gorman et al. 1982)

were transfected per 100 mm dish. Cells were transfected for 6 hrs followed by a 2 min 10% glycerol shock. For GH transient expression assays, 0.5 ml of culture media was collected at 12-hour intervals from 36 to 72 hours post-transfection. The level of growth hormone secreted in the culture media was determined by radioimmunoassay (Nichols Diagnostics, San Jaun, Capastrano CA). Generally, 100 μ l of culture media of the transfected cells was preincubated with an equal volume of ^{125} I-labeled monoclonal hGH antibody and biotin coupled monoclonal hGH antibody. Then avidin coated plastic beads were added to each reaction mixture and incubated on a horizontal rotator (set to 170 ± 10 rpm) for 1.5 hr at room temperature. After the beads were washed twice with 2.0 ml of wash solution, they were counted in a gamma counter for one minute. For CAT transient expression assays, cells were harvested at 72 hrs post-transfection. 10 μ g of cell extract was employed in a single CAT standard reaction (125 μ l). CAT enzymatic activity was monitored by a liquid scintillation counting (LSC) assay (Promega). Generally, after incubating at 37°C for 20 min, the reaction was stopped by adding 300 μ l of xylenes. The mixture was vortexed for 30 seconds, followed by centrifugation for 3 min. The upper phase was transferred to a fresh tube and extracted with 100 μ l of 0.25 M Tris-HCl pH 8.0 buffer. For maximum sensitivity, the extraction was repeated. 200 μ l of upper phase solution was mixed with 5 ml scintillation fluid in a scintillation vial, followed by counting.

Immunofluorescent staining of the cultured cells

Immunolocalizations was performed basically as described by Odenwald et al. (Odenwald et al. 1987). Transfected cells were fixed with 2% paraformaldehyde in PBS (pH 7.4) for 30 min. After rinsing with PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min. Following rinsing with PBS, the cells were blocked with 20% normal goat serum, 0.02% Biotin, and 0.1% Triton X-100 in PGBA (0.1% gelatin, 1% BSA, and 0.05% sodium azide in PBS) for 30 min. The rabbit anti-TAG antiserum (a gift from Dr. J. Keene) was diluted in block solution (1:200). The primary antibody

incubation was performed overnight at 4°C. Cells were then washed for 20 min with three changes of 0.1% Triton X-100 in PBS, repeating three times. The donkey biotinylated anti-rabbit Ig antiserum (Amersham) was diluted 1:200 with 0.1% Triton X-100 in PGBA. After incubation with the secondary antibody for 1 hr, cells were washed as described above. The secondary antibodies were labeled with streptavidin-rhodamine B (Molecular Probes, 12 ng/ml in 0.1% Triton X-100, PGBA) for 1 hr. After three 10-min rinses in the buffer of 0.1% Triton X-100 and PBS, DAPI was used to stain the cellular DNA. Cells were stained with 1 µg/ml of DAPI in 0.1% Triton X-100 and PGBA for 40 min, followed by three 10-min destaining in 0.1% Triton X-100 and PBS.

Transgenic flies and heat shocks

The experimental procedures are basically the same as described in the Materials and Methods of Chapter 2.

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CHAPTER 4

Introduction II

The segmentation pattern of *Drosophila melanogaster* is generated by a hierarchical series of pattern forming events. A large number of genes, identified by mutations that disrupt various aspects of development, are responsible for this process (Akam 1987). To summarize, maternal genes establish anterior-posterior gradients within the egg. The maternal and zygotic gap genes are activated at different positions along the anterior-posterior axis and establish large spatial domains within the embryo. The pair rule genes are required to further subdivide the embryo into 14 separate parasegments. The homeotic genes of two major complexes, the *Antennapedia* complex (ANT-C) and *bithorax* complex (BX-C), are responsible for conferring specific identities to each parasegment. Elucidating the mechanism of action of these genes may help us to understand how a fertilized egg develops to a complex organism.

So far, virtually all abnormalities in *Drosophila* embryogenesis have been observed on the basis of the existence of genetic mutants or using gene transfer for ectopic expression. Because most mutations in genes involved in development are expected to be recessive-lethal mutations with little or no heterozygous effect, zygotic-lethal mutations with abnormal phenotypes have been sought. Although these approaches have identified many genes and have revealed the functions of these genes in the process of determination of segmental identity, there are still a number of problems: (1) at the time segmental identity is initially determined (the cellular blastoderm stage), the zygotic genome has just begun transcription, and the maternal contribution of genetic information is probably still very important. As many zygotic-lethal mutations exhibit a significant maternal contribution, maternal effects in the determination of segmental identity are not a trivial concern (Struhl 1981; Lawrence et al. 1983). (2) The second problem can arise if a gene product is required for segmental identity but is also required for some other developmental process. Then, the phenotype of a mutation may be

complex and not easily discernible with respect to later function. A good illustration of this problem is the *Drosophila fushi tarazu (ftz)* gene. The *ftz* protein is detectable at two stages. In the cellular blastoderm stage, it is distributed in a pattern of seven transverse stripes of nuclei around the anterior-posterior axis of the embryo that disappear before the end of germ band extension. Later, during germ band shortening, it reappears in the central nervous system with a segmentally repeated pattern (Carroll et al. 1985; Doe et al. 1988). The loss of *ftz* function leads to such a severe phenotype that the nervous system phenotype cannot be discerned from the segmentation phenotype and was missed in an early genetic analysis of the *ftz* mutant phenotype (Wakimoto et al. 1984).

(3) Many genes encode more than one transcript. It has been shown that alternative splicing plays a key role in the process of sex determination (Nagashi et al. 1990). In contrast, one report strongly argues that the three different forms of *Ultrabithorax* proteins generated by alternative splicing are functionally equivalent (Busturia et al. 1990). In most cases, however, the functional significance of multiple transcripts of a gene is unknown.

(4) To assess the combinatorial effects of different genes which are involved in determination, first of all, particular mutations and strains should be obtained. Even for a skillful experienced researcher, this still is time consuming and labor intensive work. In addition, there are no guarantees of obtaining the sorts of mutant strains desired.

In Chapter 5, I present a novel strategy to address all these problems by employing an artificial ribozyme without requiring a specific genetic background.

The hammerhead ribozyme: a catalytic RNA endonuclease

RNAs with catalytic cleavage activity are termed "ribozymes" (Cech 1987). Genomic RNAs of a certain number of plant viruses undergo self-catalyzed cleavage (Hutchins et al. 1986; Prody et al. 1986; Forster et al. 1987a). The ability to self-cleave is imparted by distal consensus sequences that interact to form a highly structured RNA configuration

prior to cleavage. Although the natural cleavage is entirely intramolecular, a careful analysis of the sequences and structures involved led Uhlenbeck to the realization that the ribozyme can act in a trans position. They can cleave single-strand RNA targets other than natural substrates specifically (Uhlenbeck 1987). He demonstrated that these synthetic RNAs act enzymatically.

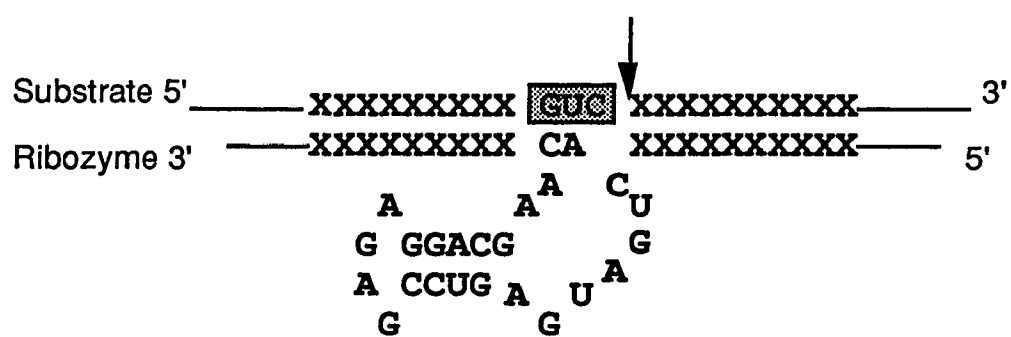
Based upon sequence comparison and truncation experiments, a consensus catalytic domain required for cleavage among plant RNA viruses was identified, termed as "hammerhead" (Forster et al. 1987b). The secondary structure of a simple useful form of hammerhead ribozyme with high cleavage specificity has been described (Haseloff et al. 1988). It consists of two principle motifs: a 24-nucleotide-long catalytic center and a recognition sequence (Figure 4-1). The only sequence requirement for the cleavage site is that it contain the trinucleotide GUC (or perhaps GUA, GUU, CUC or UUC). Since the minimum requirement for efficient cleavage within the target sequence is a triplet, there are many potential cleavage sites in RNAs. The model provides the basis for *de novo* design of ribozymes with desirable sequence-specific endonuclease activities.

Haseloff and Gerlach (1988) have designed three ribozymes against a chloramphenicol acetyl transferase (CAT) gene for use *in vitro*. The ribozymes and CAT gene were transcribed *in vitro* by T7 RNA polymerase. When the CAT mRNA was incubated with any one of the three ribozymes in 50 mM Tris-HCl, 20 mM MgCl₂ at 50°C for 60 minutes, efficient cleavage occurred producing two RNA fragments. RNA sequencing and terminal nucleotide analyses of these fragments demonstrated that cleavage had occurred at the expected locations precisely after triplets (GUC). The *in vivo* suppression of gene expression by artificial ribozymes was first demonstrated by Cameran and Jennings (Cameron et al. 1989). Two plasmids containing ribozyme and CAT gene were cotransfected into monkey (COS1) cells. At a high molar excess (about

1,000-fold) over the CAT mRNA this ribozyme was able to constantly and specifically suppress CAT expression (up to 60%), as measured by the CAT assay. It has also been shown that ribozymes function as a potential anti-viral agent against the human immunodeficiency virus-1 (HIV-1) (Sarver et al. 1990). A ribozyme motif targeted HIV-1 gag RNA was cloned into a mammalian expression vector under the control of a constitutive human beta-actin promoter. The ribozyme was successfully expressed in CD4⁺ HeLa cells with a 5' GpppG cap and a 3' poly(A) tail. When these cells were challenged with HIV-1, a substantial reduction in the level of HIV-1 gag RNA relative to that in non-ribozyme -expressing cells was observed. The reduction in gag RNA was reflected in a reduction in antigen p24 level, which is encoded by gag RNA.

Before designing of an effective ribozyme, there are a number of factors that have to be considered. (a) The cleavage reactions catalyzed by ribozymes are slower than those catalyzed by proteins, especially under physiological conditions. This low catalytic efficiency may be due to the formation of an active ribozyme conformation and subsequent dissociation from substrates (Jeffries et al. 1989; Hampel et al. 1990). Therefore, to achieve a significant biological effects a high intracellular level of ribozyme is required, as demonstrated by Cameron and Jennings (1989). (b) Ribozyme species transcribed *in vivo* should be relatively stable, since unprotected ribozymes will be degraded rapidly by abundant cellular nucleases (Cameron et al. 1989). To increase the stability, ribozymes can be modified by adding accessory structures such as a 5' GpppG cap and 3' poly(A) tail (Sarver et al. 1990) or inserted into a gene (Cameron et al. 1989). It has been shown that the catalytic activity of modified ribozymes was not impeded and that they were relatively stable in cell. (c) The target region should be exposed exteriorly and free of cellular protein interaction. This will allow the ribozyme to interact with target and assemble into an active structure (Cotten et al. 1989).

Figure 4-1. A ribozyme action model. A ribozyme hybridized to a substrate strand. The arrow indicates the site in the substrate where cleavage will occur. This site is immediately adjacent to the required GUC triplet sequence(also could be GUA, GUU, CUC or UUC). A 23-nucleotide long " catalytic domain " is in the middle of the ribozyme, which contains the consensus sequences that confer the ability to cleave the substrates. This region is common to all "hammerhead" ribozymes. Around the catalytic domain is the flanking sequences, which are chosen to complement to the target sequence surrounding the cleavage site and are thus able to interact specifically with a preselected cleavage site.



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CHAPTER 5

Generating Loss-of-Function Phenotypes of the fushi tarazu Gene With A Targeted Ribozyme In Drosophila

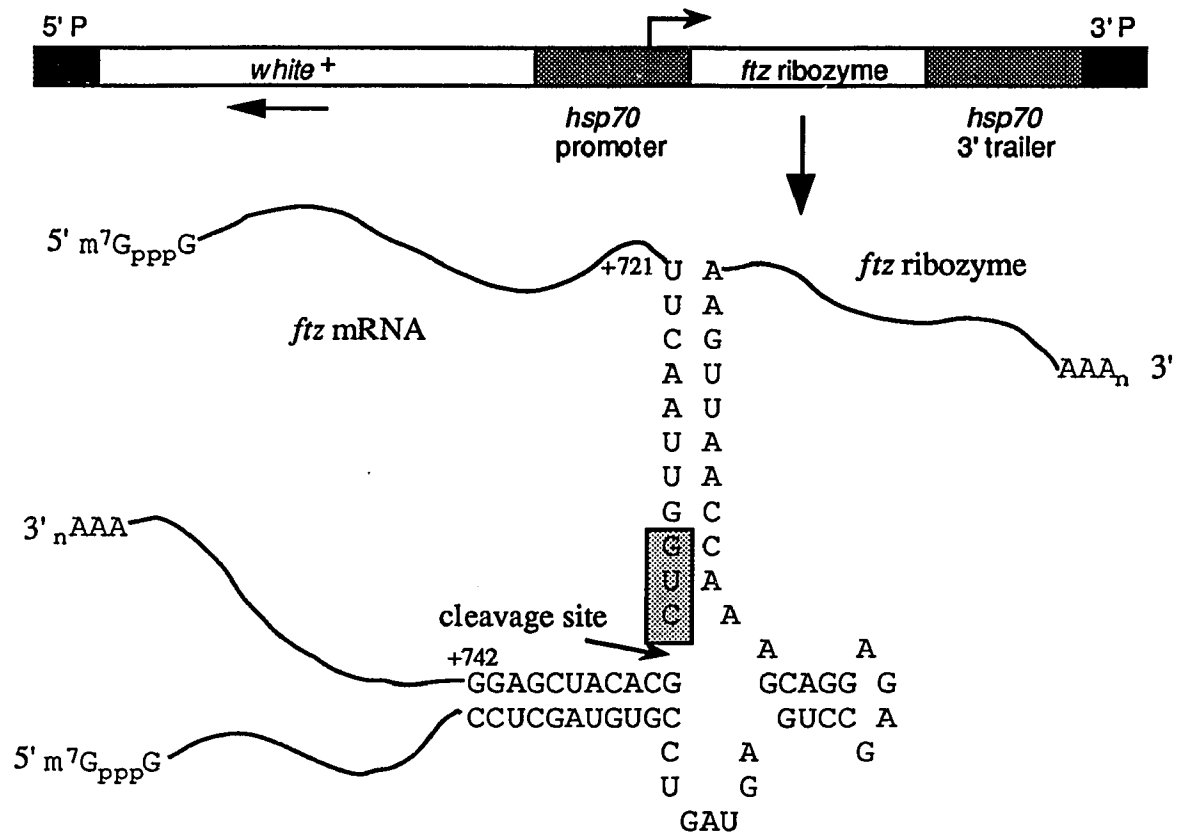
(under review for Nature)

THE ability to isolate gene sequences and to analyze their expression patterns has generated an urgent demand for creating mutations to assess their biological functions. In *Drosophila melanogaster*, although this can be achieved by traditional mutagenesis, the approach is time-consuming, labor-intensive, and often unsuccessful. Moreover, for genes expressed at multiple times during development, their functions in later stages are often obscured because of disruptions caused by the absence of early gene function. Here, we propose a new strategy to create conditional knock-out mutations using a targeted heat-inducible ribozyme. We created transgenic flies bearing a ribozyme against the *Drosophila fushi tarazu (ftz)* gene. The two phases of *ftz* function could be distinguished by timed induction of the ribozyme. Activation of the ribozyme in the blastoderm disrupts the *ftz* seven-stripe pattern and produces *ftz*-like pair-rule defects in larvae. The involvement of *ftz* in neurogenesis was verified by activation of the ribozyme during the early phase of CNS formation.

The ribozyme, an RNA molecule, can specifically cleave RNAs in a catalytic manner¹. Several different types of ribozymes have been identified by their primary and secondary structures. One of the most well-studied ribozymes is the "hammerhead" type derived from plant viral RNAs². The secondary structure of a simple useful form of hammerhead ribozyme consists of two separable functional motifs: a 24-nucleotide-long catalytic domain possessing the cleavage activity and a recognition motif which is complementary to a target RNA sequence^{3, 4}. Since the only sequence requirement for the cleavage of a target is that it contain the trinucleotide NUX (N=G, C, or U; X=C, A, or U), almost any RNA molecule can be targeted by properly designed ribozymes.

Figure 5-1. The *ftz* ribozyme transgene tested in *Drosophila* embryos. The *ftz* ribozyme is under the control of a *Drosophila* heat shock promoter (*hsp70*). The transcripts consist of the *hsp70* 5' leader (~207 bases) and 3' trailer (~525 bases) with a poly A tail (not drawn to scale). These structural motifs protect the ribozyme from degradation by intracellular RNases. This ribozyme is targeted to cleave *ftz* RNA at the nucleotide +730, which is upstream of the homeobox¹⁰. The lower part of the figure is a diagram of the predicated interaction between the *ftz* ribozyme and targeted *ftz* mRNA.

METHODS. A synthetic oligonucleotide containing the *ftz* ribozyme sequence 5'-TCGACCTCGATGTGCCTGATGAGTCCGAGAGGACGAAA CCAATTGAAT-3' with its complementary oligonucleotide was initially cloned into the *Sall* and *XbaI* sites of a pGEM7Zf(-) derived vector which contained a 0.9 Kb *EcoRI* fragment from HZ50PL²⁰. Then, the fragment containing 65 nucleotides of the 3' end of the *E. coli LacZ* gene derived from HZ50PL and the *ftz* ribozyme DNA was subcloned into the *EcoRI* and *XbaI* sites of a *Drosophila* P element vector, CaSpeR-hs²¹. This vector contains the *hsp70* promoter, *hsp70* 3' trailer, and the mini *white*⁺ gene (an eye-color marker). The construct was microinjected into *Df(1)w* eggs along with the helper plasmid p π 25.1wc²². Four independent germline transformants were obtained based on the selection of eye color²³. They are viable under normal growth conditions.



Several studies have demonstrated that ribozymes can destroy various RNAs in cells⁵⁻⁷ and even block human immunodeficiency virus (HIV) infection in cultured cells^{8, 9}. Although ribozymes may have some potential to act as anti-viral agents, they are also likely to be useful reagents for studying gene function during animal development. Unlike viral genes, critical regulatory genes are usually expressed at a relatively low level, therefore, the reduction of their transcripts is likely to have considerable effects. Furthermore, the ribozyme can be delivered to a specific region or at a particular developmental stage by using a region-specific or inducible promoter.

The *Drosophila ftz* gene is a good candidate for testing this notion. *ftz* has been well characterized at both genetic and molecular levels¹⁰⁻¹⁴. Thus, we can easily compare known loss-of-function *ftz* mutant phenotypes with the defects generated by a *ftz*-specific ribozyme. Like several other regulatory genes, *ftz* has an early function in segmentation and also participates in neurogenesis at a later stage of *Drosophila* embryogenesis^{11, 15}. In principle, it should be possible to interfere with each of these processes separately by timed induction of a *ftz* ribozyme.

We have constructed a *ftz* ribozyme that consists of the hammerhead catalytic center and a 21-nucleotide-long flanking sequence complementary to *ftz* mRNA at positions +721 to +742, near the 5' end of *ftz* homeobox and including a GUC cleavage site¹⁰. The *ftz* ribozyme DNA template was embedded between the *hsp70* promoter and 3' untranslated sequence (Figure 5-1) and inserted into the fly genome via P-element mediated transformation.

To determine whether the *ftz* ribozyme could efficiently block *ftz* protein synthesis by acting on *ftz* transcripts in embryos, we induced expression of the ribozyme in embryos between 2 and 2.5 hr after egg laying (AEL), corresponding to the first period of *ftz* gene expression^{11, 14}. In the wild-type blastoderm, *ftz* protein is expressed strongly

in a pattern of seven transverse stripes (Figure 5-2A). As we expected, the amount of *ftz* protein was dramatically reduced in embryos expressing the *ftz* ribozyme after heat induction (Figure 5-2 B and C). The reduction of *ftz* protein was not uniform within a batch of embryos. In some, *ftz* protein was depleted more completely (Fig. 5-2B); in some, just partially (Figure 5-2C). Since the stripes that appeared to be the most resistant to the ribozyme are those that emerge earliest (stripes 1, 2, and then 5^{16, 17}), it is likely that small differences in the exact age of different embryos within the population account for the variable sensitivities observed.

Embryos homozygous for known *ftz* mutations die prior to hatching and lack alternate segments evidenced by loss of odd numbered abdominal denticle belts in the larval cuticle¹². Expression of the *ftz* ribozyme at the blastoderm stage caused *ftz*-like pair-rule morphological defects in the resulting first instar larval cuticles (Figure 5-3). The second thoracic segment (T2) and odd-numbered abdominal segments (A1, A3, A5, and A7 segments) were completely deleted in the most severely affected cuticles (Figure 5-3D). We also often observed cuticles with less severe defects in the same preparations (Figure 5-3B and C). Generally, A5 is the most sensitive to the *ftz* ribozyme, followed by A3, A7, and A1 segments, while T2 is the most resistant to ribozyme effects. This range of phenotypes was also observed with a *ftz* temperature sensitive mutant¹². Further, just as the A5 denticle was the most sensitive to loss of *ftz* activity due to the ribozyme, it was the region of the cuticle most difficult to rescue with altered *ftz* proteins¹⁸. Thus, the different sensitivities to the *ftz* ribozyme are most likely due to differential requirements for active *ftz* protein in different stripes.

The *ftz* gene is also expressed later in the developing nervous system^{11, 15}. However, the loss of striped expression of *ftz* alters the fate of blastoderm cells, and causes such severe defects in segmentation and abnormalities in the neuroectoderm that an analysis of *ftz* function in neurogenesis is precluded. The role of *ftz* in the CNS

was assessed using a *ftz* transgene expressed in stripes but not detectable in the nervous system¹⁵. This study suggested a role for *ftz*⁺ product in the specification of neuron RP2, and for the positive regulation of the homeobox-containing genes *even-skipped* (*eve*) in RP2 neurons and *Ultrabithorax* in sibling neurons RP1 and RP2¹⁵.

To test the ability of the *ftz* ribozyme to distinguish the roles of *ftz* in segmentation and in neural development, we induced ribozyme expression at 4.5 hr to 9 hr AEL. The expression pattern of *eve* in the developing CNS was used as a marker to monitor neuronal abnormalities. In wild type embryos, *eve* protein is expressed in the RP2 neuron and a cluster of neurons composed of aCC, pCC, and a pair of CQ neurons in each hemisegment^{15, 19} (Figure 5-4A and B). In embryos expressing the *ftz* ribozyme, however, *eve* expression was greatly reduced in RP2 neurons (Figure 5-4C and D), as seen in *ftz* neurogenic mutants¹⁵. Moreover, we observed that the *eve* pattern in the cluster (aCC, pCC, and CQ neurons) was also altered in some embryos (Figure 5-4C and D). This additional effect may not be surprising since *ftz* is expressed in this region¹⁵. Thus, although loss of *ftz* function did not alter *eve* expression in the cluster, it appeared to affect the morphology and/or migration pattern of these neurons. No segmentation defects were observed in cuticles of animals exposed to the ribozyme at this later stage of development.

These experiments demonstrate that induction of a *ftz*-specific ribozyme mimicked the effects of known *ftz* mutations. Moreover, the timed induction of the ribozyme was able to distinguish the functional properties of two separate phases of *ftz* expression: first, in segmentation and later, in neurogenesis. In addition, the range of phenotypes induced by the ribozyme (see Figure 5-3) allow for an assessment of both "hypomorphic" and "amorphic" effects of a gene without the necessity for generating multiple mutations of a given gene.

Figure 5-2. Inhibition of *ftz* protein synthesis by the inducible expression of the *ftz* ribozyme in transgenic embryos. Embryos were given two short heat pulses starting at the age of 2.0 to 2.5 hours AEL. Lateral views of whole-mount embryos; anterior is left; dorsal is up. A, A wild type embryo, the *ftz* protein expression pattern is indistinguishable from a non-heat shocked embryo. B, C, Embryos bearing the *ftz* ribozyme displayed various degrees of reduction of *ftz* protein. In B, most transverse stripes were depleted. In C, the level of *ftz* protein expression was much lower than the wild type (compare to A); low levels of the stripes, especially the first and second stripes were detectable.

METHODS. Embryos were collected for 30 min and matured to 2.0-2.5 hr at 25°C. Then, embryos were treated by two heat pulses each combined a 5 min heat shock in a 37°C water bath followed by 20 min recovery at 25°C. The age of embryos was verified after immunostaining by the progression of nuclear elongation during blastoderm formation. Immunostaining was carried out by a modified standard protocol (T. Gutjahr and M. Noll, personal communication) using a monoclonal mouse anti-*ftz* antibody (kindly provided by D. Mattson and I. Duncan). Since the antibody is directed against the amino terminal portion of the protein, residual protein may reflect a low level of translation of the truncated *ftz* RNA after ribozyme cleavage.

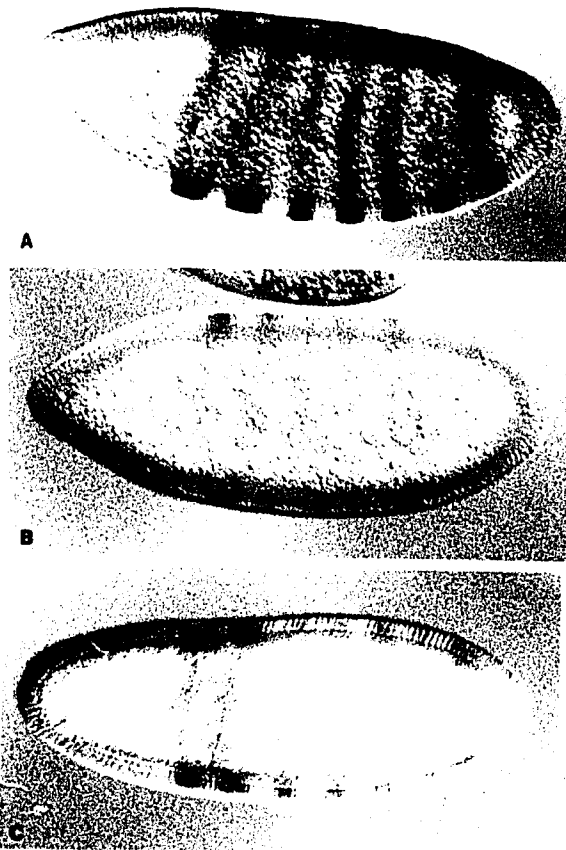


Figure 5-3. Pair-rule phenotypes generated in *ftz* ribozyme transformant animals. A, Cuticular pattern of a wild type first instar larva. From anterior to posterior (top to bottom), the visible segments consist of three thoracic segments (T1-T3) and eight abdominal segments (A1-A8), each characterized by a specific distribution pattern of ventral denticle belts. B, The weakest pair-rule phenotype observed in embryos expressing the *ftz* ribozyme. Only the A5 denticle belt was missing. C, A mild mutant phenotype which displayed a partial loss of A5, A3 and A1 denticle belts and a fusion between A6 and A7 segments. These weak pair-rule phenotypes (having partial gaps and/or fusions of some denticle belts) are extremely similar to the abnormalities observed in *ftz* mutant embryos hemizygous for the *ftz^{f47ts}* allele¹² or *ftz^{Rpl}* allele¹³. D, A strong pair-rule phenotype. A1, A3 and A5 segments were completely fused to T3, A2 and A4 respectively, while the A7 segment was partially deleted or fused. E, The typical pair-rule defect in the cuticle of a *ftz* null mutant larva (homozygous for *ftz* 9093 allele²⁴). The mutant larva retains six denticle belts (T1, T3, A2, A4, A6, and A8), the other five denticle belts (T2, A1, A3, A5, and A7) are deleted.

METHODS. A, B, C, D, embryos were collected over one hour and allowed to develop at 25°C. The 20 min heat-shock was delivered to the embryos in a 37°C water bath at the age of 2.5~3.5 hr AEL. The treated embryos were allowed to further mature to first instar larvae at 25°C. The cuticular preparation was based on a modified procedure by Zhao et. al.²⁵ and analyzed and photographed using dark-field microscopy.

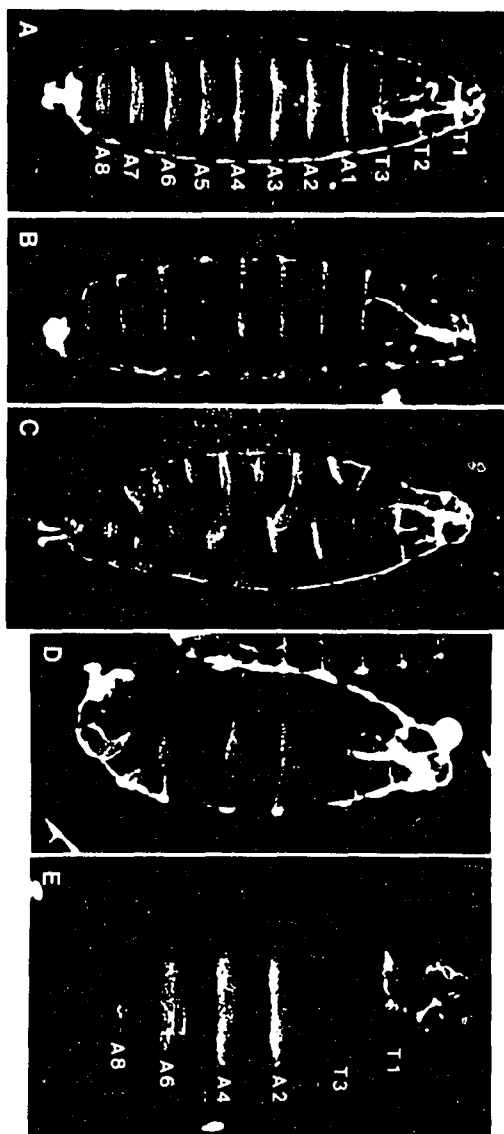
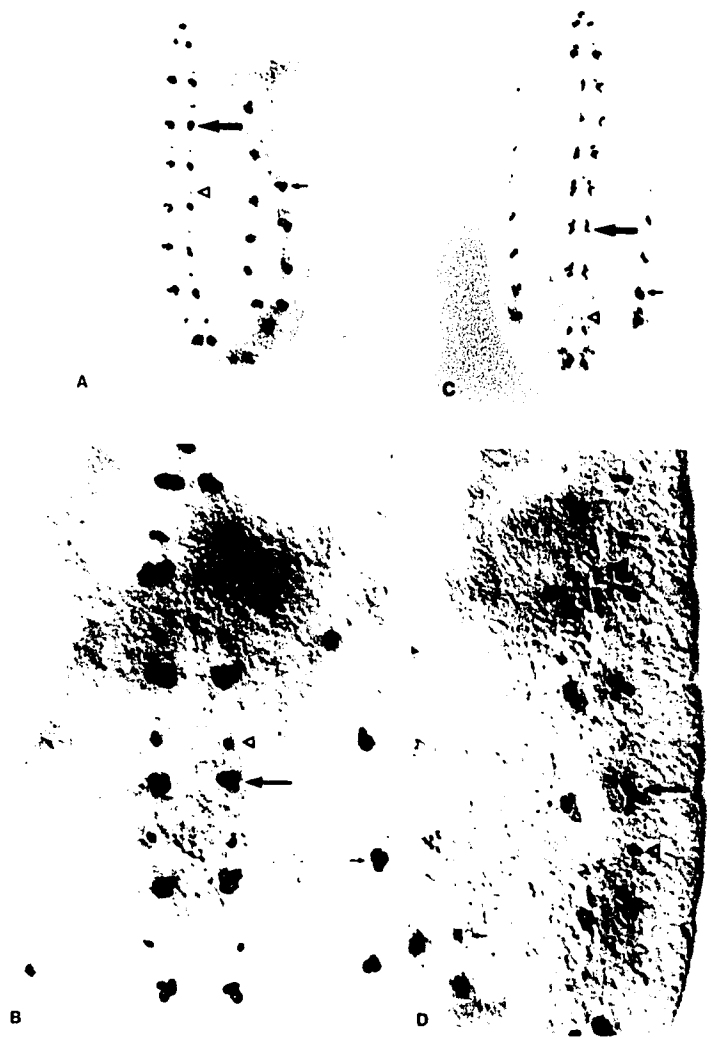


Figure 5-4. Altered *eve* expression in the *Drosophila* CNS induced by the *ftz* ribozyme. The *eve* staining patterns are shown in wild type embryos (A, B) and in embryos expressing the *ftz* ribozyme (C, D) at age 8-9 hr AEL with a whole mount view (A, C) and a high power view (50X, B, D). In all panels, embryos are viewed from the ventral side with the anterior at the top. The triangles indicate RP2 neurons. The cluster composed of aCC, pCC, and CQ neurons is indicated by a long arrow. The pericardial cells (non-neuron cells) are indicated by a small arrow. A, B, In a wild type embryo, before finishing germ-band retraction, each hemi-segment contains five neurons expressing equal levels of *eve* protein¹⁹. Among them, aCC, pCC and a pair of CQ neurons are clustered (indicated by a long arrow), while the RP2 neuron is located separately from the cluster (indicated by a triangle). C, D, *eve* expression was disrupted in RP2 neurons after the induced expression of the *ftz* ribozyme during neurogenesis. In most segments, no *eve* protein is detectable in RP2 neurons. In some segments, low levels are still detectable (indicated by a triangle). Similar effects on *eve* expression were observed in a *ftz* neurogenic mutant¹⁵. In the clusters, the level of *eve* expression seems to be relatively normal, however, the pattern of the cluster was altered (compare D to B).

METHODS. Embryos aged to 4.5~5.5 hr AEL were heat shocked three times for 20 min at 37°C with a 40 min interval at 25°C for recovery between the heat shocks. The treated embryos were fixed one hour after the final heat pulse and immunostained with an anti-*eve* antibody (kindly provided by M. Frasch).



To our knowledge, this report presents the first successful demonstration that a ribozyme can act as a conditional knock out reagent to investigate potential biological functions of a gene in a living animal. In principle, this technique can be used to probe the following questions without specific requirements for genetic manipulation: maternal effects; gene functions at multiple stages; functions of alternatively spliced transcripts; the hierarchical order of effects of different regulatory genes during development.

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