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Homebox genes in mouse fetal thymocyte development

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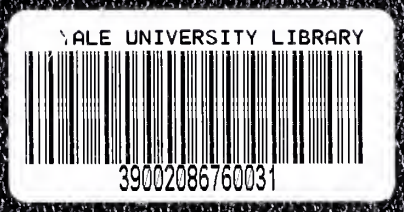

Homeobox Genes in Mouse Fetal Thymocyte Development

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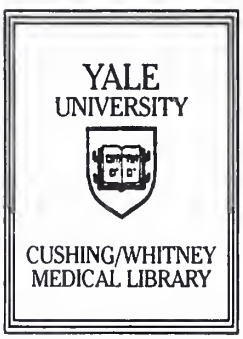
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Homeobox Genes in Mouse Fetal Thymocyte Development

A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

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HOMEOBOX GENES IN MOUSE FETAL THYMOCYTE DEVELOPMENT

Christiana Muntzel*, Connie Sommers, Jennifer Lee, and Paul E. Love, Laboratory of Mammalian Genes and Development, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland, 20892. *1995-1996 Howard Hughes Medical Institute-National Institutes of Health Research Scholar (Sponsored by Agnes Vignery, Department of Cell Biology, Yale University School of Medicine)

ABSTRACT

The development of mature, functional T-cells that recognize foreign antigens but not self-peptides is a complicated process involving multiple steps. Immature T-cells originate in the fetal liver and adult bone marrow and migrate to the thymus, where they undergo negative and positive selection, events that depend upon the specificity of their T-cell antigen receptor (TCR). During development, thymocytes differentiate from $TCR^{-}CD4^{-}CD8^{-}$ to $TCR^{lo}CD4^{+}CD8^{+}$, and finally to mature $TCR^{hi}CD4^{+}CD8^{-}$ (helper) and $TCR^{hi}CD8^{+}CD4^{-}$ (killer) T cells, whereupon they migrate to the periphery to carry out effector functions. Our laboratory seeks to identify and characterize molecules involved in the T-cell development process.

Using an RT-PCR approach on fetal thymus mRNA, we have identified several potentially novel genes in the homeobox superfamily, which are expressed during fetal thymic development in 13.5 day mouse embryos. Homeobox genes are ubiquitous developmental regulating genes thought to act as transcription factors. While they have been extensively studied in other systems, and are known to play important roles in determining cell fates along the anterior-posterior body axis, their role in T-cell development has not yet been thoroughly explored. We therefore seek to characterize potentially novel homeobox genes and determine how they influence T-cell development. Initial studies are geared toward identifying their expression pattern in fetal and adult tissues using such techniques as RT-PCR and Northern Blot.

ACKNOWLEDGMENTS

This project was made possible by funding from the Howard Hughes Medical Institute-National Institutes of Health Research Scholars Program, in which I participated during 1995-1996. I would like to thank Dr. Donald Harter, Director, and the staff of the "Cloister" program for providing a wonderful research opportunity.

My sincere thanks go to Paul E. Love, who welcomed me into his laboratory at the National Institute of Child Health and Human Development and oversaw my project, and to Connie Sommers, the postdoctoral fellow with whom I worked most closely. I could not have carried out this project without Connie's support, advice, and technical assistance. Thanks are also due to the other members of the Love lab (Hala Azzam, Howie Shen, Alex Grinberg, Kun Huang) and to Karl Pfeifer, for technical and moral support. My part of the project would not have been possible without the earlier work of Connie Sommers and Jennifer Lee, and is currently being continued by Howard Shen.

I would like also to extend my utmost appreciation to Agnes Vignery, my sponsor at Yale University. Without her encouragement I would never have considered applying for the HHMI-NIH Research Scholars Program. Thanks also go to Robert Tigelaar of the Section of Immunobiology, for reading my thesis and helping me to improve the finished product.

Finally, I thank my family for a lifetime of love and support.

TABLE OF CONTENTS

INTRODUCTION AND REVIEW OF THE LITERATURE

Homeobox genes

Homeobox genes comprise a highly conserved, nearly ubiquitous superfamily of developmental regulating genes. A *homeobox gene* is defined as any gene which contains a homeobox. The *homeobox* itself is a conserved DNA sequence consisting of approximately 180 base pairs that encode a DNA binding domain of around 60 amino acids. This amino acid domain is known as the *homeodomain*. Homeobox genes were first discovered in Drosophila, but have been found to exist in a variety of organisms, including yeast, *C. elegans*, insects, *Xenopus*, zebrafish, and mammals, including humans.[1-3] They are believed to act as transcription factors, with the homeodomain acting as the DNA binding domain, it's 3-dimensional structure consisting of a helix-turn-helix motif.[4]

Homeobox genes are being extensively studied in both invertebrates and vertebrates. In Drosophila, two clusters of "master control genes" play a central role in determining the process of development in the fruit fly. These clusters, known as the Antennapedia complex (ANT-C) and bithorax complex (BX-C), most likely evolved from an ancestral gene by the process of tandem duplication and subsequent divergence.[5] They have been shown in Drosophila to be important in determining anterior-posterior body axis, controlling the number and pattern of segments, and establishing the identity of a given segment. In vertebrates homeobox genes have an analogous function in determining developmental fates along the anterior-posterior body axis.[2]

A mutation in a homeobox gene may result in a *homeotic transformation*, a developmental anomaly in which one part of the body develops in the likeness of another in the absence of a particular homeobox

gene function.[1] It was the discovery and study of homeotic transformations in Drosophila, begun by Edward B. Lewis in 1948, which gave an early indication of the importance of these developmental regulating genes, before the advent of molecular biology.[2]

Homeobox genes occur in many families: in vertebrates, these include Hox, Pax, and POU.[6] The Hox Genes are a family of vertebrate homeobox genes similar to the Drosophila Antennapedia gene (Antp). These Hox genes occur in four clusters (on mouse chromosomes 2, 6, 11, and 15[7] or human chromosomes 7, 17, 12, and 2[8]), which appear to be closely related evolutionarily based upon the high degree of conservation of their sequences.[9] Each cluster is made up of up to 13 genes. The genes within each complex are transcribed in the same direction. The order of these genes along the chromosome is conserved and reflects where they are expressed along the body axis. When hox genes were first discovered, they were given numerical names based upon their chronological order of discovery. Because it became known that the order of genes along the chromosome had meaning, in 1992 a new vertebrate nomenclature was agreed upon based on the linear arrangement of the genes.[10] The four major hox clusters are now known as A, B, C, and D (Figure 1). The genes within each cluster are numbered in the order they fall along the chromosome, 1 through 13, with lower numbers being located 3' to the higher numbers. In the new nomenclature, hox genes with the same number, for example HoxA2 and HoxB2, are known as paralogs, because of their high degree of homology and probable evolutionary relatedness. Lower numbers correspond to more anterior expression along the anterior-posterior body axis of the organism.[10]

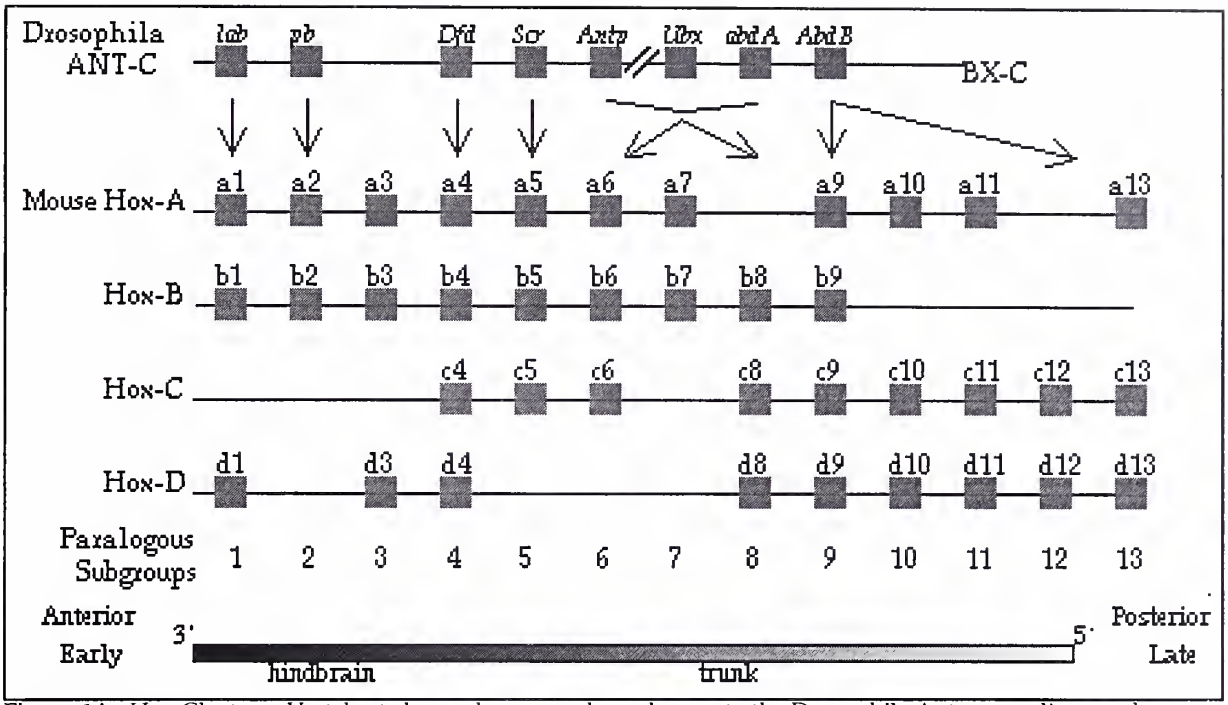


Figure 1A. Hox Clusters. Vertebrate homeobox genes homologous to the Drosophila Antennapedia complex (ANT-C) are thought to have arisen through tandem duplication and subsequent divergence. They occur in four paralogous groups on separate chromosomes (HoxA, HoxB, HoxC, HoxD). The order along the chromosome and the sequence of expression are conserved. Genes are numbered in order from 3' to 5'. (After reference 11).

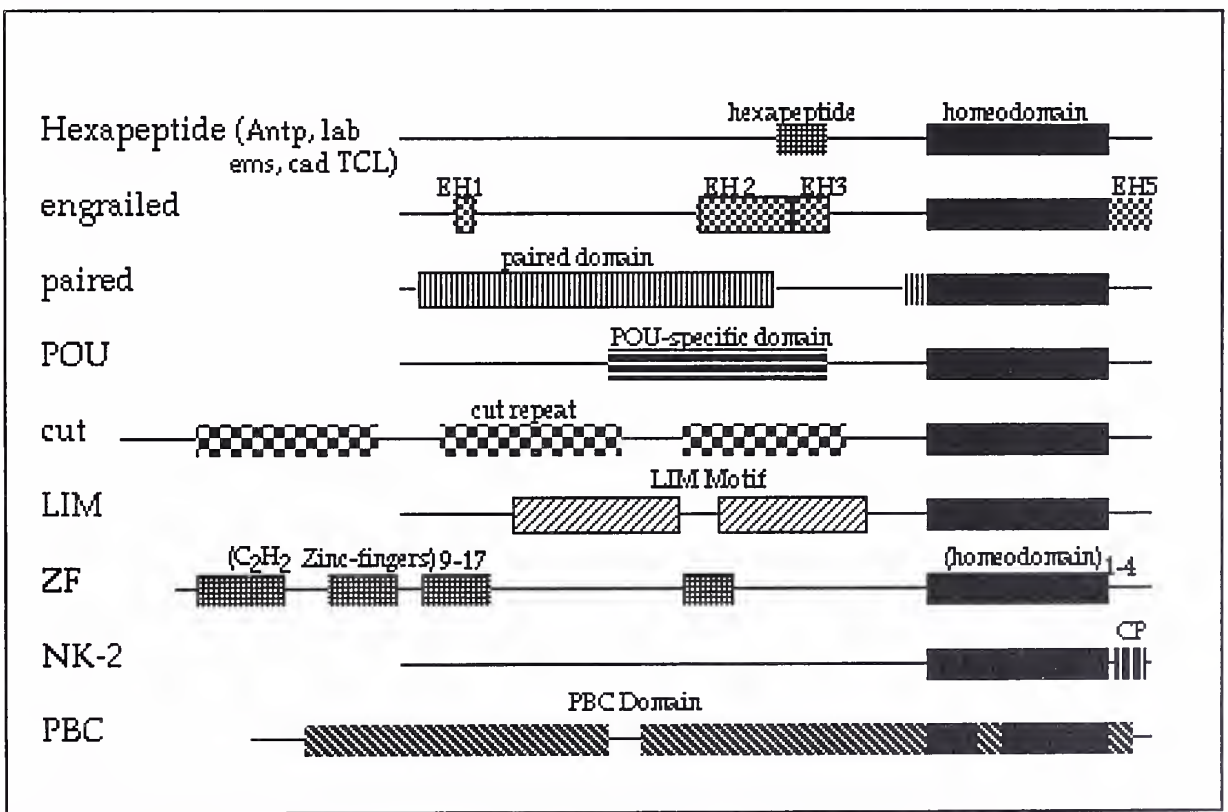


Figure 1B. Homeobox genes occur in many classes. Homeobox genes are grouped into classes and families based upon the homology of their homeodomains, as well as other conserved domains upstream of the homeodomain. These include paired, POU, LIM, cut, and zinc finger domains, which may play roles in DNA binding (After reference 12).

This discussion will make use of the new nomenclature exclusively, regardless of whether the articles reviewed were written prior to its inception.

One area which has not been extensively studied is the role of homeobox genes in the development of the immune system.

T-Cell development in the Thymus

The development of mature, functional T-cells that recognize foreign antigens but not self-peptides is a complicated process involving multiple steps. Immature T-cells originate in the fetal liver or adult bone marrow and migrate to the thymus, where they are known as thymocytes. Here, they undergo negative and positive selection, events that depend upon the specificity of their T-cell antigen receptors (TCRs). During development, thymocytes differentiate from $TCR^{-}CD4^{-}CD8^{-}$ "double negative" to $TCR^{lo}CD4^{+}CD8^{+}$ "double positive", and finally to mature "single positive" $TCR^{hi}CD4^{+}CD8^{-}$ (helper) and $TCR^{hi}CD8^{+}CD4^{-}$ (killer) T cells, whereupon they migrate to the periphery to carry out effector functions (Figure 2). While revolutionary strides have been made in elucidating mechanisms of T-cell development, there is much that is still unknown, and this is the subject of broad research and heated debate. Our laboratory seeks to identify and characterize molecules involved in the T-cell development process. Homeobox genes were chosen to study because they have been implicated in the development of nearly all organ systems across almost all phyla, but have not been widely studied in the thymus.

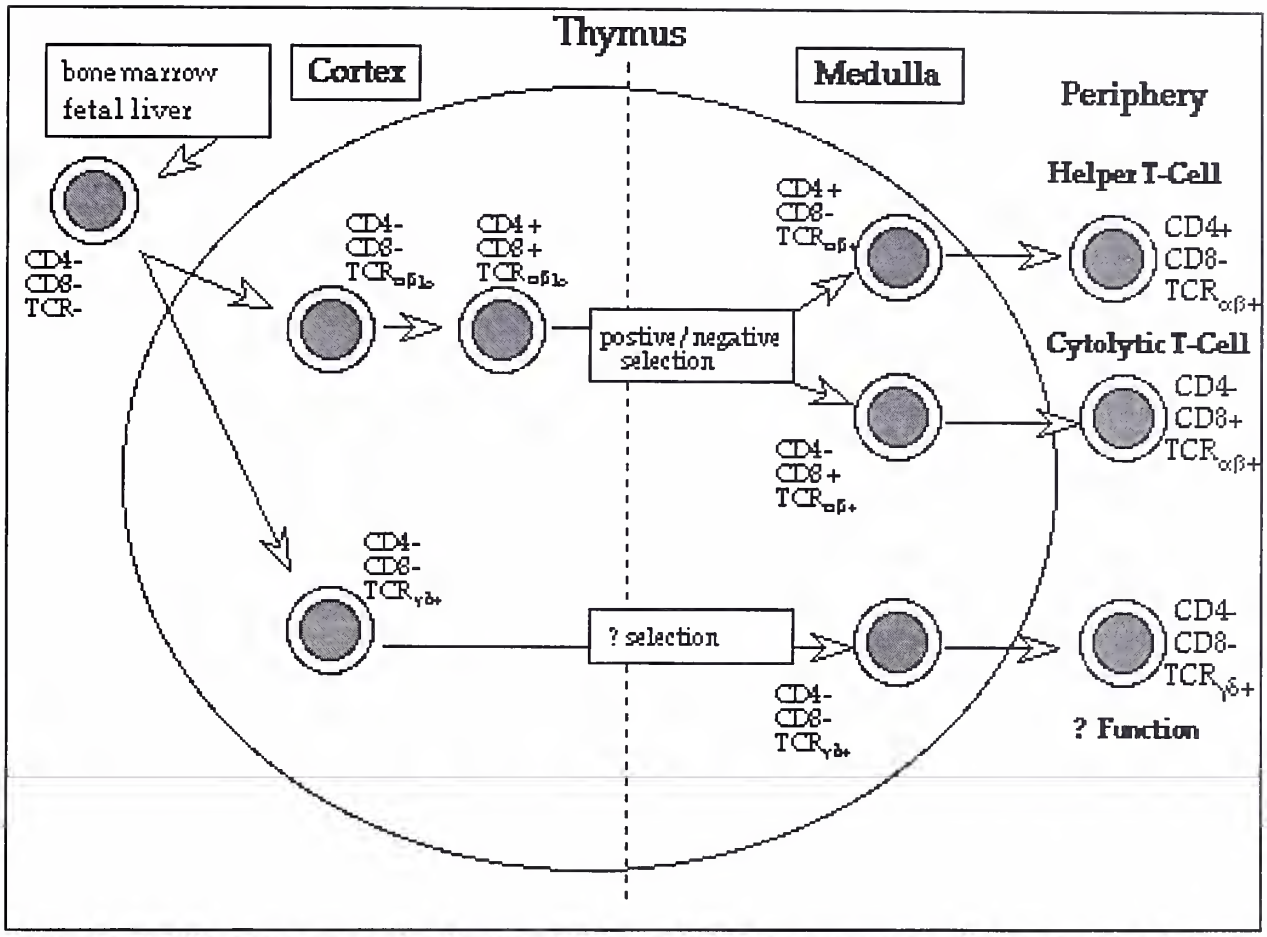


Figure 2 T-Cell maturation in the thymus. $TCR_{\gamma\delta}$ and $TCR_{\alpha\beta}$ expressing cells are separate lineages that develop from a common precursor, which migrates to the thymus from the fetal liver or adult bone marrow. In the thymus, $TCR_{\alpha\beta}$ thymocytes undergo negative and positive selection, and differentiate from $TCR^{-}CD4^{-}CD8^{-}$ to $TCR^{lo}CD4^{+}CD8^{+}$ and finally to mature $TCR^{hi}CD4^{+}CD8^{-}$ (helper) and $TCR^{hi}CD4^{-}CD8^{+}$ (cytolytic) T-cells, whereupon they migrate to the periphery to carry out effector functions (After reference 13).

Evidence Suggests that Homeobox Genes May Play A Role in Immune System Development and Regulation

Mice lacking HoxA3 are athymic. In 1995, Manley and Capecchi analyzed mice in which they had engineered a targeted deletion of the Hox A3 gene.[14-15] HoxA3 knockout mice have a number of defects in tissues and structures derived from the mesenchymal neural crest. In addition to thyroid hypoplasia, these mice lack thymi. The spectrum of abnormalities closely

resembles those found in the human disease DiGeorge syndrome (haploinsufficiency, hypoparathyroidism, thymic and thyroid hypoplasia, cardiac deficiencies, craniofacial abnormalities). Occasionally some HoxA3 knockout mice have remnants of thymi. In double mutants where both HoxA3 and HoxD3 genes had been knocked out, all mutants were athymic. This demonstrates that paralogous genes may have partially redundant functions and act in synergy.[16]

Pax-1 mutant mice have thymic hypoplasia.[17] Mice homozygous for a deletion or a point mutation in Pax-1, a vertebrate paired-box gene, have a thymus that is 2/3 smaller than wild type. Mutant thymic tissue is interspersed with cell free cysts that are lined with endothelium.

While the above gene mutations cause actual deficiency in the gross morphological structure of the thymus, our laboratory is interested in homeobox genes which may play a more subtle role in the development of T-cell immunity, perhaps in regulating the maturation of T-cell precursors. There are several homeobox genes which are expressed preferentially in hematopoietic cell lines or are elevated during lymphocyte activation. These studies may help shed light on the role homeobox genes may play in T-cell or immune system development.

Of the few studies focusing on homeobox activity in lymphocytes, most have been done on transformed cell lines, because in normal blood and bone marrow, expression of transcription factors is very low, and it is likely that Hox transcripts are restricted to small subsets of cells.[4] However, homeobox gene expression has been shown in nonmalignant hematopoietic cells. Of the four vertebrate Hox clusters, most studies have found members of HoxA,

HoxB, and HoxC clusters expressed in hematopoietic cells or cell lines, but not HoxD.[18-19,8]

The HoxB cluster appears to be important in hematopoietic cells. Deguchi[20] isolated a cDNA from the HoxB7 gene by using a degenerate oligonucleotide probe from a conserved region in the homeobox to screen a human B-cell library. He found by Northern blot and RNase protection assays that the HoxB7 expression was induced in virally transformed T- and B-cell lines, as well as B-cells activated by staphylococcus aureus Cowan strain 1 and phorbol myristate acetate (PMA), and T-cells activated by phytohemagglutinin and PMA. No transcripts were found in inactivated normal B- and T- lymphocytes.

Care et al[21] , in their study of the role of HoxB cluster genes in adult T-cells, found a similar expression pattern with all genes of the HoxB cluster--no expression in resting T-cells, and rapid induction when stimulated with PHA. This induction occurred in a 3' → 5' wave (i.e. HoxB1→HoxB9). Treatment of T-lymphocytes or leukemic T-cell lines with antisense oligos to HoxB2 or HoxB4 resulted in a drastic reduction in T-cell activation and proliferation.

When HoxB4 was overexpressed in murine bone marrow cells[19] both in vitro and in vivo transplantation studies showed an increased ability of the bone marrow to regenerate the most primitive hematopoietic stem cell compartment, but this was not accompanied by identifiable anomalies in the peripheral blood. Hence HoxB4 may be an important regulator of early but not late hematopoietic cell proliferation.

When Inamori[22] et al. used degenerate homeobox primers from the homeodomain of Antp class genes in reverse transcriptase-polymerase chain reaction (RT-PCR) on a Jurkat-T-cell-leukemia line, 10 different homeobox

genes, including 1 novel sequence were detected, with HoxB7 being the most abundantly represented. HoxB7 was shown by RT-PCR using specific primers to be expressed in 12 of 15 T-cell lines and normal peripheral blood mononuclear cells. Fluorescence activated cell sorter (FACS) analysis showed this signal to be enriched in $CD4^+$ vs. $CD8^+$ T-cells, and to increase with T-cell activation.

HoxC4 gene product has immunoregulatory activity that is not restricted to T-cell lineage-specific genes. Meazza et al.[23] studied the expression of homeobox genes in resting and activated peripheral blood lymphocytes (PBLs) using monoclonal antibodies and RNase protection experiments. They found that HoxC4 protein is expressed in activated and/or proliferating lymphocytes of the B-, T-, and natural killer (NK)-cell lineages. It is only weakly expressed in a few resting cells. Furthermore they found that while resting and activated lymphocytes had similar levels of HoxC4 mRNA, it was only upon activation that the HoxC4 protein was present, suggesting that regulation is at the translational level.

***Hlx* appears to play an important role in the maturation of B-lymphocytes and myeloid cells.** Numerous studies have focused on *Hlx*, a diverged mouse homeobox gene not linked to the Hox gene clusters, and its human homologue HB24. *Hlx* has been shown to be expressed in myeloid cells, especially mature macrophages and granulocytes, and also in immature B-cells, but not mast, erythroid, or T-lymphoid cells.[24-25] Expression of *Hlx* was shown by Allen and Adams[25] to increase with myelomonocytic maturation, and when HB24 was overexpressed in a myeloid cell line, it was accompanied by a decrease in two markers of immaturity (Thy-1 and CD34),

and showed a phenotype of differentiation. While Deguchi[26] reported that enforced expression of HB24 in Jurkat cells induced genes involved in T-cell activation, this finding was not reflected in Allen and Adam's T-cell lines expressing *Hlx*.

Transgenic mice with ectopic HB24 expression under the control of the TCR-Beta promoter and enhancer were engineered to study the effects of HB24 on lymphocyte activation.[27] In these transgenic mice, there was an increase in activated thymocytes and peripheral T-cells (based upon size and IL-2 expression) and a drastic decrease in $CD4^+$ T-cells in both the thymus and periphery, and impaired involution of the aging thymus. Transgenic mice engineered with both B and T-cells expressing an *Hlx* transgene (under the IgHμ enhancer and CD2 enhancer) showed abnormalities in maturation of these cells.[24] In these mice, the thymus lacked almost all mature single positive T-cells. Nearly one half of all T-cells in the periphery were double positive (a phenotype normally restricted to the thymus), and had some but not all features of mature T-cells. B-cells appeared to be arrested at the pro-B stage.

While *Hlx*/HB24 is not normally expressed in T-lymphoid cells at any stage of differentiation, its apparent importance in the maturation of B- and myeloid cells suggests that there may be such a gene, as yet undiscovered, that plays a similar role in T-cell maturation.

LH-2 appears to be differentially expressed in the γδ and αβ T-cell lineages. LH-2, a member of the LIM homeobox family and the mouse homologue of the Drosophila gene Apterous, is expressed in fetal liver, pre-B, B-cell lines, and pre-T cells, but is not expressed in the thymus.[28-29] Moreover, it is expressed in mature γδ-T-cells and in subdermal regions of embryonic skin where intra-epithelial (mostly γδ) lymphocytes reside. Thus

far, neither LH-2 nor HB24 have been shown to regulate or directly bind T-cell specific genes.[28]

CBF is a candidate for an early lineage determining gene in T-cells.[28] Core Binding Factor (CBF, AKA SL3 -3, PEBP2, SL3) is a T-cell restricted transcription factor complex made of at least two proteins. One of these two proteins, the alpha subunit, appears to be identical or related to a human homologue of the Drosophila homeobox protein *runt* (AML1). AML1 is involved in a chromosomal translocation in patients with the M2 subtype of acute myelogenous leukemia (AML).

***Prh* is differentially expressed in hematopoietic cells.** Another diverged homeobox gene, sharing 46% identity with Antp and 56% identity with HB24, is human *prh*, a novel proline-rich homeobox recently discovered to be differentially expressed in hematopoietic cells. Its expression appears to be primarily in B-cells, and not present in T-cells or T-leukemic lines. Its expression is higher in early B-cell differentiation than terminal B-cell differentiation, suggesting that dysregulated expression might play a role in leukemogenesis of specific cell lineages.[18]

Homeobox genes have been shown to play a role in acute leukemias. A recurring theme in the pathogenesis of acute leukemias is the acquisition of chromosomal aberrations which activate cellular proto-oncogenes. Most commonly this occurs through chromosomal translocations.[30] Two well-documented examples of human leukemia exist where a chromosomal translocation involves a homeobox gene. The first, t(10;14), a translocation seen in 5-7% of T-cell acute lymphocytic leukemias (ALL) involves a head-to-

head juxtaposition of a homeobox gene, HOX-11 (a.k.a. TCL-3), with a region of the TCR-δ gene. This translocation causes an overexpression of HOX-11 in affected T-cells. HOX-11 was shown by Lu et al.[31] to be present in 5-10X lower levels in two other leukemic cell lines that do not carry the t(10;14) translocation (Jurkat and HPB-ALL), and in very low endogenous levels in normal human T-cells. This allowed Lu to conclude that HOX-11 plays an important role in gene regulation in normal T-cells, and that its dysregulation is an important step towards leukemia formation.

The second translocation involving a human homeobox gene is t(1;19), a translocation seen in 30% of cases of pre-B-cell ALL.[4] In this translocation, the 5' end of the E2A gene, which encodes the enhancer-binding proteins E12/E47, is fused to a homeobox gene called *pbx* (or *prl*). The resulting chimeric homeobox, containing the amino 2/3 of the E2A protein with a homeodomain replacing its normal DNA binding region, was shown by Dedera et al. to induce both thymocyte and B-lineage progenitors to undergo proliferation and apoptosis, as well as to develop lymphoid malignancies, in transgenic mice with the fusion gene E2A-*pbx*1 under the control of the Ig heavy chain enhancer.[30] *Meis*1, a murine *pbx*1-related homeobox gene was shown by Moskow et al. to be involved in myeloid leukemia in BXH-2 mice. This strain is prone to develop leukemias as a result of the expression of an ectopic leukemia virus that serves as an insertional mutagen to alter the expression of cellular proto-oncogenes.[32] *Meis*1 acts as a site of insertion for the virus in 15% of BXH-2 mice.

Statement of Purpose

Homeobox gene expression has been identified in many hematopoietic cell lineages, and clearly plays an important role in the maturation and activation of cells involved in mammalian immune system function. The

studies on *Hlx*/HB24 have identified an important homeobox gene in B-cell and myeloid lineage cells. However, this gene is not normally expressed in T-cells. That so much remains to be understood in the development of T-cells, and their dysregulation in acute leukemias, is an indication for further study.

We hypothesize that while homeobox gene expression in the thymus has not been extensively characterized, these nearly ubiquitous genes most likely play an important role in the development of T-cell mediated immunity. The aims of this study are (1) to screen for homeobox genes that are expressed in the developing thymus using RT-PCR, and then to study their overall expression in the developing organism, and (2) to characterize any novel homeobox genes found. Once homeobox genes have been identified that are expressed during thymic development, it will be important to further study their function, in order to ultimately determine whether they play a significant role in thymic development.

SUMMARY OF PARTICIPANTS IN THESIS PROJECT

Initial cloning of homeobox genes from thymus

Done prior to my entering the lab

- Preparation of fetal thymus RNA
- First strand cDNA synthesis
- RT-PCR
- Cloning of products (ligation, transformation of competent cells, minipreps)

Sequencing: 34 clones were sequenced prior to my entering the laboratory; approximately half were re-sequenced due to ambiguities; this was done by me after I joined the lab; I entered repeat sequences into the computer and completed the comparison to know homeobox sequences; I also made maxipreps of potentially novel clones.

Northern Blot Analyses--mostly me with some help from lab members

- Fetal tissues-obtained by Alex Grinberg and Connie Sommers
- RNA preparation--mostly Connie, also me after being taught by Connie
- Probes--random-, specific-primed, and end-labeled probes--me
- Hybridizations, washes, stripping blots--me after being shown once by Connie

Fetal Tissue PCR--mostly me

- Fetal tissues and RNA--same as above
- PCR primers--designed by me
- Template--cDNA made by me from RNA
- Parameters/methodology--me with input from Connie
- Blotting--Southerns--me after being shown by Howard Shen
- PAGE and electrophoretic transfer--me after being shown by Connie
- Hybridization, washes, and exposure--me

3'RACE--mostly me

- 5' primers--designed by me
- 3' primers--gift from another lab or designed by me
- Template--cDNA made by me from RNA made by me or Connie, or fetal thymus cDNA library made by Connie
- Parameters/methodology--me with input from Connie
- Cloning of products--me
- Sequencing:
  - 2/3 dideoxynucleotide (Sequenase)--me
  - 1/3 automated--helped by another lab

MATERIALS AND METHODS

Preparation of RNA from tissues

Fetal organs and total embryo: Organs and tissues were dissected from several litters of fetal day 13.5 FVB/n mice. Tissues were homogenized for 30 seconds at top speed in a Polytron homogenizer in 2 ml RNAzol (Tel-Test Inc., Friendswood, TX). The total volume was brought to 4 ml with RNAzol and split into 4 eppendorf tubes.

Adult thymus: 1 FVB/n adult mouse thymus was dissected and placed in tissue culture medium. The thymus was then placed between two autoclaved pieces of nylon mesh (Bellco Glass Inc., Vineland, NJ) in a small petri dish in media (RPMI 1640 (Biofluids Inc., Rockville, MD) + 10% heat inactivated fetal bovine serum). Thymus was "smooshed" with sterile pipette tips to free thymocytes into the media. Cells and media were then put through another piece of mesh into a conical tube, and the petri dish was rinsed with 5 ml of RNase-free phosphate-buffered saline (PBS) which was also put through the mesh. The cells and media were spun for 10 minutes at 1200 RPM to pellet the cells, the supernatant was decanted, and the cells were resuspended in 0.5 ml PBS. RNAzol (Tel-Test) was added until the mix was a proper consistency (not too viscous).

All tissues 1/10 volume $CHCl_3$ was added to each tube and vortexed vigorously. The mixture was incubated on ice for 15 to 30 minutes, then spun at top speed, 15 minutes in a microfuge or 30 minutes in a centrifuge depending on the size of the tube. The top (aqueous) layer was collected and an equal volume of isopropanol was added. The mixture was incubated at -20°C overnight to precipitate the RNA. The next day it was centrifuged at 4°C for 30 minutes at 3800 RPM (large tubes) (Beckman GPR Centrifuge/GH3.7 Rotor) or 15 minutes at top speed (microfuge tubes), washed twice with 70% RNase-free ethanol (spinning 8 minutes in-between

washes), and air dried. Pellets were resuspended in DEPC (diethyl pyrocarbonate)-treated water and optical density (OD_{260} and OD_{280}) was measured using a Model DV-30 UV/vis spectrophotometer (Beckman Instruments Inc., Irvine, CA).

First strand synthesis of cDNA from RNA

RNA was reverse transcribed using oligo$(dT)_{12-18}$ primers following the Superscript RT (Gibco BRL, Gaithersburg, MD) protocol: 1 µg RNA was added to 13 µl DEPC-treated water. Next, 1 µl of oligo(dT) solution (0.5 µg/µl) was added and the mixture was heated to 70°C for 10 minutes and incubated on ice for 1 minute to allow for primer annealing. To this were added 1 µl 10X synthesis buffer (200mM Tris-HCl pH 8.4, 500mM KCl, 25mM $MgCl_2$, 1 µg/µl BSA), 1 µl 10 mM dNTP mix, 2 µl 0.1M DTT, and 1 µl Superscript II RT (200 U/µl). The reaction was mixed and incubated at room temperature for 10 minutes, then 42°C for 50 minutes. The reaction was then terminated by incubating at 70°C for 15 minutes and placed on ice. Finally, the reaction mix was treated with 1 µl RNase H for 20 minutes at 37°C.

Reverse transcriptase polymerase chain reaction (RT-PCR) amplification of homeobox clones from fetal thymus cDNA

5µl of mouse 13.5-day fetal thymus cDNA from above protocol was used per PCR reaction. Degenerate PCR primers were designed to two highly conserved regions of the homeodomain, from several homeobox families. PCR conditions were as follows: 94°C 15"--45°C 30"--2 minute ramp to 72°C--72°C 30" for 5 cycles; 94°C 15"--55°C 30"--1 minute ramp to 72°C--72°C 30" for 24 cycles; 94°C 15"--55°C 30"--72°C 1 minute for 1 cycle. Sense (ELEKEF) and antisense (KIWFQN) primers from only one class (Antennapedia) generated

the predicted 120 basepair product when run on 3% MetaPhor (FMC Bioproducts, Rockland, ME) agarose gels in TAE buffer. Agarose gels were stained with ethidium bromide (100 µg/L final concentration), visualized by ultraviolet illumination, and photographed while on the transilluminator using a Stratagene Eagle Eye camera (Stratagene Inc., La Jolla, CA).

All PCR oligonucleotide primers were synthesized, column purified, and lyophilized by Bio-Synthesis, Inc. Upon receipt, oligos were resuspended in sterile water to a final concentration of 50-200 µM using the following formula:

$$[\ ]\mu M = OD_{260} \times 10^5 / \varepsilon \text{ when dilution factor} = 100$$

$$\varepsilon = 15.6 \times (\#A) + 11.8\ (\#C) + 7.4\ (\#G) + 9.3\ (\#T)$$

Cloning PCR products into plasmid vectors

5'EcoR1 and 3'Xba1 linkers had been added to PCR primers to aid in cloning. PCR products were digested with EcoR1 (Boehringer Mannheim, Inc.,)and Xba1 (Boehringer, Mannheim, Germany), run on 3% agarose gels, and gel purified using Spin Bind (FMC). 120 basepair PCR products were ligated under sticky-end conditions into pGEM-11zf(-) plasmids (Promega, Madison, WI) that had been linearized with EcoR1 and Xba1.

Transformation of competent cells

Maximum efficiency DH5α cells were purchased from Gibco/Life Technologies and transformed using a modified Gibco/Life Technologies protocol: Competent cells (stored at -80°C) were thawed on ice, mixed gently, and placed into chilled polypropylene (Falcon 2059) tubes at approximately 70 µl cells per tube. 2 µl undiluted ligation reaction was added to the cells and mixed gently by tapping the tubes. Cells were incubated on ice for 30 minutes, then heat shocked for 45 seconds in a 42°C water bath and placed on ice for 2

minutes. 0.9 ml of S.O.C. medium (Gibco BRL) was added to each tube and shaken at 225 RPM at 37°C for 1 hour. Cells were plated at varying concentrations on LB plates containing 100 µg/ml ampicillin. At least one hour prior to plating, plates had been spread with 100 µl IPTG/XGAL (10 mg/ml) (Sigma, St. Louis, MO) for blue-white selection. After overnight incubation at 37°C, white colonies were picked and miniprep cultures were grown in 2 ml LB + 50 mg/ml ampicillin (Sigma) at 37°C shaking (225 RPM) overnight. (Blue-white selection was not used for clones under 200 base pairs because the Beta-lactamase gene is not interrupted effectively with small inserts.) Minipreps were done using QIAPREP (Qiagen, Chatsworth, CA).

Minipreps of homeobox clones

[QIAPREP (Qiagen)] 1.5 ml Eppendorf tubes were filled with miniprep cultures and spun down at high speed for 2 minutes using a tabletop microcentrifuge. Supernatants were discarded and pellets were resuspended in 250 µL buffer P1 (with RNase A) (Qiagen). To this was added 250 µL lysis buffer P2 and mixed by inversion 6 times. Next, 350 µl neutralization buffer N3 was added and again mixed by inversion. This mixture was spun at high speed for 10 minutes in a tabletop microcentrifuge at room temperature. Supernatants were removed and applied to Qiaprep columns, and spun for 1 minute. Flowthroughs were discarded. Columns were washed with approximately 1 ml wash buffer, spun 1 minute to remove all traces of buffer, and eluted in 40-60 µL sterile water. Columns were again spun 1 minute to collect eluate in clean 1.5 ml Eppendorf tubes.

Maxipreps of clones (Wizard, Promega)

Approximately 500 ml overnight cultures of E.coli transformed with our clones were grown, shaking at 225 RPM overnight at 37°C in LB containing 100 µg/ml ampicillin. In the morning, cells were centrifuged at 5000X G for 10 minutes at room temperature. Cells were resuspended in 15 ml cell resuspension solution (50mM Tris-HCl, pH 7.5, 10 mM EDTA, 100µg/ml RNase A). To this was added 15 ml cell lysis solution (0.2M NaOH, 1% sodium dodecyl sulfate (SDS)) and mixed by inversion until the solution became clear and viscous. 15 ml of neutralization solution (1.32 M potassium acetate, pH 4.8) was added and immediately mixed by inversion. The solution was then centrifuged at 14,000 G at room temperature for 15 minutes. The supernatant was filtered through cheesecloth, measured, and transferred to a centrifuge bottle. 0.5 volumes of room temperature isopropanol was added and mixed by inversion. Next, it was centrifuged again at 14,000 G for 15 minutes. The supernatant was discarded and the DNA pellet resuspended in 2 ml TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA).

10 ml Wizard (Promega) maxiprep DNA purification resin was added to the DNA and swirled to mix. A Wizard maxicolumn tip was inserted into a vacuum manifold and the resin/DNA mix was added to the column. The bottle was rinsed with 13 ml column wash solution (200 mM NaCl, 20 mM Tris-HCl pH 7.5, 5 mM EDTA, diluted with ethanol to a final EtOH concentration of 55%), and this was added to the column. A vacuum was applied to draw the solution through the column. An additional 12 ml column wash was added and drawn through the resin. The resin was washed with 5 ml of 80% ethanol and the vacuum was allowed to draw for an additional minute.

The maxicolumn was inserted into a 50 ml screw cap tube and spun in a swinging bucket rotor at 2500 RPM for 5 minutes to dry the resin. The column was placed back in the vacuum source and dried for an additional 5 minutes. The column was next removed and placed again in a 50 ml tube. 1.5 ml preheated (65-70°C) water was added to the column. After one minute, the DNA was eluted by centrifuging at 2500 RPM for 5 minutes in a swinging bucket rotor (Beckman GPR Centrifuge/GH3.7 Rotor). The DNA concentration was measured by spectrophotometer at OD_{260} (Beckman Model DV-30).

Sequencing of homeobox clones

DNA from minipreps was sequenced by using the Sequenase II dideoxynucleotide sequencing kit (Stratagene). The dGTP reagents were used, and ^{35}S-labeled dATP (ICN Biomedicals, San Francisco, CA, 1000 Ci/mmol) was used as the radioactive isotope. Sequencing primers used for clones in pGEM-11zf(-) plasmids were T7 and Sp6.

Prior to running on gel, samples were heated for 3 minutes at 85°C to denature, then placed immediately on ice. Sequencing reactions were run on 6% polyacrylamide gels (GelMix6, Gibco/Life Technologies). Upper chamber buffer was 0.5X TBE (Biofluids), lower chamber buffer was 300 ml 1X TBE (Biofluids). Gels were electrophoresed at 60W for 45 minutes to 1 hour; then 150 ml (1/2 volume) 3MNaOAc, pH5.2, was added to lower chamber, and electrophoresis was continued for an additional 45 minutes to 1 hour. Gels were then dried for 1 hour (or until completely dry) at 80°C and exposed to XAR-5 film (Eastman Kodak, Rochester, NY) overnight at room temperature.

Fluorographs were read and sequences entered into the computer, translated to amino acid sequences, and compared with published databases using the NCBI (National Center for Biotechnology Information) BLAST

search program[33]. Ambiguous sequences were re-sequenced using dITP reagents or run for shorter amounts of time (45 minutes rather than 1 hour) to read nearer the primers.

DNA extraction from agarose gels

One of three methods was used for all cases of gel extraction, using the protocols supplied by FMC (Spin Bind Kit), Bio 101 (Geneclean II), and QIAGEN (QIAEX).

Random primer labeling of homeobox clones

Stratagene Prime It II Kit was used to ^{32}P-label the approximately 100 basepair clones. To prepare the clones, large scale cultures were made of our two clones using the Wizard maxiprep protocol; clones were restriction digested with EcoR1 and Xba1, run on 3% MetaPhor agarose gels, and gel purified using Spin Bind. To 24 µl DNA (12 ng) each of clones 3 and 8 were added 10µl primers (Stratagene) (random 9-mers at 27 OD units/ml). This mix was heated for 5 minutes at 100°C, then spun down briefly. To this was added 10µl dCTP buffer (0.1mM each of dATP, dGTP, and dTTP), 5µl (3000µCi/mmole) α^{32}PdCTP (ICN), and 1µl exo(-)Klenow (5 U/µl). After incubating at 37°C for 10 minutes, 2µl Stop mix (0.5M EDTA, pH 8) and 5.8 µl 10X STE (10mM Tris, 100mM NaCl, 1mM EDTA, pH 8) were added. The final product was placed over a G-50 spin column (Boehringer Mannheim) which had been drained of buffer, and spun at 2200 RPM for 5 minutes (Beckman GPR Centrifuge/GH3.7 Rotor).

Specific primer labeling of homeobox clones

Procedure was identical to random primer labeling except that the specific 3' primer (5' CCA TAT TTT GAA CTG CCT G) or the slightly

degenerate primer located at the 3' end of the clones (KIWFQN) was used in place of random 9-mer mix, to ensure that the entire length of the probes had incorporated radioactive isotope.

End-labeling of 30 basepair oligonucleotides

To 15.5 µl distilled water was added 1 µl of oligo (10 pmol/µl) (either probe #1: 5' CTA GAG ATC AGC CAA AAC CTT GAC CTA ACA, or probe #3: 5' CTG GAG ATG CGG GGT TTG CGT CAT CTC CAA). This was incubated at 70°C for one minute and placed on ice. Next were added 2.5 µl 10X kinase buffer (0.5M Tris HCl, pH 7.6, 0.1 M $MgCl_2$, 50 mM DTT, 1 mM Spermidine, and 1 mM EDTA), 5 µl γ^{32}PdATP (ICN, 4500 Ci/mmol), and 1 µl T_4 (polynucleotide) kinase (Boehringer Mannheim). The reagents were mixed by pipetting up and down, and incubated at 37°C for 30 minutes. The reaction was stopped by adding 1 µl 0.5M EDTA. 5µl 10X STE (0.1 M NaCl, 10 mM Tris-HCl pH 8, 1 mM EDTA pH 8) was added and the final volume was brought up to 50 µl with distilled water. This was applied to a G25 spin column (Boehringer Mannheim) which had been drained of buffer and spun for 5 minutes at 2200 RPM (Beckman GPR Centrifuge/GH3.7 Rotor) to collect the probe.

Northern Blot

RNA from various fetal and newborn mouse tissues (fetal day 13.5 gut, heart, limbs, heads, bodies, liver, thymus; 9-day total embryo; and newborn thymus, spleen, and liver) were run in 1XMOPS on an RNA gel (1% agarose/1% formaldehyde/1X MOPS). RNA's were standardized so that approximately equal amounts (5µg) were run in each lane. RNA's were run in duplicate and blotted onto nylon Gene Screen filters (New England Nuclear (NEN) Research Products, Boston, MA) overnight: filters were cut to

size of gel and wet with distilled water; a small stack (1") of blotting paper was placed in a shallow dish and wet with 10X SSC (175.3g NaCl and 88.2g NaCitrate in 2 L H_2O, pH 7); after soaking in 10X SSC for 30 minutes, the gel was placed upside-down on stack and surrounded with parafilm; filter was placed on gel and covered with 3-4 inches more blotting paper; any bubbles were removed with a roller; a weight was placed atop the stack of blotting paper; the dish was filled with 10X SSC, and RNA was allowed to transfer overnight. The next day the wells and ribosomal bands were marked and the blot removed and rinsed with distilled water. Next, the blot was UV crosslinked at 1200μJ using a UV Stratalinker (Stratagene).

Hybridization of Northern Blots with Homeobox Probes

Since RNA had been run in duplicate, the filter was cut into two identical pieces. Blots were hybridized with one or the other of our two potentially novel clones (#3 and #8). Homemade prehybridization and hybridization solutions consisted of 1XSSPE (Biofluids) (20X = 175.3gNaCl, 27.6g NaH_2PO_4, and 7.4g EDTA, in 1 L H_2O, pH 7.4), 2X Denhardts, 1% nonfat dry milk, 10% dextran sulfate, 2% SDS, 200 μg/ml salmon sperm DNA, 200 μg/ml yeast tRNA, and 200 μg/ml polyadenylic acid; alternately, QuikHyb (Stratagene) solution was used. To prepare probe, 100μl of fish sperm DNA (10 mg/ml) was added and the mix was heated to 100°C for 5 minutes and then placed directly on ice. The probes were added to hybridization tubes and incubated in rotary incubators overnight at 62-68°C. Blots were washed (in 2XSSC/1%SDS for 15 minutes x 2 shaking at room temperature), then checked with a Geiger counter and exposed to Kodak XAR-5 film at -80°C overnight and for 1- and 2-week periods with intensifying screens.

Stripping Northern Blots

Approximately 1.5 liters of 0.1XSSC/0.1% SDS in DEPC water was brought to a boil. Northern blots were immersed and allowed to boil for 1 minute, then checked with a Geiger counter. The solution was removed from the heat source and allowed to cool to room temperature. Stripped Northern blots were rehybridized or stored at 4°C between 2 wet pieces of filter paper.

PCR from Fetal Tissue cDNAs

Sense and antisense oligonucleotide primers were designed within the region of two of our clones (#1 and #3). [sense primer, clone 1: 5' CCT TAC AAC ATG TAT TTA TCG; clone 3: 5' TAC AAC ATG TAT TTA TCG AAA CA; antisense primer, clone 1: 5' CCA TAT TTT GAA CTG CCT G; clone 3: 5' CCA TAT TTT GAT TTG TCT C] PCR was done on numerous tissue cDNAs (10 µl each 9-day total embryo; fetal-day 13.5 legs, heads, gut, heart, liver thymus; 1day newborn thymus and adult thymus) as well as adult and fetal (day 13.5) thymus libraries. PCR reactions were done using a PTC-100 Programmable Thermal Controller (MJ Research, Inc., Watertown, MA) in 0.5 µl Eppendorf tubes in 100 µl volume, and contained 1µl 10 mM dNTPs, 10 µl 10X PCR buffer (100mM Tris-HCl, 500mM KCl, 15mM $MgCl_2$, pH 8.3), 0.5 µl Taq polymerase, and 1µl of each primer (50µM). PCR parameters were as follows: 94°C 1 min/47°C 1 min/72°C 1 min for 30 cycles.

Southern Blot of PCR products from agarose gels[34]

After agarose gels had been run and pictures taken, the DNA in the gel was denatured for 30-45 minutes by soaking in denaturing solution (1.2 M NaCl, 20 ml 0.6 N NaOH) and then neutralized for 30-45 minutes in

neutralizing solution (1.2 M NaCl, 0.8 M Tris). For each Southern blot, a stack of blotting paper was placed in a shallow plastic dish, which was filled almost to the top with 10X SSC. The gel was placed face down on top of the blotting paper. Exposed parts of blotting paper were covered with Saran wrap. Prewet nylon membrane was placed on top of gel, and air bubbles were removed with a roller. The membrane was then covered with another stack of dry blotting paper, and a weight (e.g. 500 ml bottle) was placed on top. Transfer occurred overnight. In the morning, nylon membrane was marked and rinsed with distilled water, then UV crosslinked at 1200μJ (UV Stratalinker, Stratagene).

Electrophoretic transfer from polyacrylamide gels

(from Current Protocols in Molecular Biology volume 1 section 2.9.9) The 10% polyacrylamide gel was Ethidium Bromide (Sigma) stained by immersion in a dilute EtBr (10 mg per liter) solution on a rocker bath for approximately 30 minutes, then destained with distilled water. A UV transilluminator was used to determine whether the gel had been appropriately stained and destained, and the gel was photographed. Gene Screen (NEN Research Products) was cut to same size as the gel and wet with distilled water; then the gel and filter were equilibrated with 0.5X TBE (Biofluids). Electrophoresis apparatus (Transblot Cell, Biorad, Hercules, CA) was assembled with the gel and nylon filter sandwiched between filter paper and sponges within a cassette, all prewet with 0.5X TBE. The cassette was placed in the Transblot container filled with 0.5X TBE, with the white side of the cassette next to the positive electrode, so that the DNA could be transferred from the gel to the filter. The electrophoresis took place overnight at 120V-hr (8 V overnight). In the morning, the apparatus was disassembled, and wells were marked on the membrane. It was then

denatured for 10 minutes on filter paper soaked in 0.4M NaOH. Next, the membrane was rinsed in 2XSSC, then placed on a filter paper wet with 2XSSC and crosslinked at 1200 μJ (UV Stratalinker, Stratagene).

Hybridization of Southern Blots

Southern blots were prehybridized and hybridized in QuikHyb buffer to which probe was added (approximately 10^6 cpm/ml final) overnight in rotary incubators. Hyb- and prehyb- temperatures ranged from 55 to 68 and were calculated based on the following formula: hyb temperature 5-8°C below T_m; T_m = 16.6 (log [Na] + 81.5 + 0.41 (% G + C)) - 675/# bases; Na conc is 0.15 for hyb and pre hyb, 0.015 for washes. Washes consisted of 1X SSPE (Biofluids)/0.5% SDS (Digene Diagnostics, Rockville, MD)/0.1% nonfat dry milk for two 30 minute rinses at room temperature, 0.2X SSPE/1% SDS for 30 minutes at room temperature, and finally 0.1XSSPE/0.5% SDS for 30 minutes at a temperature based upon the above formula. Blots were monitored with a Geiger counter between washes, and washes were terminated early or extended based upon how hot the filters were. Once they had been washed, blots were exposed to film at -80°C overnight and for 2-day and 1-week exposures.

3'RACE

This is a two-step "nested" PCR process. In the first round of PCR, the upstream (sense) primer was designed to match a specific region of one of our homeobox clones. (clone 1: 5'CCT TAC AAC ATG TAT TTA TCG 3' and clone 3: 5' TCC ACT TCA GCC GTT ACC TGT 3') The antisense primer was an oligo(dT) primer and contained an "anchor sequence" for use in the 2nd round of PCR. [5' CCT CTG AAG GTT CCA GAA TCG ATA GGA ATT CTT TTT TTT TTT TTT TTT(A/G/C)(G/A/T/C) 3'] cDNA from 13.5 day fetal

thymus was used as a template for the first round. First round parameters were: 94°C 15 sec/55 °C 30 sec/72°C for 2 min; 27 cycles. For the second round of PCR, an aliquot of the first round product was used as template. The sense primer was a "nested" specific primer. [clone 1: 5' CGC TCT AGA CTA GAG ATC AGC CAA AAC CT 3' and clone 3: 5' CGC TCT AGA GAG ATG GCC GGT TTG CTG CAT], and the antisense primer matched the "anchor sequence" from the first round oligo(dT) primer [5' CTG GTT CGG CCC ACC TCT GAA GGT TCC AGA ATC GAT AG]. Parameters were 94°C 15"/50°C 30"/72°C 2 min for 28 cycles.

3'RACE products were run on 1% agarose (Gibco BRL) gels in TAE (Biofluids). Results, however, showed smears or multiple bands. Attempts were made to optimize PCR by varying the annealing temperatures and times and primer concentrations, but this was not successful. Only one 600 bp band appeared prominent enough to pursue. The product was gel purified and cloned into a linearized pGEM-11Zf(+/-) plasmid using a Rapid DNA Ligation Kit (Boehringer Mannheim). Three minipreps were shown to contain the 600 basepair insert when digested with EcoR1 and Xba1. These were sequenced using automated sequencing (Perkin Elmer Cetus, Forster City, CA).

New 3'RACE sense and antisense primers were designed with higher melting temperatures. [sense, round 1: 5' TAC AAC ATG TAT TTA TCG AAA CA (clone 1), and round 2: 5' CGC TCT AGA CGC CGA GAG ATC AGC CAA AAC CT (clone 1), 5' GCT CTA GAG AGA TGG CCG GTT TGC TGC AT (clone 3); antisense, round 1: 5' CTG AAG GTT CCA GAA TCG ATA GGA ATT CTT TTT TTT TTT TTT TTT TTT TTT T(A/G/C)(G/A/T/C) 3'] The above 3'RACE procedure was repeated almost exactly, except under more stringent 2nd round conditions (first round 94°C 15"/52°C 30"/72°C 2 min for 28 cycles; second round 94°C 15"/60°C 30"/72°C 2 min for 28 cycles).

A modified 3'RACE procedure involved making use of fetal and adult cDNA libraries readily available in the lab[35]. Libraries were oligo(dT) primed cDNA from adult or fetal-day 13.5 thymi. The phage vector was UniZapXR containing linearized plasmid Bluescript; primer sites on the plasmid were T7 (3') and T3 (5'). cDNAs were cloned into an EcoR1/Xho1 site (5' to 3') within the Bluescript plasmid. For the PCR round 1 and 2 sense primers, specific homeobox primers (listed above) were used. For the antisense primers, two methods were used. In the first, T7 primer was used as the antisense primer in both rounds of PCR. In the second, round 1 antisense oligos were engineered to match the sequence of the bluescript vector 3' to the cloned insert (5' TTG TAA AAC GAC GGC CAG TGA). Round 2 primers were nested inside the round 1 primers and contained restriction site linkers for cloning (5' GCG AAT TCG ACT CAC TAT AGG GCG AAT TGG). PCR was altered slightly, using "hot start" procedure (Clontech, Palo Alto, CA) that includes TaqStart antibody, a neutralizing monoclonal antibody which blocks polymerase activity during set-up at room temperature but is completely neutralized once temperature rises above 70°C. Parameters are as follows: 94°C for 5 min; 94°C 30"/56°C 30"/68° for 5 min for 35 cycles, then 94°C 30"/56°C 30"/68°C 10 min for 1 cycle. Prior to starting PCR, TaqStart antibody was diluted in buffer (4.4 µl antibody per 17.6 µl dilution buffer--50mM KCl, 10mM Tris-HCl, pH 7, 50% glycerol) and then to a 28:1 molar ratio with Taq polymerase (4.4 µl Taq plus 22 µl diluted TaqStart antibody), and 2.4 µl of this mix (approximately 2 units of Taq) was used per reaction. As with the prior RACE strategy, products were subcloned using the TA cloning protocol (Invitrogen) and sequenced. In all, approximately 30 bands were cloned and sequenced.

Subcloning using TA Cloning (Invitrogen, Carlsbad, CA)

1 µl PCR product was added to 5 µl sterile water, 1 µl 10X ligation buffer, 2 µl pRC vector (25ng/µl), and 1 µl T4 DNA ligase. The reaction was incubated overnight at 12°C. Transformation is similar to that of DH5α cells in the Gibco protocol, except 50µl OneShot (Invitrogen) cells were used. 2 µl β-mercapto-ethanol was added to OneShot cells and mixed by tapping. The rest of the procedure was identical to that described earlier, except 450 µl S.O.C. (Gibco, BRL) media was used. White or light colonies were picked for minipreps.

Rapid Ligation (Boeringer Mannheim)

DNA insert and vector were dissolved in 1X concentrated DNA dilution buffer in an approximately 3:1 insert:vector ratio to a total volume of 10µl. To this was added 10 µl T4 DNA ligase buffer and 1µl T4 DNA ligase. The reagents were mixed thoroughly and incubated at room temperature for 5 minutes. The ligation reaction mixture was used to directly transform DH5α competent cells (Gibco, BRL) as described earlier.

RESULTS

Generation of Homeobox Clones from Fetal Thymus

Since the homeodomains of many known homeobox genes have been published[12], and contain areas that are very highly conserved, it was possible to design degenerate sense and antisense oligonucleotide PCR primers from two such regions in the domain, for a number of homeobox gene families. One set of primers, from the Antennapedia class of homeobox genes, [ELEKEF (sense) and KIWFQN(antisense)] successfully generated products of the predicted length (approximately 120 base pairs) when 13.5 day thymic cDNA was used as template. PCR primers had been designed with restriction site linkers for ease with cloning. PCR products were digested with EcoR1 and Xba1 and ligated into plasmid vectors for sequencing. Thirty four clones were sequenced using the Sequenase II kit (Stratagene), with ^{35}S as the radioactive isotope.

Summary of Sequence Data

Sequences were entered into the computer and translated to amino acid sequences. Ambiguous regions were re-sequenced. Nucleotide and amino acid searches were performed using the NCBI (National Center for Biotechnology Information) BLAST network service.[33] Of 34 clones sequenced, 31 were matched with genes from Hox or Antennapedia class homeobox genes. Over a span of approximately 25 amino acids, clones having five or more mismatches from their nearest computer match (less than 80 % identity) were arbitrarily called "potentially novel" and flagged for further characterization (Table 1).

Table 1: Summary of Homeobox Clones

*** 34 clones generated and sequenced**

*** 31 are homeobox genes**

*** 5 "potentially novel"** (i.e. >5 mismatches from known Hox genes)
4 (nos. 1, 8, 9, and 12) most closely match Hox D10,ll Hox C10,11
1 (no. 3) most closely matches Hox D3

*** 26 known** (0 to 4 mismatches)
7 closely match Hox A4 *(14, 16, 23, 24, 32, 33, 34)*
4 closely or exactly match Hox B5 *(2, 21, 27, 29)*
3 closely or exactly match Hox A6, C6 *(10, 15, 17)*
3 closely match Hox A9, C8, C9 *(22, 26, 28)*
2 closely match Hox B7 *(7, 31)*
2 closely match Hox D8 *(4, 25)*
1 closely matches Hox A1 *(13)*
1 exactly matches Hox A2, B2 *(30)*
2 closely or exactly match Hox D3 *(5, 11)*
1 exactly matches Hox A10 *(20)*

Homeodomains between classes of homeobox genes usually have less than 53-57% identity, but among members of a single family, different homeobox genes share approximately 70-80% identity.[12] Of our 31 homeobox clones, 26 had fewer than 5 mismatches (usually only 1-2 mismatches) from known Hox genes (Tables 1 and 2-A). We felt this small number of mismatches could be explained by sequencing errors or intraspecies differences. The other five potentially novel genes had between seven and eleven mismatches from any known mouse homeobox gene (56-72% identity). These clones fell into two groups based upon amino acid homology. Four most closely matched HoxD10, and one most closely matched HoxD3 (Tables 1 and 2-B).

Table 2-A: Amino Acid Sequences of Homeobox Clones, and their Homology With Known Mouse Hox Genes

Name	Amino Acid Sequence	# mismatches from closest Hox match
HoxA1	**HFNKYLTRARRVEIAASLQLNETQV**	-----
clone 13	HFNKY**B**TRARRVEIAA**G**LQLNETQV	2
HoxA2,B2	**HFNKYLTRARRVEIAASLQLNETQV**	-----
clone 30	HFNKYLTRARRVEIAASLQLNETQV	exact
HoxD3	**HFNRYLCRPRRVEMANLLNLTERQI**	-----
clone 5	HFNRYLCRPRRVEMANLL**S**LTERQI	1
clone 11	HFNRYLCRPRRVEMANLLNLTERQI	exact
HoxA4	**HFNRYLTRRRRIEIAHTLCLSERQV**	-----
clone 14	HFNRYLTRRRRIEIAHTL**V**LSERQI	2
clone 16	HFNRYLTRRRRIEIAHTLCLSERQI	1
clone 23	HFNRYL**S**RR**A**RIEIAHTLCL**N**ERQI	4
clone 24	HFNRYL**S**RRRRIEIAHTLCL**N**ERQI	3
clone 32	HFNRYLTRRRRIEIAHTL**V**LSERQI	2
clone 33	HFNRYLTRRRRIEIAHTL**V**LSE**A**QI	3
clone 34	**R**FNRYLTRRRRIEIAHTLCL**N**ERQI	3
HoxB5	**HFNRYLTRRRRIEIAHALCLSERQI**	-----
clone 2	HFNRYLTRRRRIEIAHALCLSERQI	exact
clone 21	HFNRYLTRRRRIEIAHALCLSERQI	exact
clone 27	HFNRYLTRR**P**RIEIAHALCLSERQI	1
clone 29	HFNRYLTRRRRIEIAHALCLSERQI	exact
HoxA6,C6	**HFNRYLTRRRRIEIAHALCLTERQI**	-----
clone 10	HFN**H**YLTRRRRIEIA**N**ALCLTERQI	2
clone 15	HFNRYLTRRRRIEIA**N**ALCLTERQI	1
clone 17	HFN**H**YLTRRRRIEIA**N**ALCLTERQI	2
HoxB7	**HYNRYLTRRRRIEIAHTLCLTERQI**	-----
clone 7	**L**YNRYLTRRRR**V**EIAHTLCL**S**ERQI	3
clone 31	HYNRYLTRRRRIEIAHTL**V**L**S**ERQI	2
HoxD8	**LFNPYLTRKRRIEVSHALALTERQV**	-----
clone 4	LFNPYLTRKRRIEVSHALALTERQV	exact
clone 25	LFNPYLTRKRRIEVSHALALTE**K**QV	1
HoxA9	**LFNMYLTRDRRYEVARLLNLTERQV**	-----
clone 22	L**Y**NMYLTRDRRYEVARILSLTERQV	3
clone 26	LFNMYL**P**RDRRYEVAR**Q**LNLTERQ**F**	3
clone 28	LFNMYL**P**RDRRYEVAR**Q**LNLTERQV	2
Hox A10	**LFNMYLTRERRLEISRSVHLTDRQV**	-----
clone 20	LFNMYLTRERRLEISRSVHLTDRQV	exact

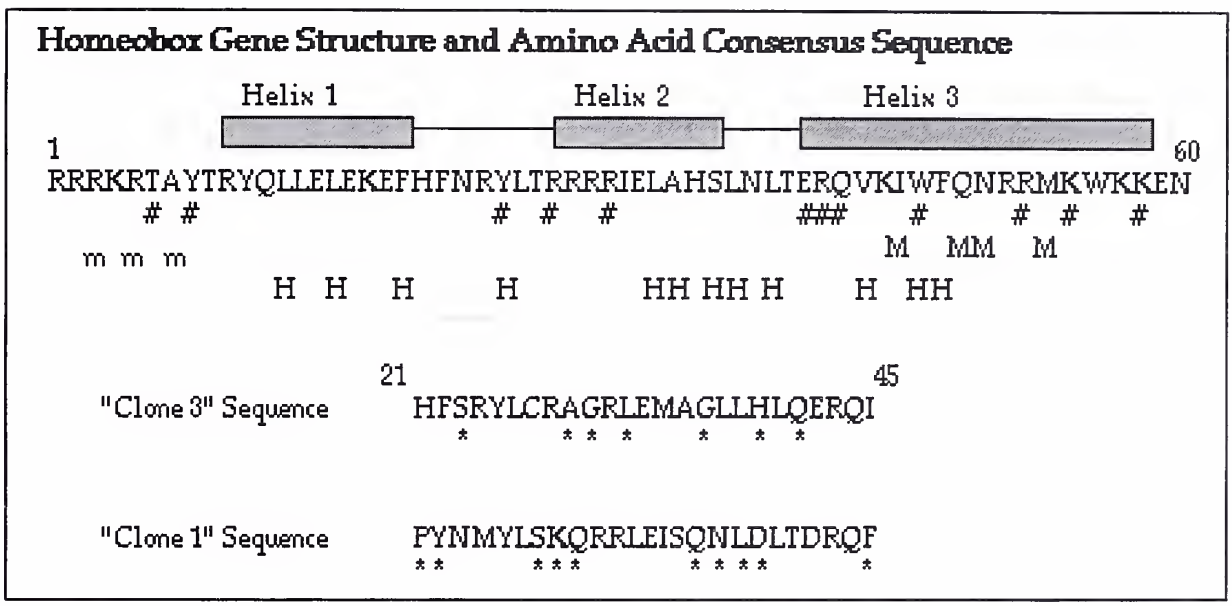
Table 2-B: "Potentially Novel" Clones

Name	Amino Acid Sequence	# of mismatches from closest Hox match
HoxD3	**HFNRYLCRPRRVEMANLLNLTERQI**	-----
clone 3	HFSRYLCRAGRLEMAGLLHLQERQI	7
HoxD10 (D11,C10,C11)	**LFNMYLTRERRLEISKSVNLTDRQV**	-----
clone 1	PYNMYLSKQRRLEISQNLDLTDRQF	10
clone 8	PYNIYLSKQRRLEISQNLDLTDRQF	11
clone 9	PYNMYLSKQRRLKISQNLDLTDRQF	11
clone 12	PYNMYLSKQRRLEISQNLDLTDRQF	10

An amino acid consensus sequence and a schematic of the structure of the homeodomain is shown in Figure 3. The amino acid residues which contribute to the tertiary structure of the DNA binding domain by forming the hydrophobic core are marked with an "H", while residues which make contact with the major and minor groove of the DNA double helix are marked "M" and "m" respectively. The amino acid numbering scheme depicted is that used in all published descriptions of homeodomain structure.[12]

The amino acid sequences of two clones (one clone from each group of potentially novel homeobox genes) are shown below the homeodomain consensus for comparison, in order to exhibit where differences between known and potentially novel homeobox genes fall in relation to the structure of the homeodomain. Mismatched residues (from most closely matched Hox class homeodomain) are marked with an asterisk. It is interesting to note that with one exception (clone 1, position 37), the mismatches do not fall in hydrophobic areas most responsible for the homeodomain's tertiary structure, strengthening the belief that the differences are real rather than sequencing artifacts, which should have a more random distribution. Most of

the mismatches fall in the region of the second alpha helix or on either side of it. Since two of the most highly conserved areas of the homeodomain consensus (ELEKEF at positions 15-20 and KIWFQN at positions 46-51) had been used to engineer PCR primers, the potentially novel clones begin at position 21 and end at position 45. Thus they do not include the residues which would come in direct contact with the DNA double helix during protein-DNA binding.



Legend

#---residues that contact sugar-phosphate backbone
m--residues that contact minor groove
M--residues that contact major groove and are responsible for sequence specific DNA contacts
H---residues that contribute to the hydrophobic core that is responsible for the tertiary structure of the homeodomain
*----amino acid mismatches from closest mouse homeobox sequence in potentially novel clones #3 and #1

One Letter Amino Acid Abbreviations

A--Ala	R--Arg	N--Asn	D--Asp	C--Cys	Q--Gln
E--Glu	G--Gly	H--His	I--Ile	L--Leu	K--Lys
M--Met	F--Phe	P--Pro	S--Ser	T--Thr	W--Trp
Y--Tyr	V--Val				

Figure 3. Consensus sequence of the homeodomain, with schematic of its structure and DNA-binding domains (after reference 12). Helices 2 and 3 form the familiar helix-turn-helix motif common in DNA binding proteins. The NMR study of Antennapedia class homeodomains identified a kink at position 52-53 of helix 3, such that it can be considered as two separate helices, helix 3 and helix 4.[12] Included for comparison are amino acid sequences for potentially novel clones #3 and #1, with mismatches marked with an asterisk. The numbering scheme for amino acid position is that used in all the published descriptions of the homeodomain structure.[12]

Once the sequencing of all homeobox clones had been completed and interpreted, the decision was made to attempt to characterize potentially novel homeobox genes. One clone from each group (clone #8 and clone #3) was selected to further characterize expression by Northern blot analysis.

Assay for Potentially Novel Homeobox Gene Expression by Northern Blot Analysis

Our first step in characterizing the potentially novel clones was to verify their expression in fetal thymus by Northern Blot analysis, and identify other fetal and adult tissues in which our clones might also be expressed. RNA from various fetal and newborn mouse tissues (fetal day 13.5 gut, heart, limbs, heads, bodies, liver, thymus, 9-day total embryo, and newborn thymus, spleen, and liver) were run on an RNA gel (1% agarose/1% formaldehyde/1X MOPS). RNA's were standardized so that equal amounts (5µg) were run in each lane. Obtaining larger amounts of RNA from tiny embryonic organs was very difficult, especially thymus. Fetal day 13.5 is the earliest that embryonic thymi can be dissected, when they are barely larger than the head of a pin. RNA's were run in duplicate and blotted onto nylon Gene Screen filters overnight, then UV crosslinked at 1200µJ. Blots were hybridized with one or the other of our two potentially novel clones (probes #3 and #8) which had been made by random-primer labeling the approximately 100 basepair clones with ^{32}PdCTP using the Stratagene Prime It II kit. Blots were washed and exposed to film at -80°C overnight and for 1- and 2-week periods with intensifying screens.

Results of the Northern Blot were disappointing: even after two week exposure, there was little or no signal. Our most promising results showed a possible band in fetal thymus but no other tissues when probed with clone #8,

but even this was inconclusive due to low signal compared to background. Probe #3 produced no signal at all (data not shown).

When homeobox probes were labeled using specific rather than random primers, and hybridized to new Northern blots, blots showed similar, inconclusive results, with signal too low to interpret. This was also the case when γ-^{32}PdATP-end-labeled oligonucleotide probes were used. (data not shown). Our results led us to believe that our approximately 100 basepair clones were very difficult to work with, most likely due to their small size.

Homeobox Expression in Fetal Tissues RT-PCR Assay

Because Northern blot results were inconclusive, we sought another method to determine the expression patterns of our potentially novel clones. A PCR experiment to assay for homeobox expression in a number of fetal and adult tissues was devised. Sense and antisense oligonucleotide primers were designed within the region of two of our clones [fetal thymus (Ft) clones #1 and #3], shown in Figures 4 and 5. Primers were tested on serially diluted plasmid stocks of the two clones, where they showed a band of the predicted size (approximately 84 basepairs) when run on 3% Metaphor (FMC) agarose gels. There was no crossreactivity between the two sets of primers, i.e. primers for clone 1 did not amplify a band when clone 3 DNA was used as template, and vice versa. Because of the small size of the product and possible overlap with primer dimers, it was very difficult to resolve the bands even on high percentage agarose gels. Therefore the PCR products were run on nondenaturing 10% polyacrylamide gels. Also, because of the very low levels of homeobox gene expression, and because there were consistently multiple bands in lanes of PCR products when thymic cDNA was used as template, a second step was added to improve sensitivity and specificity: an internal 30-mer single-stranded oligonucleotide probe was designed for each

clone, falling in the region between the two PCR primers (Figures 4 and 5). PCR products were Southern blotted (for agarose gels) or electrophoretically transferred (for polyacrylamide gels) onto nylon filters and hybridized with γ^{32}PdATP end-labeled probes to check for specific homeobox products. Prior to experimental use, both probes were tested separately on plasmids containing clones 1 and 3 to make sure there was no crosshybridization. It was demonstrated that each probe was specific to the homeobox clone with which it had been designed to hybridize (data not shown).

PCR was done on numerous tissue cDNAs (9-day total embryo; fetal-day 13.5 legs, heads, gut, heart, liver, thymus; 1-day newborn thymus and adult thymus) as well as adult and fetal (day 13.5) thymus libraries. Products run on PAGE gels showed that many tissues appeared to have faint bands of the predicted size, but when these were probed with the specific oligos, signals on autoradiographs did not correlate with bands on the gel, making results uninterpretable (data not shown). Furthermore, signals appeared in negative control lanes, and no attempts at identifying contaminated reagents were successful. Therefore this part of the project was set aside, and the decision was made to try to find larger cDNAs of our two clones, to use as probes for Northern Blots, in situ hybridization, and library screening.

Figure 4. PCR Assay for Clone Ft-1 Expression in Fetal Tissues

Figure 4. PCR primers were synthesized based upon the sequence of clone Ft-1 as pictured above. PCR was performed on cDNAs from fetal day 13.5 legs, heads, gut, liver, thymus, 9-day total embryo, and newborn and adult thymus. Products were run on PAGE gels, transferred to nylon membranes, and hybridized with radiolabeled oligonucleotide probe depicted above (data not shown).

Figure 5. PCR Assay for Clone Ft-3 Expression in Fetal Tissues

Figure 5. PCR primers specific to clone Ft-3 were synthesized and PCR was performed as in figure 3. Blots were hybridized with the Ft-3-specific oligonucleotide probe shown above.

3'RACE to Amplify Downstream Regions of Homeobox Clones

A two-step "nested" PCR process known as 3'RACE was entertained in order to search for larger pieces of our potentially novel clones from fetal thymic cDNA. cDNA from 13.5 day fetal thymus was used as a template for the first round of PCR. In this first round, the upstream (sense) primer was designed to match a specific region of one of our homeobox clones. The antisense primer was an oligo(dT) primer to amplify starting at the polyA tail of the template. This primer contained an "anchor sequence" for use in the second round of PCR. For the second round of PCR, an aliquot of the first round product was used as template. The sense primer was a "nested" specific primer which was downstream of the round 1 sense primer, and the antisense primer matched the "anchor sequence" from the first round oligo(dT) primer. Second-round primers were engineered with restriction-site linkers for use in cloning.

3'RACE products were run on agarose gels. Since the expected products were larger than the original homeobox clones, they were run on 1% rather than 3% agarose gels in TAE. The two step, nested PCR process should have increased both the sensitivity and specificity of the amplified product.

Results, however, showed smears or multiple bands in all of the lanes in which there were any products, while other lanes were empty. Only one band appeared prominent enough to pursue, and further attempts to optimize PCR conditions were unsuccessful. The single band, generated by the primers for clone #3, was approximately 600 basepairs in length. The product was gel purified and cloned into a linearized pGEM-11Zf(+/-) plasmid using a Rapid DNA Ligation Kit (Boehringer Mannheim). Three minipreps of transformed colonies were shown to contain the 600 basepair insert when digested with EcoR1 and Xba1. These were sequenced using automated sequencing (Perkin Elmer). Unfortunately, results showed the products were not homeobox genes, but the ubiquitous and highly expressed genes actin and spectrin.

Next, new 3'RACE sense and antisense primers were designed for more stringent second round PCR conditions, in order to reduce the chance of nonspecific products resulting from the amplification of abundant genes. The above procedure was repeated almost exactly, except higher melting temperatures were used in the second round of PCR, and several bands were generated between 200 and 600 basepairs in length, which were subcloned using TA Cloning (Invitrogen) and sequenced using the Sequenase II kit (Stratagene). Again, the clones proved to be abundant but nonspecific genes rather than homeobox genes.

A second approach to obtaining larger pieces of the homeobox clones involved a modification of the 3'RACE procedure. This involved making use of fetal and adult cDNA libraries readily available in the lab, as template for the PCR reaction. Libraries contained oligo(dT) primed cDNA from adult or fetal-day 13.5 thymi. The phage vector was UniZapXR containing the plasmid Bluescript; primer sites on the plasmid were T7 (3') and T3 (5'). cDNAs were cloned into an EcoR1/Xho1 site (5' to 3') within the Bluescript plasmid. For the PCR round 1 and 2 sense primers, specific homeobox

primers were used as in conventional 3'RACE. For the antisense primers, two methods were used: in the first, T7 primer was used as the antisense primer in both rounds of PCR. When this method failed, round 1 antisense oligos were engineered to match the sequence of the bluescript vector 3' to the cloned insert. Round 2 primers were nested inside the round 1 primers and contained restriction site linkers for cloning. As with the prior RACE strategy, products , which appeared to be approximately 1.2 kilobases in length for both clones, were subcloned and sequenced. In all, approximately 30 bands from 3'RACE were sequenced. None of them proved to be homeobox genes; most were nonspecific but highly expressed genes such as actin, or nonspecific bits of cloning vector (data not shown).

Discussion

While homeobox genes have not been extensively studied in thymocyte development, the literature that exists on homeobox genes' role in hematopoietic cell development and regulation points to the important role that homeobox genes are likely to play. The *Hlx* gene, for instance, appears to play a significant role in the maturation of B-lineage cells and myelocyte/macrophage lines, but does not appear to be expressed in T-lineage cells.[24-25] It is possible that equivalent genes in T-cells remain to be discovered. For these reason we were encouraged when initial RT-PCR on fetal thymus RNA amplified so many homeobox genes, especially since the levels of expression of transcription factors are generally very low. In agreement with the literature, our screening found members of the HoxA, HoxB, and HoxC clusters (table 1) expressed in the developing thymus, but also appeared to identify HoxD genes, which have not been seen in hematopoietic cells.[18-19] It is possible that the HoxD genes we identified were expressed in the thymic stroma. Our most exciting finding was that of five potentially novel homeobox clones, which appeared to fall into two groups based upon homology.

The problem with our results were that the clones we obtained from our initial RT-PCR were small (100 base pairs). Attempts at Northern Blots were unsuccessful, in part because the small size of our DNA clones made them difficult to manipulate. A second factor which contributed to our frustration was the high degree of homology between different homeobox genes within the 60 amino acid homeodomain. Therefore, hybridization conditions had to be very stringent or the background remained too high to interpret the results.

Because of the problems with our initial Northern Blots, we attempted several things to optimize results. Using specific- rather than random-labeled primers should have improved the strength of our signal, since this would ensure that the entire length of the probe had incorporated radioactive isotope. However, signal was still not high enough. The same was true when end-labeled oligonucleotide probes were used instead of the entire 100 basepair clone. It is possible that probes and conditions were satisfactory, but because the amount of RNA isolated from fetal thymi is very small, and the abundance of homeobox genes is generally very low, the level of RNA expression of homeobox genes was below the limit of detection for this technique.

3'RACE, the two-step nested PCR process, was attempted in order to obtain larger pieces of our clones. This was done using both thymic cDNA and thymic libraries as template. Despite the fact that 3'RACE should increase the specificity of the product, in all cases, the products we cloned were nonspecific but abundant genes such as actin.

Because our efforts to obtain more sequence were unsuccessful, we developed a PCR assay using cDNAs from various tissues in order to find out whether our potentially novel genes were expressed preferentially in thymus. If our genes were expressed everywhere, the likelihood that they played a significant role in thymocyte development would have been low. Finding such a pattern of expression would have lessened our interest in these potentially novel clones, since our laboratory revolves around the study of the developing immune system. Unfortunately, the results to this assay were also inconclusive. Low signal and high background, coupled with problems with contamination, made our results uninterpretable.

One method of obtaining the entire cDNA of our clones would have been to screen a cDNA library using the small fragments we had initially

cloned. We had hoped to avoid screening a library until we isolated a larger piece of a clone, because of the high degree of homology between homeodomains. Using only our 100 basepair clones as probes to screen a library could have resulted in many false positives, making the process labor intensive and risky. Unfortunately, the failure of our efforts to obtain larger pieces of our clones, and our attempts to characterize their expression using just the 100 basepair clones, leaves us with few other options. Future attempts at understanding the importance of our homeobox clones in thymic development will probably focus on screening a cDNA library and obtaining the full-length sequence. If this is unsuccessful, a genomic library could be screened.

Once a larger piece or full-length sequence of either of our clones has been isolated, it will be interesting to characterize the clone and find out its role in thymic development. Comparison of the amino acid sequence to known homeobox genes could elucidate whether differences are located at residues which directly participate in DNA binding, perhaps shedding light on function. Repeating Northern blots of various tissues will give a better understanding of the differential expression of our potentially novel clones. In Situ hybridization of whole embryos would provide another method of obtaining information about expression, and this could be repeated for embryos of different stages of development. Finally, if it appears likely that one of our clones may play a role in thymocyte development, transgenic and knockout mice could be engineered for further study. In this way, the importance of a potentially novel gene could be studied by observing T-cell development in an organism in which the gene is absent or dysregulated. Fluorescence activated cell sorter (FACS) analysis of T-cell precursor subpopulations within the developing thymus of transgenic or knockout mice could identify subtle changes in these population sizes. Comparison of

thymic and peripheral T-cell populations in wild type versus transgenic or knockout mice might shed light on what level of development is affected by our homeobox clones.

Alternatively, the RT-PCR process which initially identified our homeobox clones could be repeated using different degenerate primers. As previously mentioned, only the primers for the Antennapedia (Hox) class of homeobox genes successfully amplified products under the conditions we used. There are many other homeobox families which remain to be explored.

If the main problem in identifying thymic homeobox genes is one of abundance, clones obtained from RT-PCR could be screened in order to find some that had detectable levels of expression. One way to do this would be to make cDNA from fetal day 13.5 thymus using a radioactive nucleotide (^{32}PdCTP) and use this as a probe against a filter with the different clones gridded onto it. This method might identify both novel and previously characterized homeobox genes that are expressed abundantly in the thymus, and could be used to discover which (if any) homeobox genes are important in thymic development.

The study of homeobox genes is a young and exciting field. It is clear from the literature that homeobox genes are expressed in the T-cell, but their exact role remains to be identified. Our preliminary results have identified both known and potentially novel members of the Antennapedia class of homeobox genes in the developing thymus. It is hoped that further study will elucidate the role these genes play in the development of T-cell mediated immunity.

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