IDENTIFICATION AND CHARACTERIZATION OF Hoxa2 TARGET GENES BY ChIP

A Thesis Submitted to the College of Graduate Studies and Research in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the College of Pharmacy and Nutrition

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ABSTRACT

Hox genes are evolutionarily conserved transcription factors which act to control important developmental pathways involved in morphogenesis of the embryo. *Hoxa2* is expressed in the developing CNS in rhombomeres 2-7 in the presumptive hindbrain. During development *Hoxa2* expression extends caudally throughout the spinal cord and persists into adulthood. Although previous analysis of Hoxa2 expression indicates its possible role in neuronal circuit specification and/or dorsal-ventral patterning within the spinal cord, the precise genetic pathways through which Hoxa2 affects spinal cord development have not been characterized. We have used immunoprecipitation of Hoxa2-target DNA complexes from chromatin preparations of E18 mouse spinal cord and hindbrain tissue to isolate *in vivo* downstream target genes of Hoxa2. Seven DNA fragments were isolated, sequenced and were shown to exhibit *in vitro* DNA binding by Hoxa2. A search of sequence databases for the target sequences revealed that of these, two displayed high identity with novel mouse genes: *toll-associated serine protease* (*Tasp*) and the murine homolog of the human *dual specificity tyrosine phosphorylation regulated kinase 4* (*Dyrk4*). Also, two of the isolated clones are presumably bacterial sequences containing the canonical homeodomain binding site TAAT, and the remaining three clones have not yet been mapped in the mouse genome. A potential core Hoxa2 binding motif consisting of 5' CCATCA/T 3', which is based on a previously characterized Hoxa2-Pbx consensus sequence (Lampe et al., 2004), has been identified in both the *Tasp* and *Dyrk4* intronic elements. Both *Dyrk4* and *Tasp* mRNA have been detected within the developing mouse from E10-18 and in the adult CNS. Analysis by RT-PCR of *Tasp* expression in *Hoxa2*$^{-/-}$ newborn mice hindbrain and spinal cord tissues

showed an upregulation of *Tasp*, and transient transfection experiments indicated that Hoxa2 may act as a transcriptional repressor of *Tasp* through an intronic regulatory element. Transfection studies using the intronic sequence of *Dyrk4* indicated that it may function as an enhancer of transcription of *Dyrk4* in the presence of Hoxa2. Both Dyrk4 and Tasp belong to large protein subfamilies whose members play a role in numerous developmental pathways in several organisms. Tasp, also known as HtrA3, interacts with TGFβ signaling molecules which are known to be key regulators of development, dorsoventral patterning and are involved in various neuronal pathways. Although the function of Dyrk4 is not known, many of its family members are involved in the regulation of transcription factors and signaling molecules via phosphorylation that are involved in neuronal pathways also. Hoxa2 may act in specifying neuronal subtypes and dorsoventral patterning in the CNS through down and upregulation of its downstream targets *Dyrk4* and *Tasp*, respectively.

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DEDICATION

This is dedicated to my parents, Jim and Sebahat Akin, whose strength and faith in me, as well as their financial and emotional support made it possible for me to pursue Graduate Studies.

TABLE OF CONTENTS

TABLE OF FIGURES

LIST OF TABLES

TABLE OF APPENDICES

LIST OF ABBREVIATIONS

abd-A:	abdominal-A homeobox
Abd-B:	Abdominal-B homeobox
AD:	Activation Domain
ANOVA:	Analysis of Variance
ANT-C:	Antennapedia cluster of the *Drosophila* homeotic complex
Antp or Ant-p:	Antennapedia type homeodomain
A-P:	Anteroposterior
APP:	Amyloid Precursor Protein
b1-ARE:	Hoxb1 autoregulatory element
bHLH:	basic Helix-loop-Helix transcription factor
bp:	base pairs
BLAST:	Basic Local Alignment Sequence Tool
BMP:	Bone Morphogenic Protein
BSA:	Bovine Serum Albumin
BX-C:	Bithorax cluster of the *Drosophila* homeotic complex
$CaCl_2$:	calcium chloride
Cad:	Cadherin
CAM:	Cell Adhesion Molecule
CD:	Circular Dichroism
cDNA:	complementary DNA
Cdx:	Caudal related homeobox
ChIP:	Chromatin Immunoprecipitation
CMV:	Cyto-Megalo Virus promoter
CNS:	Central Nervous System
CRE:	cAMP Response Element
CREB:	cAMP Response Element Binding Protein
DNA:	Deoxyribonucleic acid
dpc:	days post coitum
DTT:	Dithiothreitol
D-V:	Dorsoventral

Dyrk:	Dual specificity tyrosine kinase
E:	Embryonic stage
EC:	embryonic carcinoma
EDTA:	Ethylenediamine Tetraacetic Acid
EGTA:	Ethylene Glycol Bis-2-Aminoethyl Ether Tetraacetic Acid
EMSA:	Electrophoretic Mobility Shift Assay
En:	Engrailed homeodomain protein
Eph:	Ephrin
Exd:	Extradenticle homeodomain protein
FISH:	Fluorescence *In Situ* Hybridization
FLAG:	A protein expression tag with DYKDDDDK residues
Ftz:	Fishu tarazu homeodomain protein
GATA:	Protein family of (A/T) GATA (A/G) binding zinc fingers
Gbx:	Gastrulation brain homeobox
GFP:	Green Fluorescent protein
GR:	Glucocorticoid Receptor
GST:	Glutathione-S-Transferase protein tag
Gsx:	Goosicoid-like homeobox
HAT:	Histone acetyltransferase
HB9:	Human *homeobox gene 9*
HBS:	Homeodomain binding site
HDAC:	Histone deacetyltransferase
HEMGN:	HEPES, EDTA, Mg^{2+}, Guanidine and NP-40 protein extraction buffer
HEPES:	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
HIPK:	Homeodomain Interacting Protein Kinase
HNF:	Hepatocyte nuclear factor
HOM-C:	Homeotic Complex of *homeobox* genes in *Drosophila*
Hox:	Homeobox
hr:	hours
HTRA:	High-temperature requirement factor A

Htr-A2:	*Helobdella triserialis* homeotic protein
HRP:	Horse Radish Peroxidase
IgG:	Immunoglobulin G
IGFBP:	Insulin Growth Factor Binding Protein
IPTG:	Isopropyl-β-d-Thiogalactopyranoside
Irx:	Iroquois homeobox protein
KCl:	Potassium chloride
kDa:	kiloDaltons
KFB1:	H-2Kb enhancer binding factor 1, also called H2TF1
L-CAM:	Liver cell adhesion molecule
Lbx:	*Lady bird*-like homeobox
LIM:	Zinc-binding domain present in Lin-11, Isl-1, and Mec-3
LIM class:	LIM-homeodomain proteins
MDK1:	Mouse developmental kinase 1
Meis:	*Myeloid ecotropic insertion site* genes
$MgCl_2$:	Magnesium chloride
min:	minutes
Msh:	*Drosophila muscle segment* homeobox
Msx:	*Msh*-like homeobox
Myb:	Oncogene first identified in avian myeloblastosis virus
N-CAM:	Neural Cell Adhesion Molecule
NaCl:	Sodium chloride
NaH_2PO_4:	Sodium di-hydrogen phosphate
nM:	nanoMolar
Na PO_4:	Sodium phosphate
NaSCN:	Sodium thiocyanate
NE:	nuclear extract
NET:	*Norepinephrine transporter* gene
NFκB:	Nuclear Factor kappa B
NKL:	Homeobox gene cluster containing the *NK1*, *NK3*, *NK4*, *Lbx*, *Tlx*, *Emx*, *Vax*, *Hmx*, *NK6*, and *Msx* genes

Nkx:	Nirenberg-Kim homeobox protein
NMR:	Nuclear Magnetic Resonance
OCT:	Octamer binding protein
OD:	Optical Density
OPN:	*Osteopontin* gene
Otx:	*Orthodenticle*-like homeobox
P1:	Post-natal day 1
pcDNA3:	High-level constitutive expression plasmid
pCMV-RL:	*Renilla* luciferase encoding plasmid
pGFP:	GFP encoding plasmid
pGL3:	Firefly luciferase encoding plasmid
pRSV-Hoxa2:	Hoxa2 encoding plasmid
PAGE:	Polyacrylamide gel electrophoresis
Pax:	*Paired*-like homeobox
PBC:	class of homeodomain proteins: mammalian PBX proteins, *Drosophila* Extradenticle, and *Caenorhabditis elegans* Ceh-20
PBS:	Phosphate Buffered Saline
Pbx:	pre-leukocyte B cell homeobox
PCR:	Polymerase Chain Reaction
Pdx:	Pancreatic duodenum homeobox
PDZ:	protein domain named after: PSD95 (post synaptic density protein) DlgA (*Drosophila* disc large tumor suppressor) ZO1, a mammalian tight junction protein domain
PH:	Pbx-Hox DNA bipartite binding site
Pit1:	Pituitary 1 homeobox
PKA:	Protein kinase A
PM:	Pbx-Meis bipartite DNA consensus sequence
PMSF:	Phenylmethanesulfonyl Fluoride
POU:	Pit-Oct-Unc containing transcription factors
Prep:	Pbx regulating protein

PVDF:	Polyvinylidene Difluoride
r:	rhombomere
RA:	Retinoic Acid
RNA:	Ribonucleic acid
RP:	Repressor Domain
RSV:	Rous Sarcoma Virus promoter
RTK:	Receptor Tyrosine Kinase
RT-PCR:	Reverse Transcriptase PCR
S:	Shift complex
SDS:	Sodium Dodecyl Sulfate
siRNA:	small interference RNA
SOX:	*SRY*-like *HMG-box* genes
SS:	Supershift complex
TALE:	Three amino acid loop extension homeodomain protein
Tasp:	Toll associated serine protease
TESS:	Transcription element search system
TFE:	2,2,2-trifluoroethanol
TGF:	Transforming Growth Factor
TNT:	Transcription/Translation system
TTF:	Thyroid Transcription Factor homeodomain protein
UTR:	Untranslated region
UV:	Ultraviolet light
μM:	microMolar
μl:	micro liter
X1Hbox8:	*Xenopus* homeotic protein Hox 8
Xlox:	XlHbox8/Htr-A2 like homeobox

1. INTRODUCTION

Homeotic genes were first discovered in the fruit fly, *Drosophila* (Balkaschina, 1929; Bridges and Dobzhansky, 1933; Bridges and Morgan, 1923; Ouweneel, 1976) where they were found to play a role in determining segmental identity (reviewed in: Lewis, 1978; McGinnis and Krumlauf, 1992; Peifer and Wieschaus, 1990; Castelli-Gair, 1998; Akam, 1998a, b; Gellon and McGinnis, 1998; Rijli et al., 1998). In *Drosophila,* a single homeotic complex (HOM-C) comprised of two separate clusters [the Bithorax (BX-C*)* and Antennapedia (ANT-C) cluster] is located on chromosome 3 (Lewis, 1978; Kaufman et al., 1990; Scott et al., 1983). Many homeotic genes contain a 180 base pair (bp) homeobox sequence which encodes a conserved 60 amino acid region referred to as the homeodomain (McGinnis et al., 1984; Scott and Weiner, 1984; Gehring et al., 1994). Genes encoding the homeodomain motif are referred to as *homeobox* (*Hox*) genes. These genes are conserved during evolution and have been identified in all major metazoa, plants and fungi examined thus far (Bürglin, 1997, 2003). *Hox* genes generally function as transcription regulators that govern various aspects of morphogenesis and cell differentiation. *Homeobox*-containing genes that are homologs to the *Drosophila homeotic* genes have also been identified in vertebrates, where in many instances they function to control embryonic morphogenesis (reviewed in: McGinnis and Krumlauf, 1992; Favier and Dollé, 1997; Prince, 2002).

The *Hoxa2* gene and its paralog *Hoxb2* are homologs of the *Drosophila proboscipedia* gene. High levels of *Hoxa2* expression during embryogenesis occurs in

the neural tube and neural crest cells that contribute to the second branchial arch, as well as other tissues (Prince and Lumsden, 1994; Davenne et al., 1999; Hao et al., 1999; Barrow et al., 2000; Grammatopoulos et al., 2000). Within the neural tube, the anterior boundary of *Hoxa2* expression is situated at the rhombomere (r) 1/2 interface (Davenne et al., 1999; Barrow et al., 2000). *Hoxa2* expression extends from the hindbrain caudally throughout the spinal cord, with expression initiated at first within the ventral mantle region at embryonic day 10 (E10). However, expression of *Hoxa2* at E18.5 is predominantly found within the dorsal horn (Hao et al., 1999). Hence, *Hoxa2* may potentially contribute to both anteroposterior (A-P) positioning as well as dorsoventral (D-V) patterning (Hao et al., 1999).

Targeted disruption of *Hoxa2* results in patterning defects of the hindbrain at its most anterior domain of expression, resulting in the absence of the r1/2 boundary and an alteration of the r2/3 border (Gavalas et al., 1997; Davenne et al., 1999; Barrow et al., 2000). Morphological A-P and D-V patterning defects are observed in *Hoxa2* null mutant mice with respect to the r2 and r3 segments (Davenne et al., 1999). Additionally, external defects of the branchial arch derivatives, such as cleft palate (Barrow and Capecchi, 1999), are quite severe in *Hoxa2* mutants. Studies using haploinsufficient *Hoxa2* mutant mice reveal dose dependent mechanisms of development within the hindbrain and branchial arches (Ohnemus et al., 2001). The branchial arches, in particular the second arch, are highly sensitive to a reduction in Hoxa2 activity. In contrast, the anterior hindbrain is unaffected even by an extreme decline in Hoxa2 levels. Therefore, general A-P and D-V patterning of the CNS is maintained even at low levels of Hoxa2 activity, possibly due to functional redundancy between anterior *Hox* genes or

the presence of parallel pathways in segmental regulation (Ohnemus et al., 2001). However, at the molecular level differential sensitivity to *Hoxa2* inactivation between specific neuronal subtypes has been observed, which when considered with previous studies of neuronal expression is suggestive of *Hoxa2* involvement in the specification of neuronal phenotypes (Davenne et al., 1999; Hao et al., 1999; Ohnemus et al., 2001).

Mutations of Hoxa2 and Hoxb2 in mice demonstrated that both homeoproteins are responsible for differential regulation of several genes along the D-V axis in a rhombomere specific pattern (Davenne et al., 1999). For example, a severe reduction in *Pax6* expression and a ventral shift in the border of *Pax3* expression in r2 and r3 were observed in early stages of development (E10). *Hoxa2* and *MDK1/EphA7* have shown coinciding spatial and temporal patterns of expression within the hindbrain (Taneja et al., 1996). Studies using *Hoxa2* null mutant mice revealed a selective lack of *MDK1/EphA7* expression in r3 and alterations in expression within other rhombomeres (Taneja et al., 1996). Although direct regulation of these genes by Hoxa2 has not been determined, these genes may act together with Hoxa2 in specifying early development of the hindbrain. In later stages of development the expression of *Hoxa2* within the rostral somites and in r2 of the hindbrain is directed by an enhancer sequence located at the 3′ end of *Hoxa2* that contains a 10 bp Hox/Pbx binding element (Frasch et al., 1995; Ren et al., 2002). Activation of transcription by this element is mediated by Hoxa2 DNA binding in the presence of cofactors Pbx1a and Prep1 (Lampe et al., 2004).

The activity of *Hox* paralogs 1-4 which mediate rhombomeric patterning is not well characterized, except in regard to their highly conserved auto and crossregulatory mechanisms. Few direct downstream target genes of the anteriorly expressed *Hox* genes

have been isolated with regard to CNS development. The majority of targets isolated thus far have been genes regulated by the *labial* class of *Hox* proteins (Chen and Ruley, 1998; Studer et al., 1998; Gavalas et al., 1998; Guazzi et al., 1998). Targets identified for the *proboscipedia* homeobox genes are the *Otx1* homeobox gene regulated by Hoxb2, and the autoregulation of *Hoxa2* within the rhombomeres (Guazzi et al., 1998; Lampe et al., 2004). We have employed an increasingly popular method of target gene isolation that utilizes chromatin immunoprecipitation of Hox proteins complexed to target DNA. This method allows the identification of *in vivo* rather than *in vitro* target genes. Also, both novel targets in addition to previously characterized genes may be isolated. Chromatin preparations from E18 spinal cord and hindbrain tissues were used for immunoprecipitation of Hoxa2 target sequences.

1.1 Hypothesis

Hoxa2 is a transcription factor that is important in CNS development. I will test the hypothesis that there are several downstream target genes under the direct regulation of Hoxa2 and that Hoxa2 protein interacts with the regulatory elements of these target genes.

1.2 Goal

To isolate and characterize regulatory elements of novel genes identified as direct *in vivo* downstream targets of Hoxa2 during murine CNS development.

1.3 Objectives

1. Purification of bacterially expressed recombinant Hoxa2 protein.
2. Purification of Hoxa2 specific antibody from polyclonal antisera by affinity chromatography.
3. Immunoprecipitation of Hoxa2 specific target DNA sequences from regulatory complexes isolated from E18 mouse hindbrain and spinal cord chromatin preparations.
4. Cloning of putative Hoxa2 responsive regulatory elements and identification of potential target genes by BLAST sequence analysis.
5. Confirmation of Hoxa2 recognition of target sequences by *in vitro* DNA binding analyses.
6. An analysis of the regulatory activity exhibited by Hoxa2 on the target sequences using reporter assays in cell culture.
7. *In vivo* verification of target gene regulation by Hoxa2 through an analysis of target gene expression in *Hoxa2* wildtype and null mutant mice.

2. LITERATURE REVIEW

2.1 Organization of the Vertebrate *Hox* Gene Complex

In vertebrates, more than 200 homeobox-containing genes have been identified thus far [for a complete list of vertebrate and insect *Hox*-like genes refer to: Hox ProDB at http://www.iephb.nw.ru/hoxpro (Spirov et al., 2002) and the homeodomain resource database at http://genome.nhgri.nih.gov/homeodomain/ (Banerjee-Basu et al., 2000, 2003)]. Traditionally these genes were subdivided into the "clustered" homeobox genes known as the *Hox* genes, the "dispersed" *Hox*-like genes, as well as several distinct classes of atypical homeodomain containing genes. The "clustered" *Hox* genes were identified in mammals and other vertebrates based on their similarity to genes of the *Drosophila* HOM-C. Therefore, all genes within this group have a single Antennapedia (Antp) class homeodomain (Duboule, 1994a; Gehring et al., 1994). In mice, the Hox complex is comprised of 39 genes that are arranged into four separate chromosomal clusters designated Hox a, b, c and d (Figure 2.1). *Hox* genes are arranged in a 3′ to 5′ order in each cluster, with their position reflecting not only their homology with each other but also their similarity with genes in the *Drosophila* HOM-C. This arrangement is believed to be due to a two-step process of duplication, a lateral expansion (gene duplication within a cluster or linkage unit) followed by chromosomal duplication of the expanded cluster (Kappen et al., 1989). This two-step process is also referred to as the "2R" hypothesis, representing the double modes of duplication

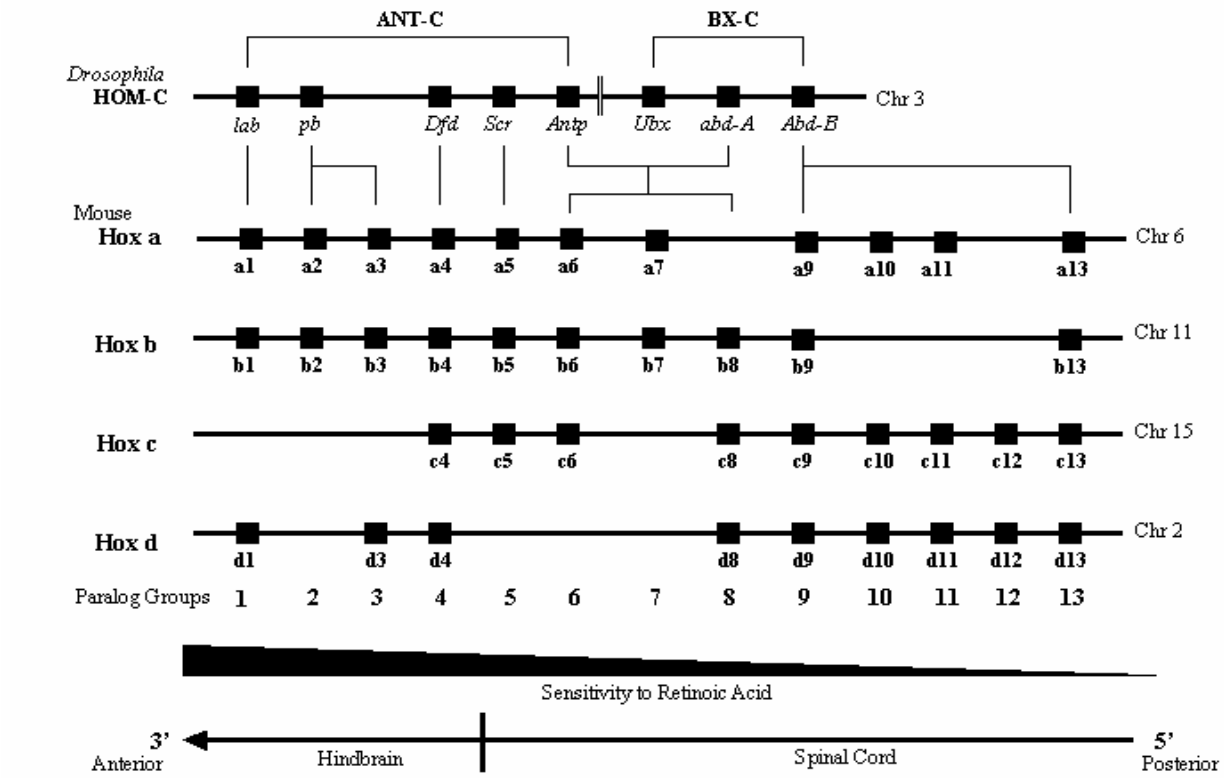


Figure 2.1 Schematic representation of Hox gene clusters

The 39 murine *Hox* genes are present on four separate chromosomal clusters (Hox a, Hox b, Hox c, Hox d). *Hox* genes (illustrated by black boxes) are arranged into 13 paralog groups based on their homology to the *Drosophila* homeotic complex (HOM-C), as well as sequence similarity between genes on different clusters, and according to their positions on their respective chromosomes (Chr). The *Drosophila* HOM-C is composed of two clusters: Antennapedia (ANT-C) and Bithorax (BX-C). ANT-C contains: *lab=labial*, *pb=proboscipedia*, *Dfd=Deformed*, *Scr=Sex Combs Reduced*, and *Antp=Antennapedia*; BX-C contains: *Ubx=Ultrabithorax*, *abd-A=abdominal-A*, *Abd-B=Abdominal-B*. The direction of transcription 5′ to 3′ is indicated, with those genes present at the 3′ end of the clusters expressed more anteriorly in the developing embryo than those at the 5′ end. The anterior *Hox* genes exhibit higher sensitivity to transactivation by retinoic acid than their 5′ neighbors along the chromosomal cluster. In general, paralogs 1-4 display anterior limits of expression within the early developing murine hindbrain, with expression extending posteriorly along the neural tube. The exception being *Hoxc4*, where its anterior limit of expression begins at the rhombomere (r) 7/spinal cord boundary approximately one rhombomere length posterior to that of its paralog *Hoxb4*, which is expressed at the r6/7 boundary (Geada et al., 1992). This figure has been adapted from Krumlauf (1993) with permission (www.elsevier.com).

involved in the evolutionary process (reviewed in: Friedman and Hughes, 2001; Sidow, 1996; Prince, 2002). The application of the "2R" hypothesis to *Hox* evolution is still under debate (Hughes et al., 2001). There is some evidence to support the evolution of *Hox* genes by chromosome polyploidization (Larhammar et al., 2003).

Genes within each cluster that occupy the same linear position along the length of the chromosome are further subdivided into 13 paralogous groups in relation to their similarity with the *HOM-C* genes (Figure 2.1). There are some irregularities in that *Hox* genes in positions 6-8 are related to more than one *HOM-C* gene, and not all *Hox* clusters have members in each paralog group; for example, Hox c has no members in groups 1-3. Paralogs exhibit greater sequence similarity between each cluster than individual genes share with adjacent genes along the same chromosomal cluster.

Many homeobox genes have since been identified that encode a homeodomain similar to the clustered *Hox* genes (Antp-type homeodomain) and have been referred to as *Hox*-like. Initially these *Hox*-like genes did not display any clustered organization, and were therefore often designated as the "dispersed" *Hox*-like genes. These genes were grouped together into various families based on a variety of criteria such as their functional and structural characteristics. For example, the *Cdx* genes were identified as the vertebrate homologs of the *Drosophila caudal* gene, which is so named because of its role in specifying development of the caudal region of the embryo. Many members of the *caudal* gene family are found to be predominantly expressed in the posterior region of the embryo or have been shown to function in the development of posterior structures such as the intestine (reviewed in: Freund et al., 1998; Lohnes, 2003). Similarly, many homeobox-containing genes were identified that did not encode

homeodomains similar to that of the *Hox* genes (i.e. Antp-type homeodomain), and these have been classified into a number of distinct groups based on sequence similarity, structural characteristics and phylogenetic analyses (e.g. Paired class, NK class, POU class, LIM class) (Banerjee-Basu and Baxevanis, 2001; Bürglin, 1994; Galliot et al., 1999; Wada et al., 2003).

Recent investigations have indicated that some of the "dispersed" *Hox*-like genes may have been organized into clustered formations as well, which have then "dispersed" to some degree in vertebrates during evolution from their ancestral source (Coulier et al., 2000a, b; Brooke et al., 1998; Popovici et al., 2001; Ferrier and Holland, 2001; Luke et al., 2003). This hypothesis has been supported by the discovery of the paraHox cluster in *Amphioxus* (Brooke et al., 1998), and the 93D/E cluster of genes or NKL cluster in *Drosophila* (Kim and Nirenberg, 1989; Jagla et al., 1994, 2001). The NKL cluster was later reported to be present as four clusters in humans and is referred to as the metaHox gene class (Coulier et al., 2000b). Four paraHox clusters bearing similarity to the *Amphioxus* paraHox cluster have also been identified in humans (Coulier et al., 2000a). In humans, the majority of the ANTP superclass genes are arranged into 14 homeobox gene clusters derived from four ancient genomic arrays and homeobox-containing genes that do not fit into these clusters were referred to as "dispersed" (or so-called orphan) genes (Pollard and Holland, 2000). These four arrays are believed to have evolved by a series of duplications from an ancestral Hox complex (reviewed in: Patel and Prince, 2000; Holland, 2001). Two of these genomic arrays consist of the paraHox (*Cdx*, *Xlox*, *Gsx*) and NKL/metaHox clusters (which includes several genes such as *Msx, NK* and *Lbx*). The third array is grouped into the ‘extended Hox’, i.e. the traditional Hox clusters

(Figure 2.1) as well as *Evx* and *Mox*, and the fourth array consists of the EHGbox genes (*En*, *HB9*, and *Gbx*) (Pollard and Holland, 2000). The 'extended Hox', paraHox, and the EHGbox subclasses can be grouped together into a larger Hox class (Pollard and Holland, 2000; Banerjee-Basu and Baxevanis, 2001; Popovici et al., 2001; Wada et al., 2003). The metaHox genes in addition to several other groups of genes make up the NK class of homeobox genes (Banerjee-Basu and Baxevanis, 2001; Wada et al., 2003).

There is a significant amount of literature on the "dispersed" *Hox*-like and other homeobox containing genes (Bendall and Abate-Shen, 2000; Panganiban and Rubenstein, 2002; Bürglin and Cassata, 2002; Tremblay and Gruss, 1994; St.-Jacques and McMahon, 1996; Stuart and Gruss, 1995, 1996; Boncinelli, 1997; Kammermeier and Reichert, 2001; Owens and Hawley, 2002; Vollmer and Clerc, 1998). These genes have many varied functions in various organisms, including the development of the central nervous system (CNS). However, since the focus of my research is on the targets of "clustered" *Hox* genes, a discussion of the dispersed or atypical homeobox genes has not been included.

2.2 Structure of the Antennapedia Homeodomain

Many homeobox genes, in particular the *Hox* genes, encode a homeodomain that belongs in the Antp-class, based on its relatedness to the homeodomain of the *Drosophila Antennapedia* gene. This class of homeodomain proteins is defined by the presence of an evolutionarily conserved helix-turn-helix motif (reviewed in Gehring et al., 1994). The solution structure of the Antp homeodomain expressed as a 68 amino acid recombinant polypeptide (Müller et al., 1988) was determined by nuclear magnetic resonance (NMR) spectroscopy (Qian et al., 1989; Billeter et al., 1990). The structure is

composed of three alpha helical regions folded into a tight globular structure, plus a more disordered and flexible fourth helix that consists of several basic amino acid side chains that appears to be a direct extension of the third helix (helix III/IV). The first helix is preceded by a flexible N-terminal arm that has a conserved hexapeptide motif which is involved in protein-protein interactions. Helix I is connected via a loop to helix II, and the second helix in conjunction with helix III forms the helix-turn-helix DNA binding motif. Helices I and II are arranged in an antiparallel fashion relative to each other, while helix III/IV is perpendicularly aligned to the first two helices. The helix-turn-helix motif formed by helices II and III is a common motif present in many prokaryotic transcription factors (reviewed in: Pabo and Sauer, 1992; Gehring et al., 1990). The backbone structure of the Antp helix-turn-helix motif is superimposable with that of several of these prokaryotic transcription factors, demonstrating the evolutionary conservation of the homeobox (Qian et al., 1989; reviewed in Gehring et al., 1990). *In vitro* analyses of DNA binding by Hox proteins have determined that all proteins encoded by the class I *homeobox* (Antp) genes recognize an element of approximately 10-12 bases consisting of a 5′ TAAT 3′ (β strand) core motif with varying flanking sequences (Kalionis and O'Farrell, 1993; Kumar and Nazarali, 2001; reviewed in: Laughon, 1991; Gehring et al., 1994). NMR spectroscopy analysis of the Antp homeodomain complexed to a DNA duplex containing this TAAT core sequence revealed that helix III (recognition helix) forms contacts with the major groove of the target DNA. The N-terminal flexible arm makes additional specific contacts to the bases in the minor groove, while the loop between helix I and helix II interacts with the DNA backbone. Sequence alignment within the *HOX* class has also revealed the conservation

of Arg-5 within the N-terminal arm, as well as residues Gln-12, Glu-15, Leu-16, Glu-17 and Glu-19 within the first helix (Banerjee-Basu and Baxevanis, 2001). It is the recognition helix that is responsible for establishing specific DNA recognition with the TAAT motif. For example, homeodomains of the Antp class have a characteristic Gln residue found at position 50 within the recognition helix. Gln-50 in addition to other conserved residues of helix III, such as Ile-47 and Met-54, are important in establishing contacts with bases within the target sequence (Kumar and Nazarali, 2001; reviewed in Gehring et al., 1994). These residues also function in the binding of DNA by HoxB1 within the HoxB1-Pbx1-DNA complex, as determined by X-ray crystallography (Piper et al., 1999). An invariant characteristic residue Asn-51, found within helix III, is essential in target site recognition for both monomeric and heterodimeric complexes. This residue is believed to mediate base contacts to the polar groups on the DNA through hydrogen bonding in both the *Drosophila* Antp and HoxB1 homeodomains (Billeter et al., 1993; Piper et al., 1999; reviewed in Billeter, 1996).

2.3 DNA-binding of Hox Proteins and their Cofactors

Although *in vitro* binding analyses have determined that Hox proteins bind to similar DNA sequences with similar affinities (Pellerin et al., 1994; Catron et al., 1993; Kumar and Nazarali, 2001), their ability to function *in vivo* as regulatory factors controlling highly specialized cellular mechanisms during morphogenesis requires the coordinate action of homeodomain proteins and cofactors within a transcriptional complex (Remacle et al., 2002; Chan and Mann, 1996; Ryoo and Mann, 1999; reviewed in: Chariot et al., 1999; Mann and Affolter, 1998). Several Hox cofactors have been identified (Pbx, Meis, Prep, Sox and Oct proteins) which modulate the action of Hox

proteins to: bind DNA with a higher specificity, increase their affinity for a particular recognition site within a regulatory element, or modify the activity of specific domains by conformational variation (Di Rocco et al., 2001; reviewed in Mann and Affolter, 1998).

In some instances the functional specificity of Hox factors is conferred by their ability to act differentially on a given target in a cell-specific manner. This is due mainly to the availability of varying interacting proteins within a transcription complex (Saleh et al., 2000; Di Rocco et al., 1997, 2001). For example, for efficient binding of HOX/PBC (homeobox/Pbx family of proteins) to targets *in vivo* recognition by SOX/OCT (SRY-like HMG-box/Octamer protein) heterodimer(s) are required. For HOXA1 to function efficiently *in vivo* on the b1-ARE (autoregulatory enhancer from the murine *Hoxb1* gene), both SOX and OCT proteins are recruited to the SOct site (Di Rocco et al., 2001).

Peifer and Wieschaus (1990) identified the homeotic cofactor Extradenticle (Exd) in *Drosophila* through the isolation of mutants displaying homeotic-like patterning defects in the absence of any disruption in *homeotic* gene expression. The mammalian homologs of Exd are the pre-B cell homeobox factors, Pbx1, Pbx2, Pbx3 and Pbx4, which collectively are referred to as the PBC family of proteins. The PBC proteins together with the MEIS protein family (myeloid ecotropic insertion site genes: MEIS1, MEIS2, MEIS3; and Pbx regulating protein genes: PREP1, PREP2) are part of a larger family of atypical homeodomain proteins that make up the TALE class of homeodomains (Bürglin, 1997), many of which have been found to act cooperatively with Hox proteins in regulation of transcription (Fognani et al., 2002; Ferretti et al., 2000; Jacobs et al., 1999).

Exd/Pbx family members have the ability to alter activity of Hox proteins *in vivo* (Rauskolb and Wieschaus, 1994). Since many Hox proteins have been found to bind DNA cooperatively with Pbx it has been postulated that this interaction increases the selectivity of the Hox proteins, allowing for regulated transcription of its target(s) in a highly specified manner (Pöpperl et al., 1995; Chang et al., 1996; Knoepfler and Kamps, 1995; Lu et al., 1995; Phelan et al., 1995; van Dijk et al., 1995; Piper et al., 1999; Jabet et al., 1999).

Although some studies have indicated that alterations in functional specificity can occur due to enhanced Hox selectivity of DNA target site recognition by Hox-Pbx heterodimers (Chang et al., 1996; Chan et al., 1997), this cannot be generalized to account for all Hox responsive elements where Pbx is involved in Hox interactions. In fact, in some instances Pbx proteins and their Hox partners display a lack of selectivity in binding, instead recognizing identical consensus sequences (Neuteboom and Murre, 1997). Rather than enhancing the selectivity of DNA binding by Hox-Pbx heterodimers, affinity of DNA target recognition by Hox proteins in the presence of a Pbx cofactor was greatly increased due to decreased specificity in DNA recognition (Neuteboom and Murre, 1997).

Initially, interactions between Pbx and Hox proteins in heterodimer formation were believed to be solely mediated through contacts between the Pbx interacting motif (located in the N-terminal region upstream of the homeodomain in Hox proteins) with the C-terminal region of Pbx that contains a Hox interacting motif (Chang et al., 1995; Phelan et al., 1995; Knoepfler et al., 1996). The N-terminal Pbx interacting motif of the Hox proteins is highly conserved and its integrity is considered to be critical for Hox-

Pbx heterodimer formation (Chang et al., 1995; Chan et al., 1996; Knoepfler and Kamps, 1995; Neuteboom et al., 1995). However, the linker joining the motif to the N-terminal end of the homeodomain is highly variable in sequence and length for different Hox family members (Knoepfler and Kamps, 1995; Bürglin, 1994; Chan et al., 1996; Chan and Mann, 1996; Mann, 1995; Pöpperl et al., 1995; Merabet et al., 2003). *In vitro* studies have shown that the Hox-Pbx dimers bind to sequences where each protein recognizes a specific DNA half site (Lu et al., 1995; van Dijk et al., 1995). Variations found in the N-terminal arm region may contribute to differential selectivity and affinity of heterodimers for different types of Hox/Pbx binding sequences (Chan and Mann, 1996; Chang et al., 1996; Chan et al., 1997; Lu and Kamps, 1997; Phelan and Featherstone, 1997).

The Hox cooperativity motif found in the C-terminal region downstream of the Pbx homeodomain that was initially reported to be required for Pbx interactions with Hox proteins was found not to be absolutely essential in the formation of cooperative complexes (Green et al., 1998). This 16 amino acid C-terminal tail however was implicated in stabilizing Pbx-DNA contacts, and therefore is required indirectly for maximal cooperative interactions with Hox factors; its contribution is perhaps variable depending on the Hox partner involved (Chang et al., 1995; Lu and Kamps, 1996; Peltenburg and Murre, 1997; Green et al., 1998).

Experimental analyses of Pbx domains have demonstrated the requirement of specific residues within the Pbx homeodomain, in addition to residues in the TALE loop between helices I and II for interaction with Hox proteins (Bertolino et al., 1995; Shen et al., 1996; Peltenburg and Murre, 1997; Green et al., 1998). This has been confirmed by

X-ray crystallography and NMR spectroscopy analyses of Pbx1-Hox heterodimers and Pbx1 proteins. Upon binding of DNA by Pbx1 (in the presence or absence of a Hox partner) residues in the C-terminal end of the third α-helix of the homeodomain become ordered, favoring folding of the C-terminal tail that follows it into a fourth α helical structure (Piper et al., 1999; Jabet et al., 1999; Sprules et al., 2000). The fourth α helical extension is not involved in DNA contact, instead it interacts with the Pbx homeodomain structure resulting in increased structural stability of the DNA bound protein (Sprules et al., 2000). This stability also contributes to the formation of the hydrophobic pocket which consists of the C-terminus of helix III and the TALE, against which helix IV is packed forming an additional side to the pocket (Sprules et al., 2000, 2003; Slupsky et al., 2001; Piper et al., 1999; Jabet et al., 1999). Hydrophobic residues form contacts when the third α helix, which is bound within the major groove of the DNA, lengthens. The result is contact between TALE residues and the DNA duplex, thereby bringing the TALE closer to residues in the third helix (Sprules et al., 2003). Insertion of the Hox protein's N-terminal Pbx interacting motif, which is itself partially folded, into the hydrophobic pocket is then favored and results in stable Hox-Pbx heterodimer formation on the target DNA (Sprules et al., 2000, 2003; Piper et al., 1999; Jabet et al., 1999; Slupsky et al., 2001).

Hox paralogs 1-10 have been shown to form heterodimeric complexes with Pbx proteins that requires DNA binding by both proteins (Chang et al., 1996). However, only the Abd-B class of Hox proteins (paralogs 9 and 10) are able to complex directly with MEIS (Shen et al., 1997). Hox paralogs 11-13 are incapable of a direct interaction with Pbx due to an absence of a Pbx interacting motif, but they are able to interact with

MEIS/PREP proteins (Shen et al., 1997). In addition, MEIS is capable of affecting the DNA binding specificity of Hox proteins by forming trimeric complexes through protein-protein interaction with Pbx (Shanmugam et al., 1999; Shen et al., 1999; Waskiewicz et al., 2001; reviewed in Owens and Hawley, 2002). The Meis-related factors Prep1 and Prep2 can also form trimeric complexes with Hox and Pbx proteins (Berthelsen et al., 1998a, b; Ferretti et al., 2000; Fognani et al., 2002).

Finally, dimerization between Hox-Pbx, Hox-Prep/Meis and Pbx-Prep/Meis may occur even when all three factors are present (Jacobs et al., 1999**;** Ferretti et al., 1999). Hox-Pbx and Hox-Meis protein interactions are mostly DNA dependent, whereas MEIS-PBC protein interactions are DNA independent (Fognani et al., 2002). Association of Hox proteins with both the MEIS and PBC proteins in heterodimers has been shown to require specific conserved regions in the respective N-terminal arms of all three proteins (Phelan and Featherstone, 1997; Shanmugam et al., 1997; Remacle et al., 2002). Trimeric Hox-Pbx-Meis/Prep complexes have been isolated and analyzed with respect to various regulatory elements and recognition sites (Swift et al., 1998; Jacobs et al., 1999; Liu et al., 2001; Shanmugam et al., 1999; Shen et al., 1999). The activity of variable Hox-cofactor complexes on transcription appear to be highly dependent on the context of the cell-type and enhancer element used in analysis (Saleh et al., 2000; Shen et al., 2001). In some instances, it is speculated that all three proteins are involved in recognition and binding of sequences within a regulatory element, while in others two proteins act as a tether for interaction with a third factor (Jacobs et al., 1999; Berthelsen et al., 1998a; Fognani et al., 2002). However, in all scenarios it is apparent that formation of these trimeric complexes enhances the functional specificity of Hox

proteins, and that these functions could potentially vary greatly between any given cell-type or tissue. Within the trimeric complexes there is also a potential for the formation of a second hydrophobic pocket by the PBC or MEIS component that is not directly interacting with the Hox protein in question, potentially favoring insertion of a Pbx interacting motif from another Hox or Hox-related protein (Shen et al., 1997; Kroon et al., 1998; Swift et al., 1998; Affolter et al., 1999).

DNA recognition is not the sole variable in determining the function of a *Hox* protein, but rather it is the interaction of the N-terminal region of these Hox proteins with other factors that is responsible for mediating their differential activities in a tissue- or cell-specific manner. Studies in *Drosophila* using *lab* and *Dfd* gene products in association with the Exd/Pbx cofactor indicated that, in the context of certain *Hox* responsive elements, the functional specificity is determined by other cofactor binding sites in a manner independent of the Hox protein or Hox-Exd heterodimer binding preferences (Li et al., 1999a). There is also evidence supporting a regulatory role for Hox proteins that is independent of DNA binding specificity, i.e. through alteration of their transcriptional activities via functional domains rather than affecting their binding to response elements. For example in *Drosophila,* Exd was necessary but not sufficient for full activation of the Dfd homeotic protein and a combination of other cofactors may be required for maximal activity (Li et al., 1999b). It seems apparent that the functional specificity of Hox proteins is determined not only by their ability to recognize specific target sequences within a regulatory element, but also by the activation of various functional domains through protein-protein interactions. The ability to identify the functional domains of mammalian Hox proteins is very much dependent on the

physiological context of a specific regulatory element *in vivo* (Vigano et al., 1998). Additional mechanisms of regulation such as phosphorylation and nuclear translocation by a variety of cofactors that are mainly independent of the DNA target specificity may also be at play (reviewed in Chariot et al., 1999a). For example, PREP/MEIS factors are required to translocate EXD/PBX to the nucleus (Abu-Shaar et al., 1999; Berthelsen et al., 1999; Jaw et al., 2000). Hence, due to the involvement of several different mechanisms involving cofactors and their *in vivo* context, the dissection of Hox activity and functional specificity is complex.

2.4 *Hox* Gene Function and Expression

A characteristic of both the vertebrate Hox and *Drosophila* HOM-C homeobox clusters is spatial colinearity, which is the expression of the *Hox* genes along the anteroposterior axis of the embryo in congruence with their arrangement along the chromosome (Figure 2.1) (Lewis, 1978; Duboule and Dollé, 1989; Dressler and Gruss, 1989; Graham et al., 1989; Izpisua-Belmonte et al., 1991; reviewed in Kmita and Duboule, 2003). Thus, the 3′ genes within a chromosomal cluster are expressed more anteriorly than the 5′ genes with respect to the rostrocaudal axis of the embryo. In general, vertebrate *Hox* genes also display temporal colinearity (Izpisua-Belmonte et al., 1991; Gaunt and Strachan, 1996; Dollé et al., 1989; Duboule, 1994b) and colinear sensitivity to RA, a known inducer of *Hox* gene expression (Acampora et al., 1989; Simeone et al., 1990; Dekker et al., 1992; Krumlauf and Gould, 1992; Papalopulu et al., 1991; reviewed in Marshall et al., 1996; Conlon, 1995; Lufkin, 1997). Hence, there is a 3′ to 5′ colinear sequence where genes at the extreme 3′ end of the individual clusters are activated the earliest, have the most anterior boundaries of expression, and exhibit the

most sensitivity to RA. A direct correlation (colinearity) appears to exist between the *Hox* gene order along the chromosomal cluster and the relative dose response of each gene to RA (reviewed in: Boncinelli et al., 1991; Simeone et al., 1991; Conlon and Rossant, 1992; Morrison et al., 1997). The precise mechanisms of temporal and spatial regulation of *Hox* genes are not entirely understood, although several upstream regulatory factors have been implicated. These involve an interplay of both positive and negative regulatory actions on the *Hox* promoter and enhancer elements by such factors as kreisler, Krox-20, trithorax and Polycomb proteins, as well as RA induced nuclear factors (Barrow et al., 2000; Bel-Vialar et al., 2000; Dupé et al., 1997; Frasch et al., 1995; Gould, 1997; Huang et al., 2002; Maconochie et al., 2001; Manzanares et al., 1997, 2002; Marshall et al., 1994, 1996; Nolte et al., 2003; Nonchev et al., 1996a; Oosterveen et al., 2003; Schumacher and Magnuson, 1997; Studer et al., 1994; 1998). Additionally, the clustered organization of *Hox* genes has been postulated to play a role in the establishment of colinear expression based on the identification of shared enhancer elements (Gould et al., 1997; Sharpe et al., 1998; Kmita et al., 2000; Sham et al., 1992). However, to what degree this influences colinearity may depend on the context of the specific tissue, or the *Hox* gene in question (Kmita et al., 2000; reviewed in: Duboule, 1998; Prince, 2002). *Hox* gene products also contribute to the maintenance of their expression domains through auto and crossregulation (Barrow et al., 2000; Faiella et al., 1994; Hooiveld et al., 1999; Maconochie et al., 1997; Manzanares et al., 2001).

Spatial and temporal colinearity of *Hox* genes is observed along a chromosomal cluster and does not extend to expression between paralog members, i.e. *Hoxa1* is not

expressed before *Hoxb1* nor does it have a more anterior domain of expression. In general most paralogous genes have identical or similar domains of expression (Gaunt et al., 1989; Manley and Capecchi, 1997; Rossel and Capecchi, 1999; Morrison et al., 1997; Greer et al., 2000 and referenced therein; reviewed in: Keynes and Krumlauf, 1994; Hunt et al., 1991a, b). The overlapping expression patterns of various paralogs, in addition to the high degree of sequence similarity between paralogous genes is indicative of some degree of functional redundancy (Rossel and Capecchi, 1999; Davenne et al., 1999; Manley and Capecchi, 1998). Thus, a gene within a paralog group may in part or entirely function to replace the activity of another member of that same group that has been disrupted (Horan et al., 1995a, b). As an example, *Hoxa3*, *Hoxb3*, and *Hoxd3* generally have very similar patterning, and gene targeting has shown that members of paralogy group 3 functionally compensate for each other (Manley and Capecchi, 1997, 1998; Greer et al., 2000). This functional equivalency has been observed for the paralog 3 Hox proteins, even though they do not exhibit a high degree of identity in amino acid sequence. It is probable that the functional specificity of each paralog 3 member is dependent on quantitative differences in expression levels since they display overlapping expression domains, and is likely determined by the actions of cis-regulatory sequences present in each gene (Greer et al., 2000).

Although *Hox* genes follow these general arrangements of expression along the anteroposterior body axis, the most common parallels between *Drosophila* and mouse *Hox* gene function can be made with regard to the development of the hindbrain and spinal cord. During early embryonic development in the mouse all *Hox* genes are expressed in the CNS and adjacent mesoderm. The division of the hindbrain into

metameric units referred to as rhombomeres (r), and the restricted expression of the *Hox* genes within the hindbrain, resembles that of the segmental organization of the *Drosophila* embryo by the *HOM-C* genes. Also, the expression of *Hox* genes in defined rostrocaudal domains in the developing spinal cord is indicative of a role for *Hox* genes in spinal cord patterning. It is also very probable that the coordinated activity of the Hox paralogs rather than an individual *Hox* gene is responsible for establishing unique domains during pattern formation of the spinal cord and hindbrain (reviewed in Carpenter, 2002).

2.6 Expression of *Hox* Genes in the Developing Hindbrain and Spinal Cord

The generation of *Hox* gene expression during embryonic development has been described as encompassing three distinct phases: initiation, establishment, and maintenance (Deschamps and Wijgerde, 1993; Deschamps et al., 1999). During gastrulation *Hox* gene transcription is initiated within the primitive streak. As development progresses specific domains of *Hox* expression are observed, such as those within the developing CNS. These restricted domains of expression are maintained through a series of molecular mechanisms that also involve *Hox* auto and crossregulatory networks, in addition to upstream factors such as kreisler and Krox-20 (Akasaka et al., 2001; Brend et al., 2003; Di Rocco et al., 2001; Kwan et al., 2001; Manzanares et al., 1997, 1999, 2002; Nonchev et al., 1996 a, b; Packer et al., 1998; Ren et al., 2002; Seitanidou et al., 1997; Sham et al., 1993). The molecular mechanisms involved in the generation of *Hox* gene expression during these phases of development in the hindbrain and spinal cord have been reviewed in Deschamps et al. (1999).

Nested expression of members of the Hox paralogs 1 through 4 in the rhombomeres has been observed in the developing murine hindbrain (Figure 2.2), where their anterior boundaries of expression coincide with that of the rhombomeric boundaries (reviewed in: Rijli et al., 1998; Krumlauf, 1993; Krumlauf et al., 1993; Wilkinson, 1995; Lumsden and Krumlauf, 1996). General colinearity among the *Hox* genes is evident, particularly in the developing spinal cord (Figure 2.3). The 3′ anterior Hox paralogs have rostral expression limits in the hindbrain, with expression extending in longitudinal domains to varying extents through the spinal cord. The 5′ Hox groups (5-13) generally have anterior boundaries of expression limited to domains within the spinal cord (Figure 2.3) although expression of some 5′ posterior *Hox* genes may extend rostrally into caudal structures of the hindbrain either in later development stages or postnatally (Sakach and Safaei, 1996; Zhang et al., 1997a; Oosterveen et al., 2003).

One exception with regard to colinear expression is the *Hoxa2* gene. In the hindbrain, the rostral boundary of *Hoxa2* expression is anterior to the *Hoxa1* boundary (r4) at the r1/r2 interface (Prince and Lumsden, 1994; reviewed in Krumlauf, 1993). At later stages of development *Hoxa2* was expressed in the diencephalon and the forebrain (Wolf et al., 2001; Tan et al., 1992). Although members of paralogs 1 and 2 do not follow strict colinearity, members of paralog groups 3 and 4 from clusters a, b, and d share rostral boundaries of expression at the r4/r5 and r6/r7 interface, respectively (reviewed in: Krumlauf, 1993; Krumlauf et al., 1993; Lumsden and Krumlauf, 1996).

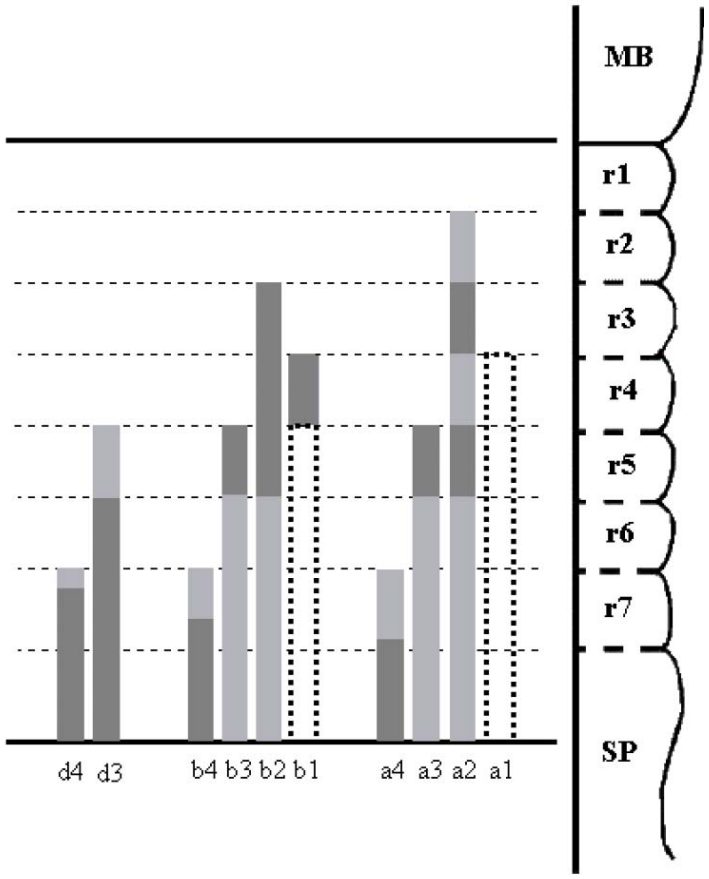


Figure 2.2 Schematic drawing of *Hox* gene expression patterns within the rhombomeres

Hox gene expression patterns of the anterior paralog members within the rhombomere segments of the early embryonic (E9.5) murine hindbrain. Expression of the clustered *Hox* genes is absent within the early embryonic forebrain and midbrain (MB) structures. In general, *Hox* genes display spatial colinearity within the rhombomeric (r) subdivisions 1-7 of the hindbrain, with the exception of the paralog 2 genes. Transient expression of *Hoxa1* and *Hoxb1* (dashed bars) is observed within the hindbrain, where expression begins prior to rhombomere formation. Light grey bars indicate low levels of expression, while dark grey bars indicate areas of high expression. Many of the more 5′ *Hox* genes also display rostral boundaries within the hindbrain, however this is not observed until later stages of embryonic development after rhombomeric segmentation is accomplished. For example, *Hoxb5*, *Hoxb6* and *Hoxb8* are expressed in the spinal cord (SP) region in early development and their expression extends into the posterior hindbrain where they form their distinctive anterior boundaries of expression by E11.5 (Oosterveen et al., 2003). This figure has been adapted from Hunt et al. (1991a) with permission (The Company of Biologists Ltd., http://www.biologists.com/).

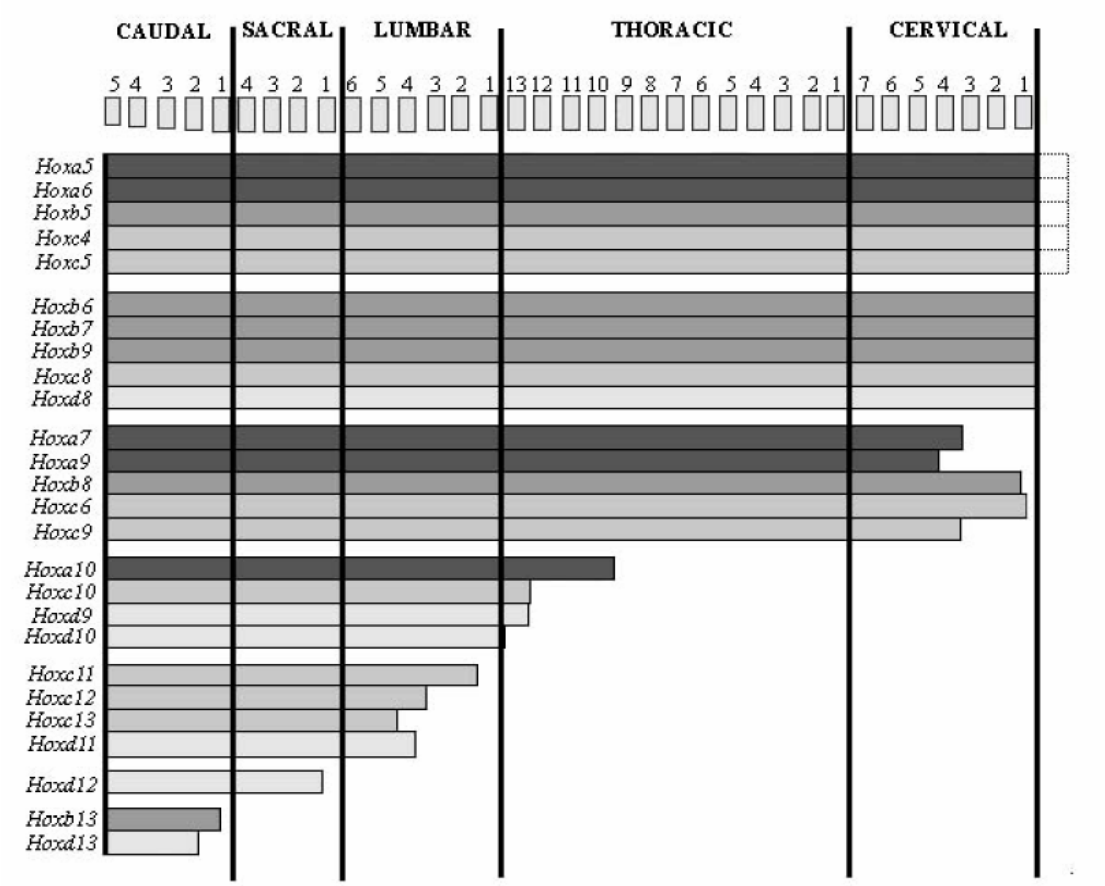


Figure 2.3 *Hox* gene expression in the spinal cord

Schematic illustration of *Hox* gene expression within the developing murine spinal cord at embryonic (E) 12.5 (Carpenter, 2002 and referenced therein). The anterior boundaries of expression for *Hoxa5*, *Hoxb5*, and *Hoxc5* at E12.5 lie within the posterior myelencephalon of the hindbrain rather than within the spinal cord region (indicated by dashed boxes) (Gaunt et al., 1990; Geada et al., 1992; Holland and Hogan, 1988; Hogan et al., 1988; Zakany et al., 1988; Nowling et al., 1999; Tuggle et al., 1990). The rostral limit of expression for *Hoxc4* also lies within the posterior hindbrain of E12.5 mice at a position slightly anterior to that of *Hoxc5* (Geada et al., 1992). Genes are grouped according to the region of their anterior boundary limit, with regions of the spinal cord separated according to the coordinate vertebrae (cervical, thoracic, lumbar, sacral, caudal). The different shades of grey do not represent intensity of expression but instead paralog members are separated by shades of grey with Hox a genes shown in the darkest shade of grey followed respectively, with decreasing shades of grey for Hox b, c and d genes. (Adapted from Carpenter, 2002 with permission from author and S. Karger AG, Basel)

Generally, the ordered expression of *Hox* genes within the hindbrain is well conserved across the vertebrate species (reviewed in Glover, 2001).

In the developing murine spinal cord spatial colinear expression of paralogs 6-13 is observed within individual clusters (Figure 2.3), but this is not the case when comparing expression between members within a paralog group (reviewed in Carpenter, 2002). Expression of *Hox* genes in positions 6-9 extends rostrally to the cervical region whereas paralogy groups 10-13 generally extend expression only into the lumbar region of the spinal cord. The *Hoxd9* gene is atypical in that it is the one member of paralog 9 that mostly has restricted expression to the lumbar level of the spinal cord (reviewed in Carpenter, 2002). However, the majority of other posterior members of the Hox c and d groups share overlapping domains of expression along the spinal cord with highly analogous boundaries of expression. The Hox b genes at positions 6-9 have the most extreme rostral boundary of expression within the cervical spinal cord in comparison to the other paralog members, including the Hox a group (reviewed in: Krumlauf et al., 1993; Carpenter, 2002). In vertebrate development, *Hox* genes exhibit overlapping domains of expression in the caudal regions, although they exert most of their functional activity at or in close proximity to their rostral boundaries of expression such that in mutant mice, abnormalities are generally observed at or near the anterior boundary of expression (reviewed in Prince, 2002). This has been attributed to the functional dominance of posterior genes over anterior genes expressed in the same domain and is called posterior prevalence in vertebrates and phenotypic suppression in *Drosophila*

(Gonzales-Reyes et al., 1990: Duboule, 1994a; Schöck et al., 2000; Kmita and Duboule, 2003).

The patterning of *Hox* gene expression within the developing spinal cord is not restricted to the anteroposterior (A-P) axis, but is also demonstrated in a dorsoventral manner along the transverse plane. At the E12.5 stage of development, *Hoxa4*, *a5*, and *a6* genes exhibit a ventral domain of expression in the spinal cord, as do *Hoxc5* and *c6* (Gaunt et al., 1990; reviewed in Gaunt, 1991). In addition, expression analyses of genes in the Hox b cluster have revealed a dynamic dorsoventral pattern of expression during development of the spinal cord (Graham et al., 1991; reviewed in: Krumlauf et al., 1991; Keynes and Krumlauf, 1994). Within the developing spinal cord and the hindbrain, *Hoxa2* expression exhibits dorsoventral patterning that may determine the location of specific groups of neurons (Davenne et al., 1999; Hao et al., 1999). The dorsally restricted expression of *Hoxb7* and *Hoxb8*, and the ventral specific expression of paralog 10 members as well as Hox c members (at position 8, 10, 11, and 12), may be indicative of *Hox* mediated dorsoventral specification of cell populations (Awgulewitsch and Jacobs, 1990; Deschamps and Wijgerde, 1993; Hostikka and Capecchi, 1998; Peterson et al., 1994; reviewed in Carpenter, 2002).

Hox genes play an important role in CNS development, especially in determining neuronal organization within the hindbrain (Studer et al., 1996; Pattyn et al., 2003; reviewed in: Lumsden and Krumlauf, 1996; Rijli et al., 1998; Pasqualetti and Rijli, 2001) and the spinal cord (reviewed in Carpenter, 2002). Although *Hox* genes may play an instructive role in specification of neuronal cell types, this most likely involves a

combinatorial effect of various signals rather than the actions of a specific *Hox* gene (reviewed in Glover, 2001).

Finally, *Hox* genes are also involved in patterning of the cranial neural crest cells, and therefore contribute to the development of the pharyngeal arches and craniofacial structures (reviewed in: Trainor and Krumlauf, 2001; Santagati and Rijli, 2003). In particular, *Hoxa2* has a clear role as a *homeotic* 'selector' gene in development of the second pharyngeal arch (Gendron–Maguire et al., 1993; Rijli et al., 1993; Pasqualetti et al., 2000; Grammatopoulos et al., 2000; Hunter and Prince, 2002). Recent data has suggested that in neural crest cells, *Hox* genes act as negative modulators of skeletogenic fate (Creuzet et al., 2002; Abzhanov et al., 2003; discussed in Santagati and Rijli, 2003).

2.7 Criteria for Direct Regulation by a Hox Protein

Promiscuous DNA binding of Hox proteins in vitro may result in the identification of false target genes. Hence, it is important that various critieria are fulfilled to demonstrate that a potential target gene is regulated by a Hox protein *in vivo* (discussed in: Andrew and Scott, 1992; Chalepakis et al., 1993; Damante et al., 2001). Overlapping patterns of expression for an induced target should be evident, whereas a repressed target gene will display restricted expression in relation to the Hox protein. This infers regulatory control of the target gene by the Hox protein *in vivo* and should be confirmed by analysis of *Hox* mutant mice, where an increase or decrease in expression of the target gene should coincide with alterations in *Hox* gene expression. Often the requirement for regulation by a Hox protein is shown through reporter assays in cell culture, where determination of the precise mode of action of a *Hox* responsive element is relatively rapid and straightforward to ascertain. However, ultimately the responsive

element needs to be assessed in a transgenic system to demonstrate repression or activation via a specific Hox binding site *in vivo*.

A prerequisite for characterization of a gene as a direct target of a Hox protein is the evidence of DNA binding either *in vitro* or *in vivo* by the Hox protein to the regulatory sequence. However, most DNA binding assays do not reveal whether the Hox protein regulates transcription by directly binding to a DNA sequence, or through protein-protein interactions with other transcription factors. Binding studies using purified Hox protein may help determine if the target is controlled by direct recognition of DNA. Additionally, if the homeodomain binding site is well characterized then mutation analyses of the regulatory region may also provide evidence of a direct Hox-DNA interaction. However, many Hox proteins are known to require additional cofactors to help stabilize their binding to various regulatory elements. More recently several DNA binding defective Hox proteins or various homeodomain truncations and mutations have been designed for use in DNA binding assays and transgenic analysis. These assays could be used to demonstrate a direct regulatory requirement of Hox proteins and their function within a transcription regulatory complex (Koizumi et al., 2003; Remacle et al., 2002; DiRocco et al., 2001; Sloop et al., 2001; Quentien et al., 2002). Transgenic mutants of the target gene should in principle exhibit phenotypes similar to that of its corresponding *Hox* mutants. This can however be complicated by functional redundancy among *Hox* paralogs. However, this problem can be overcome with advancements in transgenic technology. For example, the production of temporal and/or tissue specific mutants, as well as partially active (haploinsufficient) mutants may be able to specifically target a single Hox regulator.

2.8 Approaches to Target Gene Identification

In order to decipher pathways through which *Hox* genes function in regulating regional specification, we need to identify the downstream targets of Hox transcription factors. Vertebrate *Hox* genes are believed to specify this regional identity through regulation of common cellular processes such as cell death, adhesion, proliferation, and migration. It appears that the *Hox* genes are the 'selector' genes of Garcia-Bellido (1975), which are at the top of a genetic hierarchy controlling development by regulating the transcription of 'realizator' genes and regulatory molecules (Andrew and Scott, 1992). Genes involved in regulating cellular mechanisms such as mitotic rate, cell-cell adhesion and cell migration during morphogenesis have been identified as targets for many *homeobox* gene products (Edelman and Jones, 1995, 1998; van Oostveen et al., 1999; Boncinelli and Morgan, 2001; Dailey and Basilico, 2001; Valarche et al., 1993; Jones et al. 1997; Drouin et al., 1998; Meier et al., 1999; McWhirter et al., 1997), however the direct downstream targets of *Hox* genes have not been well defined. Most of the potential targets that have been identified for the Hox transcription factors are involved in either auto or crossregulation of other *Hox* genes (Gould et al., 1997; Maconochie et al., 1997; Manzanares et al., 2001; Packer et al., 1998; Pöpperl and Featherstone, 1992; Pöpperl et al., 1995; Wu and Wolgemuth, 1993), in addition to crossregulation between "dispersed" *homeobox* and *Hox* genes (Charite et al., 1998; Guazzi et al., 1998).

Several cell adhesion molecules such as *cytotactin*, *L-CAM*, *N-CAM*, and *E-cadherin* have been identified as potential targets (Izon et al., 1998; Jones et al., 1992a,b,

1993; Goomer et al., 1994; Boersma et al., 1999; reviewed in Edelman and Jones, 1998). However, many of these targets have not been analyzed as direct targets *in vivo*. This in part is dependent on the method used for identification of a potential downstream target gene.

Various strategies have been utilized to identify targets of homeobox proteins in both vertebrates and *Drosophila* (reviewed in: Andrew and Scott, 1992; Chalepakis et al., 1993; Pradel and White, 1998; Martinez and Amemiya, 2002). A significant amount of information available that delineates the pathways by which *homeotic* genes regulate morphogenesis is due to the availability of mutants in *Drosophila*. The earliest and most common method for target gene isolation involves genetic screening and indicative gene expression pattern analyses (Pradel and White, 1998). Thus, previously characterized genes are identified as targets based on a change in their expression pattern in *Hox* mutants (Graba et al., 1997). A similar approach is the observation of suppression or an enhancement of a homeotic phenotype in genetic mutants, implying that the genes isolated may belong to a common developmental pathway with the *homeotic* gene. However, both of these methods may also potentially identify regulators of *Hox* genes or parallel factors within the same developmental pathway. These methods also cannot preclude whether the *Hox* gene directly regulates the downstream target in question or if it is merely a downstream effector within the *Hox* genetic pathway (reviewed in Mannervik, 1999). Therefore, further molecular analysis is required to verify actual direct regulation. Identification of targets in vertebrates using these approaches is further complicated by the functional redundancy observed between several paralogous *Hox* genes. The ability to produce double and triple mutants in mice has increased our

ability to determine the specific function of an individual *Hox* gene to some extent, however isolation of direct targets based solely on this approach is still inadequate as a whole.

Subtractive hybridization, based on differential expression patterns to identify potential targets, is a useful method for identification of target genes. The method involves isolation of genes by an up or downregulation of specific mRNAs in a cell system or in tissues where a particular *Hox* gene is activated at a specific developmental stage. Different approaches have been used where the *Hox* gene in question may be ectopically expressed in tissue (Hooiveld et al., 1999; Tkatchenko et al., 2001), and overexpressed or suppressed in cell culture (Bromleigh and Freedman, 2000; Carè et al., 1996). However, these approaches are limited since the isolated gene may not be a direct target and it can be difficult to identify those targets whose relative expression patterns are altered during development rather than being entirely abolished or activated. Hence, if there is a shift in the expression pattern during development, or if there is only a minimal up/downregulation rather than a gene being turned "on" or "off", this method of target gene isolation becomes unreliable (reviewed in: Pradel and White, 1998; Martinez and Amemiya, 2002). Subtractive hybridization has progressed significantly with the use of microarrays, enabling the isolation of several potential target genes within a short time frame (Zhao and Potter, 2001; reviewed in Martinez and Amemiya, 2002). New methodologies are being employed to improve the integrity of this approach by increasing its sensitivity for low abundance mRNA (Werner, 2001). However, direct regulation of these potential targets by a particular Hox protein still needs to be

demonstrated by DNA binding analysis and Hox regulation of the target promoter *in vivo*.

Targets of *Hox* and *HOM-C* genes have been identified by various transfection assays using fusion proteins with GAL or VP16 activation domains (Mastick et al., 1995; Friedman-Einat et al., 1996). This approach can be used as a random genetic screen of mouse genomic DNA fragments for the identification of target sequences (Mastick, 1995; discussed in Pradel and White, 1998). It may also be used for investigating the transcription activites of Hox proteins on previously identified targets (Nasiadka et al., 2000), or for the isolation of transcription regulators of a previously identified promoter/enhancer element through a one-hybrid screen (Rausa et al., 1997; Chen et al., 1997; Hayashi et al., 2000). One limitation is that the presence of non-physiological concentrations of Hox proteins has been known to sometimes result in promiscuous DNA binding (Ekker et al., 1994). Also, it has been documented that the use of various forms of Hoxa2-VP16 fusion protein in cell culture resulted in VP16-mediated squelching (Matis et al., 2001). The VP-16 is the moiety from the herpes simplex viral protein that is acts as a strong transactivator when fused to various DNA binding domains. When expressed at high levels *in vitro*, strong transcription activators are believed to bind endogenous factors of transcription complexes independent of DNA thereby resulting in non-specific inhibition of transcription (Gill and Ptashne, 1988). Therefore, it is important to assess the regulatory activity of Hox proteins on their targets using *in vivo* studies as well.

All of the above methods, in addition to the demonstration of *in vitro* DNA-binding of the Hox protein to the candidate target gene, are good indicators of the

identification of an *in vivo* regulated target gene. After the initial isolation of the core TAAT binding motif of Hox proteins, characterization of specific binding sites for several homeodomain factors and identification of the potential cofactors involved in Hox binding specificity was established (discussed in section 1.2). One example is the homeobox-containing protein hepatocyte nuclear factor 1 (HNF1), which has over 50 potential target genes isolated based on the presence of its *in vitro* DNA binding site within the promoter regions of various genes (Tronche et al., 1997). Hence, a candidate target gene can first be characterized based on the presence of a Hox binding site within its regulatory element. These targets can then be further analyzed for a regulatory requirement for a particular Ho*x* protein by reporter assays in cell culture. As with other methods, this approach is restricted in that only well-characterized genes may be identified. Additionally, it is difficult to assess the relevance of an *in vitro* DNA binding site in relation to an *in vivo* environment due to potential promiscuous binding of Hox proteins *in vitro.* The absence of appropriate cofactors may affect the DNA binding affinities or the type of consensus sequence required for Hox recognition and should therefore be taken into consideration (reviewed in Pradel and White, 1998).

In *Drosophila,* Gould et al. (1990) isolated *in viv*o targets of the Ubx homeotic protein by immunoprecipitation of chromosomal-protein DNA complexes. Modifications of this method have been successfully used in the murine system for the isolation of Hoxc8 (Tomotsune et al., 1993), Hoxb5 (Safaei, 1997), and Hoxa2 (Akin and Nazarali, unpublished data) candidate target genes. This technique is believed to be advantageous over other approaches in that it allows for the isolation of more than one target gene *in vivo*. Also, isolation of targets by immunoprecipitation alleviates

difficulties presented by promiscuous DNA binding *in vitro* and allows for the identification of targets controlled by a Hox protein in conjunction with cofactors. However, it is possible that the targets isolated are actually regulated by the Hox protein via protein-protein interactions with other cofactors and not by direct DNA binding of the Hox protein. Therefore, in all approaches, the criteria presented in section 2.0 must be fulfilled in order to confirm the validity of a candidate target gene in terms of direct regulation by a Hox protein.

2.9 Targets of *Hox* genes in the Developing Murine Hindbrain and Spinal Cord

Hox proteins are important developmental regulators that interact in multiple germ layers to coordinate cell division and cellular functions such as cell migration, differentiation, proliferation and apoptosis. In order to decipher the mechanisms through which *Hox* genes regulate development it is necessary to characterize their downstream targets (reviewed in Andrew and Scott, 1992). The following section provides a review of several candidate downstream targets of Hox proteins in the developing hindbrain and spinal cord.

2.9.1 Cell Adhesion

Many of the target gene candidates for homeobox proteins that are involved in cellular processes such as migration and differentiation belong to the cellular adhesion molecule (CAM) family such as *N-CAM*, *L-CAM* and *cytotactin* (Jones et al., 1992a, b, 1993; Sorkin et al., 1993; Goomer et al., 1994; reviewed in Edelman and Jones, 1993). However, several of these target genes have been identified in systems other than the CNS (Tables 2.1 and 2.2). Various members of the CAM family have coinciding expression patterns with several homeobox genes, and homeodomain DNA binding sites

Table 2.1 Examples of candidate cell adhesion targets of divergent homeobox-containing genes in the CNS

Homeobox gene	Target Gene	Transcriptional Activity	Function	Reference
Mouse *Pax8*	Mouse *N-CAM*	+	Neuron specific cell adhesive actions during neural development	Holst et al., 1994; Edelman and Jones, 1995, 1998.
Mouse *Pax6*	Mouse *N-CAM*	+	as above	Edelman and Jones, 1995, 1998.
Mouse *Phox2* and *Cux1*	Mouse *N-CAM*	+	as above	Valarche et al., 1993.
Mouse *Cux2*	Mouse *N-CAM*	+	as above	Quaggin et al., 1996.
Mouse *Pax3*	Chicken *Ng-CAM*	-	Immunoglobulin N-CAM family cell adhesion molecule, specific for peripheral glial cells and post-mitotic neurons	Kallunki et al., 1995; Edelman and Jones, 1998.
Mouse *Barx2* and *Pax6*	Mouse *L1*	+	Immunoglobulin CAM , modulates neuron-neuron and neuron-glia interactions	Jones et al., 1997; Meech et al., 1999; Simpson and Duce, 2002.
Mouse *Otx2*	Mouse *R-Cadherin*	+	Ca2+ dependent cell adhesion, regionalization of the brain and regulation of cell intermixing	Rhinn et al., 1999.
Mouse *Pax6*	Mouse *R-Cadherin*	+	as above	Stoykova et al., 1997.
Xenopus gbx2	*Xenopus N-Cadherin*	-	Ca2+ dependent cell adhesion, implicated in neural tube morphogenesis	King et al., 1998.

Table 2.2 Examples of *Hox* gene regulation of cell adhesion molecules in non-neuronal systems

Hox gene	Target Gene	Transcriptional Activity	System/Cell Type and Function	References
Human *HOXD9*/ mouse *HNF1*	Chicken *L-CAM*	+	Ca2+ dependent cell adhesion: Liver specific, contributes to the development of epithelial and parenchymal tissues in development	Goomer et al., 1994; Sorkin et al., 1993.
Mouse *Evx1/Pax6/Pax8*	Chicken/ mouse *cytotactin* (*tenascin*)	+	Chicken embryonic fibroblast: ECM involved in processes such as counter adhesion and cell spreading, tissue patterning	Jones et al., 1992a; Chalepakis et al., 1992; Copertino et al., 1995.
Rat *Prx1*/ mouse *Prx2*	Mouse *tenascin*	+	Smooth muscle cells, implicated in vasculogenesis	Jones et al., 2001; Norris and Kern, 2001.
Mouse *Hoxa9*	Mouse *E-cadherin* (*L-CAM*)	+	Ca2+ dependent cell adhesion; involved in early T cell development; hematopoiesis	Izon et al., 1998; reviewed by van Oosteveen et al., 1999
Mouse *Hlx*	Mouse *I-CAM1*(*CD54*)	–	Myeloid cells, hematopoiesis	Allen et al., 1993; reviewed by van Oosteveen et al., 1999.
	CD44		Integral membrane CAM in T cells, involved in T cell activation, lymphopoiesis and cell migration	
Human *HOXD3*	Human *αIIb33*	+	Integrin involved in cell adhesion of various tissues during embryogenesis including hematopoiesis	Taniguchi et al., 1995; reviewed by van Oosteveen et al., 1999.
Human *OCT1*	Human *V-CAM1*	–	Immunoglobulin CAM, known as vascular cell adhesion molecule 1, found on vascular endothelial cells, involved in inflammation response	Schwachtgen et al., 1998; Iademarco et al., 1993.
Mouse *Nkx2.3*	Mouse *MAdCAM1*	–	Immunoglobulin CAM, known as mucosal addressin CAM, found on endothelial cells, involved in leukocyte homing in lymphoid organ development	Wang et al., 2000.
Human *BARX2*	Human *Cadherin6*	+	Ca2+ dependent cell adhesion; found on ovarian surface epithelium, involved in tumor suppression in ovarian cancer cell lines	Sellar et al., 2001.
Human *HOXB4*	Human *α-2-integrin*	–	Found on neonatal keratinocytes, involved in regulation of cell proliferation	Komuves et al., 2002.
	Human *CD44*			

have been identified within their regulatory elements. These proteins play an important role in nervous system development, where they may function as downstream effectors of *Hox* genes.

The four main groups of cell adhesion molecules are: the immunoglobulin (Ig) related molecules, integrins, cadherins and selectins. In addition to their involvement incell-cell/matrix adhesion these proteins are also involved in various signaling events that regulate processes such as cell growth, migration and differentiation (reviewed in Juliano, 2002). *N-CAM* (neural cell adhesion molecule), which encodes an Ig related cell-cell adhesion molecule, is expressed at the earliest stage of neural tube formation and persists into adulthood in mice (reviewed in: Chuong, 1990; Edelman and Jones, 1995; Edelman and Jones, 1998). It is predominantly located in neurons where it is involved in neuronal development, plasticity and signaling pathways (reviewed in: Ronn et al., 1998; Kiss and Muller, 2001).

Several negative and positive regulatory elements have been characterized within the promoter of *N-CAM* that were found to contain distinct *Hox* binding sites (HBS) capable of mediating transcription activity by several *Hox* genes, as well as the "dispersed" *homeobox* genes (Jones et al., 1992b, 1993; Holst et al., 1994; reviewed in Edelman and Jones, 1998). These HBS sites represent Hox consensus sequences containing one or multiple canonical TAAT sites (for example, HBS-I 5' CCTA**ATTTTATTA**A 3') flanked by varying sequences (Jones et al., 1993). Hoxb9 induces transcription by binding to HBS sequences within the *N-CAM* promoter, although cotransfection with Hoxb8 prevents this transactivation (Jones et al., 1992b; reviewed in Edelman and Jones, 1995). *Hoxc6*, which displays overlapping expression

with *N-CAM* along the body axis (Chuong et al., 1990), also induces a positive regulatory effect on the promoter of *N-CAM* that is dependent not only on the presence of intact HBS sequences, but also on the neighboring elements (Jones et al., 1993; Boersma et al., 1999). However, when taken out of context from its native promoter, these HBS sequences are insufficient in inducing transcription *in vitro*, although they appear to be required *in vivo* for proper specification of *N-CAM* expression along the dorsoventral axis of the developing spinal cord (Wang et al., 1996; reviewed in Edelman and Jones, 1998). This observation, in addition to the apparent differential effects displayed by *Hox* genes on a common target (Jones et al., 1992b), indicate that cofactors may play an important role in determining specificity of Hox activity (Di Rocco et al., 2001; Remacle et al., 2002; reviewed in Mann and Affolter, 1998).

Neuroepithelial cells in general do not cross the boundaries between adjacent rhombomeres that subdivide the hindbrain (Fraser et al., 1990; Guthrie et al., 1993; Birgbauer and Fraser, 1994; Nittenberg et al., 1997). This was believed to be due to segment-specific adhesiveness of cells within the hindbrain (Lumsden, 1990; Guthrie et al., 1993; Wingate and Lumsden, 1996; Nittenberg et al., 1997). Interestingly, even and odd numbered rhombomeres display different functional adhesive properties (Wizemann and Lumsden, 1997). Cadherins (Cad), which belong to the CAM family of proteins (reviewed in Redies, 1995) could be involved in the compartmentalization of cells (Inoue et al., 1997; Shen et al., 2000). Unlike Ig related molecules such as N-CAM, Cads function in a Ca^{2+} dependent manner (reviewed in Redies, 1995). The segregation of cells between adjacent rhombomeres is dependent on processes involving Ca^{2+} dependent molecules (Wizenmann and Lumsden, 1997).

R-Cadherin (*R-cad, cad4*) exhibits restricted expression in specific rhombomeres and *in vitro* cell aggregation assays show that R-cad^{+} cells segregate from R-cad $^{-}$ cells (Ganzler and Redies, 1995; Redies, 1995; Matsunami and Takeichi, 1995). *R-cad* and *cadherin 6 (cad6*) display alternate expression patterns that delineates the odd and even numbered rhombomeres of mice, as well as the neighboring subdivisions of the brain within the telencephalon (Inoue et al., 1997, 2001). Hence, the function of Cads may be a factor in the segregation of neuroepithelial cells of rhombomeric compartments.

Many *cads* exhibit differential expression patterns throughout the developing CNS and experimental evidence implicate them in functioning as morphoregulatory molecules during embryogenesis (reviewed in: Redies, 2000; Tepass et al., 2000). Also, several *cads* display segmentally restricted expression similar to that observed for *Hox* genes. Hence, *cads* may be potential targets of Hox proteins, especially in consideration of their role(s) in rhombomere compartmentalization. In wildtype embryos, *Hoxa1* and *cad6* exhibit overlapping spatiotemporal expression in early posterior hindbrain (Inoue et al., 1997). Although a direct mode of action has not been determined, transgenic analysis reveals rhombomere and stage specific effects of *Hoxa1*$^{-/-}$ mutation on the expression of *cad6* (Inoue et al., 1997). *Cad6* was also identified as a potential Hoxa1 target using differential hybridization and subtractive gene screening (Shen et al., 2000). Thus, Hoxa1 may either directly or indirectly be involved in the regulation of *cad6* in the posterior hindbrain.

Another cell adhesion molecule that has been identified as a potential direct target of Hoxc8 is the *β-amyloid precursor protein* (*APP*) gene (Violette et al., 1992; Schubert et al., 1989; Breen et al., 1991; Wu et al., 1997). APP is a transmembrane

protein similar to a cell-surface receptor. APP and its derivatives (formed by secretase processing) are expressed in the developing CNS, where they have multiple functions and have been associated with pathological processes such as Alzheimer's disease (reviewed in Dodart et al., 2000; Panegyres, 2001). In addition to cell adhesion properties, APP is important for cell viability and neuroprotection (Goodman and Mattson, 1994; Perez et al., 1997; Nishimura et al., 2003). APP is involved in the morphological and functional plasticity of nerve cells (reviewed in Dodart et al., 2000) and has also been implicated in cell signaling pathways (Russo et al., 2002; Mook-Jung and Saitoh, 1997; reviewed in Mattson and Furukawa, 1998). In transfection studies, *Hoxc8* exhibited repressor activity in a reporter system driven by the *APP* gene promoter, in addition to repression of endogenous *APP* expression (Violette et al., 1992). Although it has not been demonstrated *in vivo*, Hox proteins may directly regulate this response since there are at least 20 Hox binding sites present within the *APP* promoter.

Osteopontin (OPN) is a secreted noncollagenous phosphoprotein component of the extracellular matrix (Oldberg et al., 1986), which exhibits adhesive properties through interactions with integrin adhesion molecules (Liaw et al., 1998). Although OPN's role in osteoblast differentiation and bone formation is well known, its role in the developing and adult nervous system has not been well characterized. *OPN* is expressed in the developing notochord and hindbrain where it may contribute to CNS patterning (Thayer et al., 1995; Thayer and Schoenwolf, 1998). However, it is not known whether OPN functions in the nervous system by targeting integrins as it does in osteoblast differentiation (Lee et al., 2001). *OPN* exhibits neuron specific expression within the developing brainstem and adult brain (Shi et al., 1999; Lee et al., 2001) where it may

play a role in the differentiation and maturation of specific neuronal populations (Lee et al., 2001). Hoxc8 and Hoxa9 mediated repression of *OPN* transcription in osteoblast precursor cells via a *Hox* binding element within its promoter (Shi et al., 1999, 2001). This repression may be antagonized or enhanced through interactions with various Smad signaling molecules, which mediate signaling in the TGFβ and BMP pathways (Bai et al., 2000; Yang et al., 2000; Shi et al., 2001). Although the regulatory mechanisms controlling expression of *OPN* within the nervous system have not been determined, both Hoxc8 and Hoxa9 are present in the developing spinal cord where they may regulate *OPN* expression.

2.9.2 Tumor Supressor Genes

An additional target of Hoxc8 is *mgl-1*, whose function is not entirely understood but has been identified as a mammalian homolog of the *Drosophila lethal (2) giant larvae*, *l(2)gl* (Tomotsune et al., 1993). Immunoprecipitation isolation of Hoxc8 bound DNA complexes from mouse spinal cords identified *mgl-1* as a novel target that displays complementary expression patterns to *Hoxc8* in the embryonic spinal cord, and may therefore be downregulated by Hoxc8 (Tomotsune et al., 1993).

The *l(2)gl* gene is required for *Drosophila* development and a mutation of the gene results in a neoplastic overgrowth of the imaginal discs during embryogenesis (Bryant and Schmidt, 1990). The *l(2)gl* gene functions by altering cell adhesiveness and is required for cell shape remodeling of epithelial cells and regulation of cell polarity (Manfruelli et al., 1996; Strand et al., 1994; reviewed in Humbert et al., 2003). Both the *Drosophila l(2)gl* and its human homolog have been shown to act as a component of the cytoskeletal network (Strand et al., 1994; Kalmes et al., 1996), where they function to

traffick proteins in signaling pathways in regulation of polarity and organization (Manfruelli et al., 1996; Plant et al., 2003; Yamanaka et al., 2003). The role of the murine *mgl-1* (also refered to as *mlgl*) in the regulation of cell polarity has also been studied, although it was not found to associate with the cytoskeletal network (Müsch et al., 2002).

2.9.3 Cell Signaling

There are several candidate downstream targets of *Hox* genes that are involved in signal transduction pathways regulating processes such as apoptosis, cell differentiation, cell proliferation and migration. An important family of signaling molecules is the receptor tyrosine kinases (RTK), which are involved in the regulatory network that control multiple aspects of hindbrain development. Upon ligand binding, the intracellular domain of these receptors is activated, resulting in autophosphorylation of specific tyrosine residues that subsequently initiates an intracellular signaling cascade (discussed in Taneja et al., 1996).

Ephrin (Eph) receptors are an Ig CAM subfamily of the RTKs that are expressed much like *Hox* genes in characteristic rhombomeric specific patterns in the hindbrain (Taneja et al., 1996). Eph receptors and their membrane bound ligands, ephrins, display complementary expression patterns during development (Gale et al., 1996), i.e. they are expressed in odd and even numbered rhombomeres in zebrafish embryos (Xu et al., 1999). This dynamic expression pattern implicates the Eph receptors and ephrins as being important factors in regulating embryonic patterning during development by maintaining cell restrictions (Gale et al., 1996; reviewed in: Coulthard et al., 2002; Tepass et al., 2002). This is particularly evident in the developing hindbrain, where

bidirectional signaling mediated by both Eph receptors and their ligands inhibited intermixing of neuroepithelial cells at the rhombomeric boundaries (Mellitzer et al., 1999; reviewed in Lumsden, 1999). Although the precise function(s) of Eph receptors has not yet been determined, they are believed to restrict cell intermingling through repulsive cell-cell interactions much like cell adhesion molecules (reviewed in Xu et al., 2000). They have also been shown to regulate cell adhesion through interactions with integrin molecules in the regulation of the cytoskeleton (reviewed in Juliano, 2002).

Hoxa1, *Hoxb1* and the *EphA2* receptor gene are all coordinately expressed in the primitive streak during gastrulation, where *Hoxb1* and *EphA2* expression is restricted to r4 (Chen and Ruley, 1998). Additionally, several Hox-Pbx bipartite sites within an r4 specific enhancer of *EphA2* were identified that are capable of mediating transactivation only in the presence of HOXA1-Pbx1 and HOXB1-Pbx1 heterodimers (Chen and Ruley, 1998). Furthermore, *Hoxa1/Hoxb1* double knockout mice exhibited decreased *EphA2* expression, providing additional evidence that *EphA2* could be directly regulated by Hox transcription factors (Studer et al., 1998; Gavalas et al., 1998).

Studies using *Hoxa2* null mice revealed a rhombomere specific alteration in expression of another Eph family member, *MDK1/EphA7* (Taneja et al., 1996). These mice displayed a selective lack of *MDK1* expression in r3, in addition to an alteration of its normal expression pattern in other rhombomeres. Although direct regulation of *MDK1* by Hoxa2 has not been determined, further examination of Eph receptor regulation in the developing hindbrain may result in the identification of several downstream targets of *Hox* genes.

Another candidate target gene is the Purkinje cell-specific *pcp-2(L7)* gene, which is involved in modulating the G protein signaling pathway (Zhang et al., 2002; Redd et al., 2002). Specifically, pcp-2 affects GDP binding of the alpha G proteins, G_o and G_i (Luo and Denker, 1999; Natochin et al., 2001). These heterotrimeric G proteins regulate both cellular and transcription machinery, and can activate various ion channels. They induce transcription factor activity via signal transduction pathways such as the Src and Ras pathway, thereby directing cell processes specifically involved in embryogenesis (reviewed in Neves et al., 2002). Several Hox consensus binding sites (5' TAAT 3') are present within the proximal promoter of the *pcp-2* gene, which is comprised of a short regulatory element (L7ATE) found to be required for normal cerebellar expression (Sanlioglu-Crisman and Oberdick, 1997). Most homeodomain proteins can bind these sites *in vitro*, however HOXA5 and HOXB7 were capable of specific synergistic transactivation of reporter genes mediated by the L7ATE in cell culture (Sanlioglu et al., 1998). Although *HOXA5* and *HOXB7* are not expressed in the developing cerebellum, both factors are enriched in adult Purkinje cells. Transgenic analysis did however, indicate a negative regulatory effect on the *pcp-2* gene by the homeobox protein Engrailed-2 (EN-2) (Oberdick et al., 1993). Hence, a potential biphasic regulatory mechanism is in effect whereby expression of the *pcp-2* gene is repressed in embryonic cerebellar Purkinje cells by the En proteins, followed by initiation and maintenance of expression postnatally by Hox proteins (Sanlioglu et al., 1998). The function of pcp-2 in development is not well understood, although postnatally this regulatory molecule may play a role in synaptogenesis and dendritic growth within the cerebellum (Zhang et al., 2002; Redd et al., 2002).

Rap1 is a member of the small GTPase Ras superfamily that is involved in signal transduction, which like Ras is active in its GTP bound form. Although its function is not entirely understood, Rap1 is known to antagonize Ras signaling of the ERK/MAPK (extracellular signal regulated kinase/ mitogen activated protein kinase) pathway (Cook et al., 1993; Hu et al., 1997). However, Rap1 is also involved in the activation of the MAPK pathway by regulating the activity of B-Raf (York et al., 1998). In neuronal cells, consecutive stimulation of Ras and Rap1 results in a biphasic activation of the MAPK pathway (York et al., 1998; Bouschet et al., 2003). Rap1 is involved in brain development, where it is believed to contribute to neuronal differentiation through the MAPK pathway (Bouschet et al., 2003). It is also involved in the regulation of the cytoskeletal network through interactions with integrin (Ohba et al., 2001; reviewed in: Bos et al., 2001, 2003). In mice, transient expression of *Rap1* is observed within the developing CNS (Kehrer-Sawatzki et al., 2002) whereas in *Xenopus, XRap1* displays restricted expression within the presumptive forebrain and midbrain during embryogenesis (Morsi El-Kadi et al., 2002). *Hoxb4* and *XRap1* display complementary patterns of expression during embryogenesis and *XRap1* was isolated as a putative Hoxb4 downstream target by differential display (Morsi El-Kadi et al., 2002). *In vivo,* the repression of *XRap1* transcription was mediated by DNA binding of Hoxb4 to the *XRap1* regulatory sequence that is present within the 3′ UTR (Morsi El-Kadi et al., 2002).

2.9.4 Protease Inhibitors

Proteases and their cognate inhibitors have highly diversified modes of action that regulate basic cellular processes. Proteases can activate or degrade structural and/or

functional proteins involved in various signaling pathways regulating processes such as cellular communication, apoptosis, differentiation and cell growth (reviewed in: Seidah and Chretien, 1997; McFarlane, 2003; Marks and Berg, 1999; Ye and Fortini, 2000). Hence, proteases and their endogenous inhibitors play a role in regulating cellular mechanisms that are important for embryonic morphogenesis (discussed in: LeMosy et al., 1999; Ye and Fortini, 2000; Brachmann and Cagan, 2003).

Members of the serpin family of serine protease inhibitors are found in a variety of tissues and organisms (reviewed in Gettins, 2002). Serpins are expressed in the developing and adult brain and are believed to play a role in the development and plasticity of the nervous system (reviewed in: Turgeon and Houenou, 1997; Smirnova et al., 1994). The serpin *SPI3* was isolated *in vivo* as a candidate downstream target of Hoxb5. It was identified by immunoprecipitation of Hoxb5 bound to DNA chromatin prepared from embryonic mice hindbrain tissue (Safaei, 1997). Interestingly, overlapping domains of *SPI3* and *Hoxb5* expression were observed in the brain and the developing mouse embryo. Furthermore, *in vitro* binding studies indicated that Hoxb5 recognizes specific elements within the *SPI3* promoter, although their transactivation potential was not evaluated. Recently in the adult brain, SPI3 was found to interact with neuropsin, a serine protease that may be involved in synaptic plasticity (Kato et al., 2001).

2.9.5 Transcription Factors

Perhaps the most characterized targets of *Hox* gene products are genes encoding transcription factors. This is in part due to the high degree of *Hox* gene auto and crossregulation (Gould et al., 1997; Hooiveld et al., 1999; Jacobs et al., 1999; Kwan et

al., 2001; Manzanares et al., 2001; Maconochie et al., 1997; Packer et al., 1998; Pöpperl et al., 1995; Yau et al., 2002; Zhang et al., 1997a). Regulatory interactions between the Hox proteins and other homeodomain encoding genes are also quite common, as has been observed in the regulation of *Otx* during development of the brain. The *Otx* homeobox genes are vertebrate homologs of the *Drosophila orthodenticle* (*otd*), and their role in specification of the rostral CNS is well documented (reviewed in Simeone and Acampora, 2001). The *Otx* family members exhibit spatially restricted expression in the forebrain and midbrain region at the cerebellar-r1 boundary, but are absent in the rhombomeric hindbrain where the anterior *Hox* genes are expressed (reviewed in Rubenstein and Puelles, 1994). Hindbrain expansion occurs by induction of the anterior *Hox* genes coupled with severe repression of *Otx* expression in anterior brain structures indicating the presence of a crossregulatory mechanism (Simeone et al., 1995). The regulatory pathway of both *Otx* and *Hox* gene transcription is responsive to RA gradients, which initiate *Hox* gene expression while at the same time repressing *Otx* expression. This results in mutually exclusive boundaries of expression for *Otx2* and *Hox* in the developing brain (Guazzi et al., 1998). It has been postulated that RA controls the restricted boundaries of expression of both the *Hox* and *Otx* genes by crossregulatory mechanisms, which would contribute to the mutually exclusive expression of the two gene families early on in brain development. The 5′ flanking genomic sequence of *Otx2* contains negative regulatory elements responsive to RA, and this same regulatory sequence was recognized differentially at multiple sites *in vitro* by the anterior *Hox* genes (*HOXB1*, *HOXB2*, *HOXB3*). However, transactivation and not repression of *Otx2* expression was observed *in vitro* in the presence of the anterior *Hox*

genes. This activity did not extend to the more posterior *Hox* genes (*HOXC6* and *Hoxd8*), whereas transactivation can be replicated with HOXD3. Therefore, activation of *Otx2* transcription appeared to be a characteristic of the Hox proteins in Hox paralogs 1-3 (Guazzi et al., 1998). Although the stability of Hox binding is dependent on the presence of nuclear cofactors, the identity of these factors has not been determined. Hence, *Hox* genes do not appear to fit the role of *Otx* repressors as previously believed, but perhaps function in conjunction with RA to refine the early developmental boundaries of *Otx* expression.

The *Iroquois* (*Irx*) genes encode TALE homeodomain proteins and like the *Hox* genes are expressed in early development (Bosse et al., 2000; Cohen et al., 2000). They function in establishing the midbrain-hindbrain boundary (Glavic et al., 2002) and in neuronal specification of cells within the neural tube (Briscoe et al., 2000). Recently, the *Irx5* homeobox gene was identified as a direct downstream target of Hoxb4 (Theokli et al., 2003). In the developing murine CNS, *Irx5* expression displayed dorsoventral patterning within the midbrain, hindbrain and along the spinal cord (Cohen et al., 2000). *Irx5* and *Hoxb4* display overlapping patterns of expression in the developing CNS in both mice and *Xenopus* (Graham et al., 1988; Wilkinson et al., 1989; Cohen et al., 2000; Theokli et al., 2003), and upregulation of *Xenopus Irx5* transcription by Hoxb4 has been shown to be independent of protein synthesis and therefore likely involves a direct interaction (Theokli et al., 2003).

The *GATA2* and *GATA3* genes are the only members of the GATA family of transcription factors expressed in the CNS (Kornhauser et al., 1994). Both GATA2 and GATA3 have been implicated in specification of neural subtypes during development

(Karunaratne et al., 2002). In the spinal cord and hindbrain these genes are expressed in spatially restricted domains similar to that of the *Hox* genes (Pata et al., 1999; Karunaratne et al., 2002). *GATA3* expression is particularly prominent in r4 (Nardelli et al., 1999; van Doorninck et al., 1999), exhibiting overlapping domains of expression with *Hoxb1*. An analysis of *GATA3* null mutant mice showed the presence of similar abnormalities to that of *Hoxb1*$^{-/-}$ mice, both demonstrating defects in r4-derived motor neurons. Furthermore, *Hoxb1* null mutants displayed a loss in expression of *GATA2* and *GATA3* in the ventral r4. Hence, GATA3 appears to be a downstream effector of *Hoxb1,* although it is not known whether the mode of action for this regulation is direct (Pata et al., 1999). It is interesting to note that many "dispersed" *homeobox* genes and some *Hox* genes are regulated by other GATA members in developmental systems other than the CNS (Vieille-Grosjean and Huber, 1995; Searcy et al., 1998). Crossregulation between *Hox* and *GATA* genes may potentially play a role in the genetic pathways of *Hox* mediated morphogenesis.

2.9.6 Norepinephrine Transporter Gene (*NET*)

The *norepinephrine transporter* (*NET*) gene is expressed in the noradenergic neurons in many regions of the CNS and peripheral nervous system (PNS) (reviewed in: Hoffman et al., 1998; Amara, 1995; referenced in Kim et al., 2002), where it mediates norepinephrine reuptake into the presypnaptic nerve endings (Axelrod and Kopin, 1969). In early embryonic development NET is distributed in a wide range of neuronal and non-neuronal tissues (Sieber-Blum and Ren, 2000). NET is believed to have additional functions such as specification of the neuronal norardrenergic phenotype, as well as the differentiation of neural crest stem cells into sympathetic neurons (Zhang and Sieber-

Blum, 1992; Zhang et al., 1997b; Renn et al., 2001; Kim et al. 2002). Hoxa5 has been shown to recognize a HBS sequence within the promoter of the human (h) *NET* gene *in vitro*, through which it transactivates expression of a reporter gene (Kim et al., 2002). *In vitro* binding and transfection experiments indicated that this HBS sequence played a critical role in directing noradrenergic cell specific expression of *hNET*. Hence, Hoxa5 in addition to other factors may contribute to the specification of noradrenergic neurons within the CNS and PNS through regulation of the *NET* gene (Kim et al., 2002).

2.10 Mechanisms of Auto, Cross, and ParaRegulation of *Hox* Genes in the Developing Mouse Hindbrain and Spinal Cord

Many of the potential direct targets initially identified for Hox proteins in both *Drosophila* and vertebrates were found to be *Hox* genes themselves. Many auto and crossregulatory loops involved in controlling *Hox* gene expression of paralogs 1-4 in the developing hindbrain have been characterized. In particular, rhombomere restricted expression of *Hox* genes driven by neuronal enhancers have been well characterized for the anterior *Hox* genes.

2.10.1 *Hoxb1* Autoregulatory Enhancer (b1-ARE)

Experiments using transgenic animals initially indicated that both Hoxa1 and Hoxb1 function to specify r4 restricted expression of *Hoxb1* early in development (7.5-8.5 dpc.). However, *Hoxb1* is vital in the maintenance of this expression in later stages of development (Studer et al., 1996, 1998). A highly conserved neuronal enhancer (b1-ARE) was identified and found to consist of three Hox-Pbx bipartite consensus motifs (Knoepfler et al., 1996). This b1-ARE was determined to be responsive to *Hoxb1* expression by transgenic analyses (Pöpperl et al., 1992, 1995), indicating the presence of a positive autoregulatory feedback mechanism for regulation of *Hoxb1* expression in r4.

Transfection and *in vitro* binding experiments showed that although Hoxb1 is necessary to mediate b1-ARE activation, it alone is not sufficient for the initiation of transcription. Therefore, the activity of this neuronal enhancer is dependent on the presence of either cofactors of Hoxb1, or crossregulation by other Hox proteins.

The DNA-binding selectivity of Hoxb1 for the r4 enhancer is determined by cooperative recognition of the b1-ARE by heterotrimeric complexes consisting of Pbx and Meis/Prep proteins (Pöpperl et al., 1995, Di Rocco et al., 1997; Berthelsen et al., 1998a. b; Jacobs et al., 1999; Shanmugam et al., 1999; Fognani et al., 2002). Both Prep1 and Prep2 have been found to directly interact with the HOXB1-Pbx complex through association with Pbx, and did not require DNA binding by the Meis-related factors or direct interaction between the Hox and Meis protein (Berthelsen et al., 1998a, b; Fognani et al., 2002). The effect of each Prep factor on the activity of HOXB1-Pbx1 is very different with regard to the b1-ARE. The presence of Prep1 within the ternary complex stimulates transcription, and HOXB1 is an essential factor for activation by this complex (Figure 2.4) (Berthelsen et al., 1998). In contrast, the presence of Prep2 has an inhibitory effect on b1-ARE directed expression by HOXB1-Pbx (Fognani et al., 2002). Whether this differential behavior of Prep proteins is due to distinct abilities in stabilizing Hox-Pbx complexes on DNA, or due to an absence of a transactivation domain is not yet clear (Fognani et al., 2002). It is therefore apparent that the diversity of *Hox* gene function can be enhanced through protein-protein interactions with other proteins in a DNA specific manner.

Meis1 has also been identified as a cofactor within timeric complexes involving Hoxb1, but in a DNA binding dependent mechanism (Jacobs et al., 1999). A distal Meis

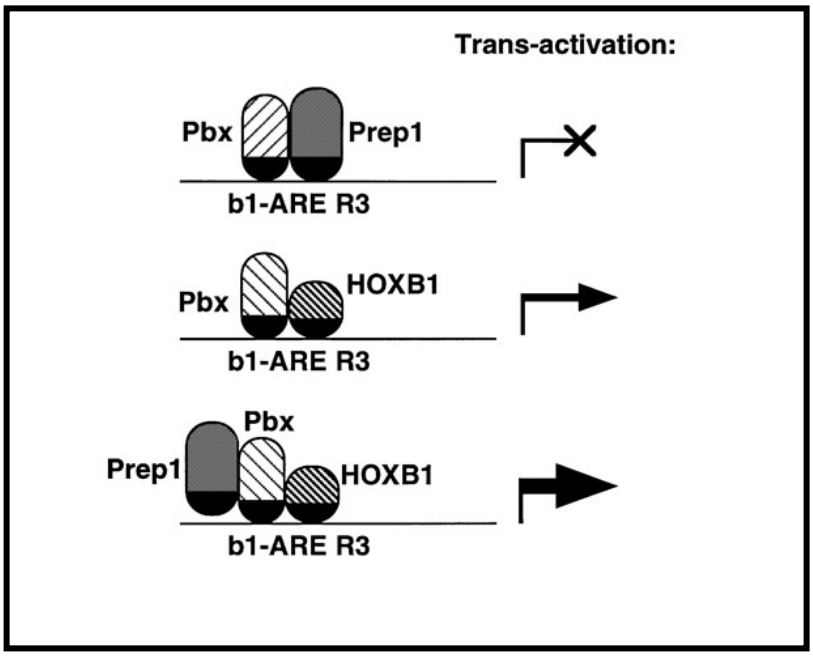


Figure 2.4 Hox, Pbx and Prep interaction on the b1-ARE

Complexes of Hox/Pbx and Meis proteins that are involved in autoregulation of *Hoxb1*, crossregulation of *Hoxb2*, and the regulatory mechanism involved in controlling the R3 Hox-Pbx site in the b1-ARE. Ternary complex formation and HOXB1-Pbx interaction resulted in the activation of *Hoxb1* transcription via the b1-ARE enhancer. This activity is dependent on the DNA binding activity of both Hox and Pbx proteins, but not for Meis/Prep. (Taken from Berthelsen et al., 1998a with permission: http://www.nature.com/)

site in relation to the Hox-Pbx recognition sequence present within the b1-ARE results in trimeric association of all three proteins (Figure 2.5). For enhanced transcription to occur DNA binding by all three proteins must occur since a mutation in the Meis binding site phenocopies the effects of mutations in the Pbx-Hox site on reporter gene expression within the hindbrain. The flexible amino terminal arms of the Meis and Pbx proteins interact, leaving their homeodomains relatively free to bind consensus sequences positioned in various configurations. This is vital in enabling the Hox-Pbx heterodimer to fulfill the stringent half site requirements for DNA binding and association, while simultaneously permitting trimeric complex formation with Meis (Jacobs et al., 1999).

In addition to autoregulation by Hoxb1, the b1-ARE is selectively activated and recognized *in vitro* only by members of Hox paralog groups 1 and 2 (*HOXA1, HOXB1, HOXB2*), reflecting their ability for differential recognition of enhancers through cooperative binding with modulatory proteins (Di Rocco et al., 1997). Cooperative binding of these Hox paralogs with Pbx1/PBX1 to an intact bipartite consensus sequence within the b1-ARE is required for stable *in vitro* binding by Hox/HOX proteins, as well as transactivation (Pöpperl et al., 1995; Di Rocco et al., 1997). This is also evident *in vivo* where *Hoxa1* transgenic mice displayed ectopic patterns of *Hoxb1* expression only in select regions of the hindbrain (r1/2), indicating that other components are required for permissiveness of the b1-ARE for pararegulatory control (Zhang et al., 1994). Genetic analyses suggest that Hoxa1 plays a role in activation of the r4 enhancer early on in development, although it is not exclusively required for initiation of *Hoxb1* expression nor is it involved in maintenance of this expression in later stages of development (Studer et al., 1998).

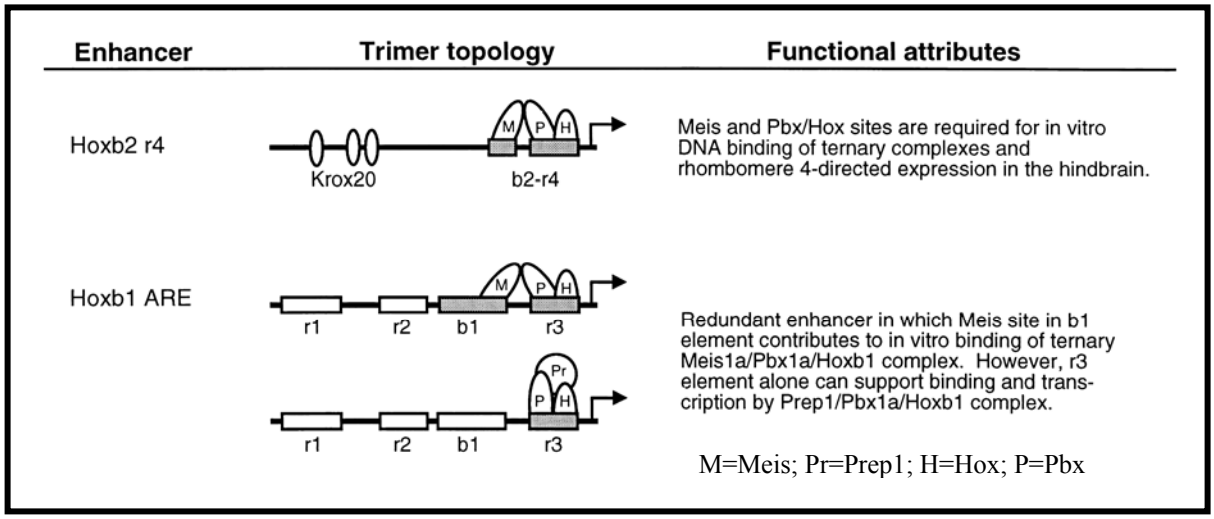


Figure 2.5 Trimeric interactions of homeobox proteins on hindbrain specific enhancers.

Schematic representation taken from Jacobs et al. (1999) with permission (http://www.journals.asm.org/), which depicts the association of Hox, Pbx and Meis trimeric complexes on various enhancers. Two contrasting proposals of trimeric complex formation: those in which the Meis/Prep protein is tethered to the DNA via protein-protein interactions, and the other involves DNA recognition by all components of the complex.

Further cell-specificity of the *Hox* gene function was observed when using mouse and human embryonic carcinoma (EC) cells in studying the transactivation potential of the b1-ARE (Di Rocco et al., 2001). These cells are originally derived from germ cell tumors, retaining many characteristics of the primitive neuroectodermal cells, such as the potential for neuronal differentiation and activation of all *Hox* clusters upon retinoic acid treatment (discussed in Di Rocco et al., 2001). Transactivation through the b1-ARE by both HOXA1 and HOXB1 proteins with PBX as a cofactor is in most cell types quite similar for both proteins. In EC cells a differential ability in transactivation between these HOX proteins was observed, with HOXA1-PBX1 activation being definitively less efficient with regard to the b1-ARE and highly dependent on the presence of a SOX/OCT bipartite sequence (Di Rocco et al., 2001). Therefore, the presence of cell specific/enriched factors that recognize this consensus must play a role in the stability/activity of the Hox-Pbx complex. Accordingly, an analysis of transgenic mice has demonstrated that recruitment of these Sox and Oct proteins to the b1-ARE are necessary for efficient transactivation by Hoxa1. Therefore, *in vivo* Hoxb1 is more readily capable of initiating and maintaining expression in the r4 via the b1-ARE relative to Hoxa1, and hence is absolutely necessary for maintaining r4 identity.

A less consistent finding is the effect of the interaction of Hox proteins with histone acetyltransferases (HATs) and histone deactelyases (HDACs) as coregulators of transcription. Both enzymes are chromatin modifiers, capable of enhancing repression or activation of transcription. HATs establish local chromatin structure that is permissive for events leading up to transcription by acetylation of the NH_2-terminal tails of histones within nucleosomes. Conversely, deactylation of these tails by HDACs

results in stabilization of chromatin higher order folding. This results in obstruction of access by the transcription machinery to their regulatory targets, resulting in repression of transcription (reviewed in Horn and Peterson, 2002).

Hox-Pbx complexes can act as repressors or activators of transcription by association with corepressors and coactivators, implying that cell signaling is a direct determinant of Hox-Pbx function in embryonic patterning during development (Saleh et al., 2000). Hoxb1-Pbx dimers have been shown to recruit HDACs to the b1-ARE resulting in repression of transcription. Cell signaling due to aggregation of cells or by the protein kinase A (PKA) pathway switches activity of this complex to activation of transcription (Saleh et al., 2000). Transcription can also be regulated by the cyclic AMP pathway, which has been shown to mediate these signaling effects via the PKA pathway and by a well-characterized HAT, CBP/p300. The activation domain of Hoxd4 recruits CBP/p300 to its Hox-Pbx complex resulting in a shift from repression to activation (Figure 2.6) (Saleh et al., 2000). This model is however disputed by others since representatives from all Hox paralog groups exhibited binding with CBP/p300, resulting in disruption of Hox-DNA binding (Shen et al., 2001). Despite the presence of Pbx, addition of CBP/p300 to the Hox protein did not result in transcription of a reporter gene. Also, a general mechanism of transcriptional repression is proposed whereby interactions of Hox proteins with CBP/p300 block the activity of these HATs, thus repressing transcription in a non-DNA dependent manner (Shen et al., 2001). The previous findings of Hoxd4-Pbx interactions by Saleh et al. (2000) could not be duplicated, even though similar cell-types and genetic constructs were used in experimentation. This is perhaps due in part to differences in orientation, number of, or

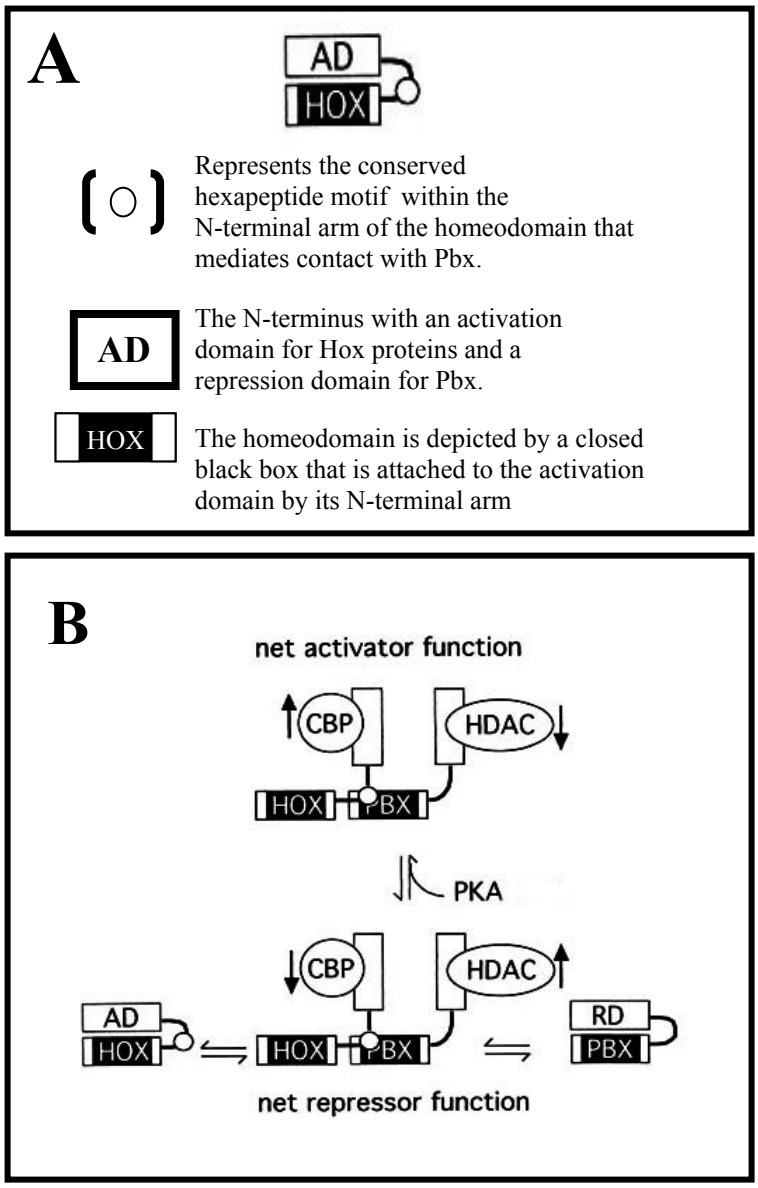


Figure 2.6 Model for activation and repression of transcription by Hox-Pbx

Figure A: A schematic diagram shows how intramolecular contacts between the AD (activation domain) and homeodomain block access of other regulatory factors to the Hox protein. Hox protein interacts with Pbx via the conserved hexapeptide motif disrupting intramolecular contacts to free the N-terminus domain to associate with other factors. Figure B: A model for activation and repression of transcription by Hox-Pbx complexes (taken from Saleh et al., 2000 with permission from American Society for Microbiology: http://www.journals.asm.org/). Corepressors (HDAC) and coactivators (CBP) are able to interact with the AD and/or RD (repressor domain) of the Hox-Pbx dimer. In response to PKA signaling, either an increase in coactivator function and/or decrease in corepressor activity occurs resulting in a shift from a net repression of transcription to a net ability to initiate activation.

distance between bipartite sequences used for the studies that resulted in varying effects on transcription in each study. Therefore, it is possible that in an enhancer and cell-specific context, both types of HDAC-Hox interactions are possible.

2.10.2 *Hoxb2* Neuronal Enhancer (b2-r4)

Expression analyses of transgenic mice for the labial class of Hox genes have identified an *in vivo* neuronal enhancer responsible for r4 expression of *Hoxb2* that was activated by Hoxa1 and Hoxb1 proteins, but was not autoregulated (Maconochie et al., 1997). Although the Meis binding site within the b1-ARE was not found to be essential *in vivo*, the Hoxb2 enhancer absolutely requires Meis binding in addition to the Hox-Pbx heterodimer for initiation of Hoxb2 r4 specific expression by a ternary complex (Figure 2.7) (Ferretti et al., 2000). This is perhaps due to the presence of only a single Pbx-Hox (PH) and Pbx-Meis (PM) element in the b2-r4. These elements have also been identified in several species (Scemama et al., 2002), where interference with either element resulted in the total abolishment of activity. The presence of a single PH and PM element is contrary to the multiple PM and PH motifs found in the b1-ARE. While the specific Meis family member involved in this regulatory complex has not been defined, both Prep1 and Meis1 were shown to interact with Pbx in the regulation of *Hoxb2* (Ferretti et al., 2000; Waskiewicz et al., 2001). The Meis family of proteins potentially has two critical functions during vertebrate hindbrain patterning; that of a DNA bound Hox partner, and as a Pbx stabilizing factor. Either Prep1 or Meis1 are candidates for Hoxa1 and Hoxb1 mediated regulation of *Hoxb2*, since both proteins are present in the hindbrain during critical developmental stages and their expression coincides with the *Hox* paralogs 1-4 during segmentation (Ferretti et al., 1999; Waskiewicz et al., 2001).

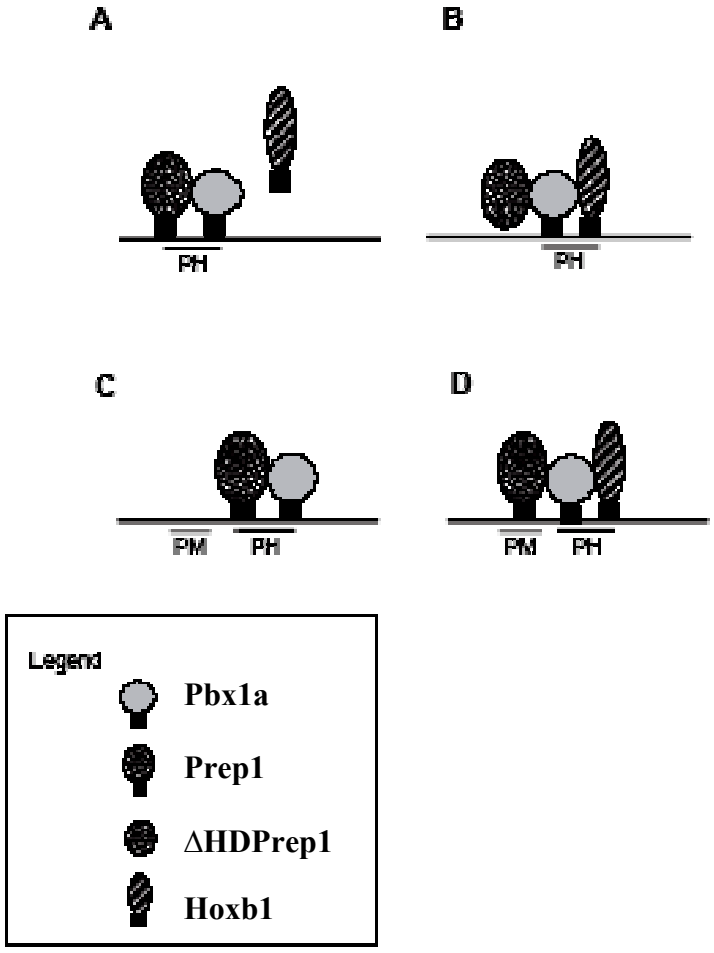


Figure 2.7 Schematic representation of homeodomain complexes on PH and PM sites.

In figures A and B a single PH (Pbx-Hox) site is bound by Prep-Pbx dimers, which may hinder ternary complex formation with Hoxb1. Removal of the HD (homeodomain) of Prep1 allows dimerization of Hoxb1 and Pbx (B). In figure C, a Prep-Pbx dimer will preferentially form on the PH site (A & C), however in the presence of an additional PM (Pbx-Meis) site it is possible for Hoxb1 to displace the Meis factor from the PH site (figure D). This results in ternary complex formation, where all three factors bind DNA (D). (Taken from Ferretti et al., 2000 with permission from The Company of Biologists Ltd., http://www.biologists.com/)

2.10.3 *Hoxb3* Element IIIa

Three major regulatory elements have been characterized that determine *Hoxb3* expression in the developing mouse hindbrain and spinal cord. Each element has a specific effect on the expression of *Hoxb3* within different domains of the neural tube (Kwan et al., 2001). The first element is the *Hoxb3* and *Hoxb4* intergenic late neuronal enhancer that is responsible for directing expression up to the r6/r7 boundary (Morrison et al., 1997; Gould et al., 1997, 1998). The second element, IIIa, involves auto/crossregulation of *Hoxb3* expression at the r5/r6 boundary (Kwan et al., 2001; Yau et al., 2002) and the third element (IVa) involves kreisler dependent regulation of *Hoxb3* expression in r5 (Manzaneres et al., 1997, 1999a, b).

In vivo, the Element IIIa was shown to direct expression of *Hoxb3* to the r5/r6 interface in the hindbrain and also the anterior spinal cord (Kwan et al., 2001). The activity of this element was dependent on the presence of a minimal enhancer consisting of two consensus *Hox* binding sites (Yau et al., 2002). These consensus sequences are recognized *in vitro* by Hoxb3 and Hoxb4, and mutation of the sequences indicated that they are required *in vivo* for *Hoxb3* expression within the neural tube (Yau et al., 2002). Interestingly, neural crest cells migrating from the same neural domain of *Hoxb3* expression were not dependent on these homeodomain binding sequences (HBS) and hence require separate elements.

2.10.4 *Hoxb3* and *Hoxb4* Late Neuronal Enhancer Element (Late NE)

Both *Hoxb3* and *Hoxb4* share overlapping expression patterns, with a common rhombomeric boundary at r6/7 (Wilkinson et al., 1989). In transgenic mice, this was shown to be controlled by an intronic neuronal enhancer present at the 3′ end of *Hoxb4*

(Gould et al., 1997). The conserved element within this enhancer (CR3) is composed of a Hox-Pbx recognition site that is bidirectional in nature. Additionally, no boundary or insulator elements have been identified within this enhancer; it is therefore not restricted in activity to only one *Hox* promoter. *In vitro* DNA binding studies and analysis of transgenic mice demonstrated a regulatory requirement for multiple Hox proteins (including Hoxb4), with proteins from Hox paralog groups 4-6 displaying activation of transcription by the CR3 (Gould et al., 1997). Analyses in mice using reporter gene assays have shown that the Late NE is able to impose an r6/7 boundary of expression for both *Hoxb4*, and also for *Hoxb3* where it acts coordinately with its promoter.

In transgenic mice, dissection of the regulatory regions that determine *Hoxb4* expression in the CNS indicated that the late NE was responsible for maintaining the r6/7 boundary of *Hoxb3* expression during segmentation, although its expression is initiated by a pre-rhombomeric enhancer (Early NE) (Gould et al., 1998). Therefore, the mechanism by which *Hoxb4* expression is regulated during the establishment and maintenance phases is initially by induction of the Early NE via paraxial mesoderm signals. Maintenance of *Hoxb4* expression at the r6/7 boundary is mediated by auto and crossregulatory Hox feedback loops.

Although an autoregulatory element for *Hoxd4* that is positively regulated by Hoxd4 in EC cells has also been identified (Pöpperl and Featherstone, 1992; Zhang et al., 1997a), *in vivo* this Hox responsive element did not appear to be essential for neuronal specific expression (Morrison et al., 1997). It is possible that this conserved autoregulatory enhancer might increase specificity or activity of the downstream mesodermal and neuronal enhancer; especially considering that this *Hoxd4* ARE is also

found in humans and *Xenopus*. Additionally, ectopic *Hoxd4* expression in *Xenopus* resulted in autoregulatory transactivation (Hoovield et al., 1999). Further studies are necessary to evaluate the precise role that autoregulation may play in the maintenance of *Hoxd4* expression in hindbrain development.

2.10.5 *Hoxa2* Autoregulation, r2 Specific 3′ Enhancer

In later stages of development the expression of *Hoxa2* within the rostral somites and in r2 of the hindbrain is directed by an enhancer sequence located at the 3′ end of *Hoxa2* (Frasch et al., 1995; Ren et al., 2002). Recently, a 10 bp Hox/Pbx binding element located within this enhancer was identified and is referred to as the E2-ARE (Lampe et al., 2004). Hoxa2/Pbx1a/Prep1 mediated activation of the E2-ARE *in vitro*, and this activity was shown to require DNA binding by Hoxa2. This enhancer sequence was also present in the zebrafish and human *Hoxa2* gene (Lampe et al., 2004).

2.10.6 *Hoxa3* Conserved r5/r6 5′ Enhancer

In r5-6, expression of *Hoxa3* and *Hoxb3* is initiated by the *kreisler* transcription factor but in later stages of development *Hoxa3* expression is maintained by conserved auto/crossregulatory mechanisms (Manzanares et al., 2001). The regulatory enhancer responsible for *Hoxa3* expression consisted of two Hox-Pbx binding motifs, HOX/PBC-A and HOX/PBC-B that were essential *in vivo* and *in vitro* for enhancer activity. Although these Hox-Pbx bipartite sites are similar to those identified in enhancers of *Hoxb1/b2* and *Hoxb4*, the reporter genes under the control of HOX/PBC-A demonstrated a distinctive r5-6 expression pattern unlike the r6/7 and r4 expression pattern observed for studies using the b1-ARE and b2-r4 bipartite sequences. Therefore, subtle differences in the bipartite sites can result in establishment of dramatically different

expression domains *in vivo* (Manzanares et al., 2001). Hence, minor alterations in the binding motifs of various Hox responsive enhancers *in vitro* may represent a striking difference in selectivity/ability of these motifs to recruit cofactors to the transcription complex. These protein-protein interactions are then ultimately responsible for determining restriction of target expression by *Hox* genes in a cell- and tissue-specific manner.

Analogous to the Hox responsive elements involved in mediating *Hoxb1* and *Hoxb2* expression, a proximal Prep/Meis binding site is present relative to the HOX/PBC-B site of the *Hoxa3* enhancer. *In vivo* and *in vitro* studies have shown that of *Hoxa3* regulation by trimeric complexes of Hox-Pbx-Meis/Prep proteins occurs through recognition of their binding sites found within the r5-6 enhancer. All Hox paralogs in group 3, in conjunction with Pbx1, are capable of recognizing this enhancer *in vitro*. In contrast to the Hoxb2 r4 enhancer, both the b1-ARE and the *Hoxa3* element did not essentially require Meis/Prep binding for transactivation *in vivo*. This is most likely a direct consequence of the presence of several HBS within the b1-ARE and *Hoxa3* r5/r6 enhancer, compared to the single Hox binding motif found in the *Hoxb2* r4 enhancer (Ferretti et al., 2000; Manzanares et al., 2001).

2.10.7 *Hoxa4* Autoregulatory Region

Potential *Hox* binding sites are present within both the promoter and intron of *Hoxa4* (Behringer et al., 1993; Wu and Wolgemuth, 1993), with both regulatory regions exhibiting *in vitro* binding by Hoxa4 and the *Drosophila* transcription factor fishu tarazu (ftz) (Wu and Wolgemuth, 1993). Although ftz mediated transactivation of reporter genes driven by the *Hoxa4* consensus sequences from these regulatory regions, Hoxa4

itself was unable to activate transcription. However, *in vivo* binding of Hoxa4 to the consensus sites did occur and resulted in disruption of ftz-potentiated transcription. This inhibitory effect on ftz activity by Hoxa4 is dependent on DNA binding since it requires the presence of an intact homeodomain (Wu and Wolgemuth, 1993). In the spinal ganglion and neural tube, the activity of this regulatory region was severly decreased in *Hoxa4* null mutants. Taken together, the results of both *in vitro* and *in vivo* studies indicate that Hoxa4 is unable to initiate its own expression (Wu and Wolgemuth, 1993). However, studies on the induction of *Hoxa4* expression by RA indicated that an autoregulatory loop is required for maintenance of this expression in some areas of the CNS (Packer et al., 1998). It is therefore possible that Hoxa4 potentially plays a role in maintenance but not initiation of its expression, although further studies using transgenic mice are necessary to determine the mechanism underlying the activity of these elements.

Other instances of potential cross/para/autoregulation have been observed (Friedman-Einat et al., 1996; Vigano et al., 1998), but not in the context of their role *in vivo* during development. Nevertheless, these studies do contribute to information concerning the functional cooperativity of Hox proteins with other factors, and their importance in the transcription activity of a particular complex. Domain-swapping experiments have demonstrated the necessity of performing experiments in a biologically relevant system. Specifically, these proteins should be studied in the appropriate context; in their native conformation on a natural homeodomain target and in the presence of appropriate cofactors. Otherwise it is highly likely that the activity of a

Hox protein will be misinterpreted due to the highly specialized manner in which they appear to function.

2.11 Conclusion

Many common characteristics are observed for the *Hox* genes, such as their conserved structure, organization and the presence of spatial and temporal restricted patterns of expression during embryogenesis. In addition, interaction of Hox proteins with their cofactors, such as Pbx and Meis, is conserved in several species. Little information exists regarding the interactions of Hox proteins with their cofactors on downstream targets. However, their coordinated actions in various auto and crossregulatory mechanisms have been well described. It is therefore likely that various cofactors play an important role in contributing to the actions of Hox proteins in the regulation of specific molecular pathways.

Hox genes are expressed in a variety of embryonic tissues and are known to function in developmental processes such as limb morphogenesis and skeletogenesis. In the early neural tube, *Hox* expression is highly restricted, particularly within the rhombomeric boundaries of the hindbrain. The role of *Hox* genes as critical morphogenetic regulators in the development of the vertebrate hindbrain is well documented. Analyses using *Hox* mutants have determined that *Hox* genes have important roles in the specification of neuronal networks within the developing spinal cord and hindbrain, but this has not clearly defined their role in determining rhombomeric identity.

Deciphering the precise genetic pathways of individual *Hox* genes by disrupting their function *in vivo* has not been entirely successful due to functional redundancy.

Understanding the precise regulatory mechanisms through which *Hox* genes achieve morphological control during development will require the identification of their downstream target genes. *Hox* genes are believed to be required for segmentation of the hindbrain and specification of rhombomeric identity; hence they may regulate genes involved in processes such as cell migration and differentiation. Therefore, their involvement in directing expression of various cell adhesion molecules and Ephrin receptors is not surprising, implicating *Hox* genes in the formation of rhombomeric boundaries within the hindbrain. Characterization of *Hox* targets, such as signaling molecules and transcription factors, may facilitate identification of the signal transduction pathways through which *Hox* genes act as effectors in the specification and differentiation of neuronal cell populations.

3. MATERIALS AND METHODS

3.1 Protein Purification

The full-length *Hoxa2* cDNA sequence (Nazarali et al., 1992; Tan et al., 1992) was previously subcloned into the pFLAG-2 vector (Sigma/Kodak) at the *EcoR* I and *Xho* I sites downstream of the FLAG epitope coding region. *E.coli* strain XL1-Blue supercompetent (Stratagene) was used for protein expression (Hao et al., 1999). Expression was induced in cultures with isopropyl-β-thiogalactoside (IPTG) as per Kumar and Nazarali (2001). FLAG-Hoxa2 recombinant protein was extracted as inclusion bodies from bacteria using the method of Hoey (1990) and described in Kumar and Nazarali (2001). In brief, the protein from inclusion bodies was extracted by dissolution in equal volumes of HEMGN buffer containing [100 mM KCl, 25 mM HEPES (pH 7.6), 0.1 mM EDTA (pH 8.0), 12.5 mM $MgCl_2$, 10% glycerol, 0.1% v/v Nonidet P-40, 0.1 mM PMSF, 0.1 mM sodium metabisulfite] and 8 M guanidine-HCl for 30 min at 4^{o}C. After centrifugation at 87,000 g for 30 min (4^{o}C), the supernatant was dialyzed against HEMGN in decreasing concentrations of guanidine-HCl (1 X in 1 M guanidine-HCl, 1 X in 0.1 M guanidine-HCl, and 2 X in HEMGN alone). Insoluble material was removed by centrifugation and the supernatant was utilized for subsequent purification by affinity chromatography with columns containing anti-FLAG M2 antibody affinity gel (Sigma). Purified recombinant Hoxa2 protein was eluted with 0.1 M glycine (pH 3.5) and then neutralized with 1 M Tris (pH 8.0).

Recombinant Hoxa2 protein was also isolated as soluble protein with B-Per™ reagent (Pierce). Briefly, 40 ml of bacterial culture (OD_{600} of 1.5-3.0) was harvested

and pelleted by centrifugation. Pellets were resuspended in 5 ml of B-Per™ reagent and incubated for 20 min at room temperature. The supernatant containing soluble proteins was isolated by centrifugation at 27,000 g for 15 min. Protein samples were dialyzed against PBS with 0.1 mM PMSF. Recombinant Hoxa2 protein was partially purified by salting out the protein from the soluble protein extract with 30% ammonium sulphate. The protein was then dialyzed against PBS. Protein samples were concentrated by centrifugation through Centricon-30 filters (Millipore-Amicon). All protein samples were analyzed on 10-12% SDS-PAGE gels for purity and identified by Western Blot analysis.

3.2 Antibody Purification

A 17 amino acid oligopeptide (J3 peptide) from the coding sequence of the Hoxa2 protein was previously used to generate Hoxa2 peptide specific polyclonal antiserum (B579) in rabbits (Hao et al., 1999). HiTrap protein A columns (Amersham Pharmacia Biotech) were used to purify IgG antibodies from the Hoxa2 antisera. B579 antiserum was diluted (1:2) in 20 mM NaH_2PO_4 (pH 7.0) and applied to the column. The IgG antibodies were eluted with 0.1 M Citric Acid (pH 4.0) and then neutralized with 1 M Tris (pH 8.0). The eluant was dialyzed against 0.1 M HEPES (pH 8.0) and quantified using the DC Protein Assay (BioRad).

Antibodies specific for Hoxa2 were purified from B579 antiserum using affinity chromatography. Both purified and partially pure recombinant Hoxa2 protein (as described above) were conjugated to AffiGel 10 affinity support (BioRad) in coupling buffer [PBS containing 80 mM $CaCl_2$] for 4 hr at 4^oC. The remaining active ester sites were blocked with 0.1 M Tris (pH 8.0) and the matrix was used to prepare an affinity

column. The B579 antiserum was heat inactivated at 56°C for 30 min and then diluted 1:2 in wash buffer [50 mM Tris (pH 7.5)]. The column was washed with alternating solutions of wash buffer alone or wash buffer containing 0.5 M NaCl. Hoxa2 specific antibodies were eluted with 0.1 M glycine and neutralized with 0.1 M triethylamine (pH 11.5). Antibodies were dialyzed against 0.1 M HEPES (pH 8.0). Eluants were tested by electrophoresis on 12% SDS-PAGE gels and subsequent western blot analysis.

3.3 Western Blot Analysis

Both 10 and 12% SDS-PAGE gels were used to separate proteins according to the manufacturer's protocol (Invitrogen). The protein was transferred and immobilized onto a PVDF membrane (NEN Dupont) by using the Xcell II Blot Module (Invitrogen) for wet transfer in 1 X transfer buffer containing 12 mM Tris, 96 mM glycine and 20% methanol, for 2 hr at 30 V/200 mAmp. Blocking of the membrane was performed in 3% skim milk (PBS) at 4°C for 16 hr. For detection of the Hoxa2 protein, the membrane was incubated for 2 hr at room temperature with the rabbit anti-Hoxa2 antiserum (B579) at a dilution of 1:5000 in 3% skim milk/PBS. The membrane was washed 3 X 20 minutes with PBS/0.8% Tween-20 and incubated for 1 hr at room temperature with the horse radish peroxidase (HRP) conjugated secondary goat anti-rabbit antibody, diluted 1:3000 in 3% skim milk (PBS). The membrane was then washed for 2 hr, 6 X in PBS/0.8% Tween-20. For detection of FLAG-Hoxa2 recombinant protein a 1:3000 dilution of mouse anti-FLAG M2 primary antibody (Eastman Kodak, New Haven, CT) was used, followed by 3 X 10 min washes. A 1:1500 dilution of HRP conjugated goat anti-mouse IgG was used as the secondary antibody and the membrane was washed 3 X for 20 min in PBS/0.08% Tween-20. Visualization of proteins was performed by a

chemiluminescence reaction (PerkinElmer Life Sciences, Inc.) as per manufacturer's instructions, followed by exposure of the membrane to Kodak X-OMAT Blue XB-1 film for 5 sec to1 min.

3.4 Chromatin Preparation and Immunoprecipitation

All mice used in experiments were obtained from the Animal Resource Center, Saskatoon, SK and embryos dissected as described in section C of *Manipulating the Mouse Embryo* (2^{nd} Ed.). Day twelve (E12) and day eighteen (E18) gestational embryos were removed from CD-1 mice and staged as per Kaufman (1992) and Theiler (1989). The spinal cord and surrounding tissue was removed from the embryos and fixed in 4% paraformaldehyde. Nuclear extracts were prepared from the tissues according to the method of Rosenberg (1996). In summary, tissues were washed and resuspended in hypotonic buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF, and 0.5 μg/ml each of leupeptin, pepstatin A, antipain, aprotinin]. The sample was homogenized and pelleted to collect the nuclear pellet. Nuclei were sonicated in binding buffer [10 mM NaH_2PO_4 (pH 7.0), 10 mM KCl, 2 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM PMSF, 15 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml antipain, and 2 μg/ml pepstatin A) and centrifuged at 8,000 g for 10 min to collect the supernatant. Chromatin preparations were digested with 1500 U of *Hae* III for 1 hr at 37°C. Hoxa2 protein-DNA complexes were isolated from the digested chromatin by affinity chromatography using an AffiGel10 column conjugated with IgG purified antibodies from anti-Hoxa2 rabbit polyclonal antiserum (described above). The column was washed three times with high salt buffer [0.1 mM NaH_2PO_4 (pH 7.5), 1 M NaCl] followed by low salt buffer [0.1 mM NaH_2PO_4 (pH 7.0)]. Target sequences were eluted

with 3 M NaSCN and dialyzed against 10 mM $NaPO_4$ (pH 7.0). Protein was removed from the target sequences by digestion with 0.1 mg/ml proteinase K, 50 mM Tris (pH 8.0), 100 mM EDTA and 0.3% SDS for 8 hrs at 37^{o}C. DNA was extracted by phenol-chloroform, precipitated, and purified using NAP-5 columns (Amersham Pharmacia Biotech). Klenow enzyme was used to blunt end the DNA before ligation into the *Sma* I site of pBluescript SKII^{+} (Stratagene).

Target DNA sequences of Hoxa2 were also isolated from E18 hindbrain and spinal cord chromatin preparations using a modified immunoprecipitation method previously utilized in isolating *in vivo* target genes for Hoxc8 and Hoxb5 (Tomotsune et al., 1993; Safaei, 1997). E18 hindbrains, spinal cords and surrounding tissues were isolated as previously described. Chromatin was prepared from the tissues by homogenization in binding buffer and sonication, as described by Safaei (1997). The chromatin containing supernatant was digested with 10^4 U of *Hae* III for 2 hr at 37^{o}C, followed by *DNase* I (10 U) digestion at 4^{o}C for 5 min and the reactions were stopped with 2.5 mM EGTA. Hoxa2-DNA complexes were immunoprecipitated from E18 mouse hindbrain and spinal cord chromatin preparations by affinity chromatography using purified anti-Hoxa2 antibodies conjugated to AffiGel10 matrix (Pharmacia Biotech). DNA was eluted from the column as previously described and treated with 0.1 mg/ml proteinase K in 10 mM Tris, 5 mM EDTA and 0.5% SDS for 16 hr at 37^{o}C. Samples were extracted with phenol-chloroform followed by passage through NAP-5 columns. The resulting DNA was cleaved with *Sma I* prior to subcloning into pBluescript SKII^{+}. DH5α *E.coli* cells were used for transformation with plasmids and subsequent DNA isolation. Target sequences were sequenced by dideoxy-chain

termination method (Sanger et al., 1977) using Sequenase-Version 2.0 (U.S. Biochemical Corp.) and also at the DNA Technologies Unit, NRC-Plant Biotechnology Institute, Saskatoon, SK.

3.5 *In vitro* translation of Hoxa2 transcript

The TNT Coupled Wheat Germ Extract System (Promega) was used to produce *in vitro* Hoxa2 protein from the *Hoxa2* cDNA using the T3 promoter of the pBluescript $SKII^{+}$-*Hoxa2* plasmid (Hao et al., 1999) as per the manufacturer's instructions. In short, Hoxa2 was produced from 1 μg of circular plasmid in 25 μl of wheat germ extract with 1 μl of T3 polymerase incubated for 60 min at 30°C.

3.6 Electromobility Shift Assays (EMSAs)

Nuclear extracts (NE) were prepared from whole E12 mouse tissue, and also from the spinal cord and hindbrain tissue of E18 and P1 mice. For extraction of nuclear protein from E12 and E18 mice, the method described in Thompson et al. (1998) was used. Briefly, embryos were homogenized in PBS, pelleted and resuspended in cell lysis buffer [10 mM HEPES (pH 7.0), 3 mM $MgCl_2$, 40 mM KCl, 0.5 mM PMSF, 1 mM DTT, 5% glycerol, 1% protease inhibitor cocktail (Sigma), and 0.2% Nonidet P-40]. Cells were lysed for 10 min at 4°C and the nuclei pelleted by centrifugation in microcentrifuge at 5,000 rpm for 5 min. Nuclei were resuspended in extraction buffer [20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 0.42 KCl, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 25% glycerol] and incubated for 1 hr at 4°C on an orbitron shaker. Supernatant was collected after centrifugation and dialyzed against a solution of 20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 0.42 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, and 20% glycerol.

For extraction of NE from hindbrain and spinal cord tissue of P1 mice a method provided by Alan Bateson (personal communication) was used for extraction of nuclei. In short, tissues from *Hoxa2*$^{-/-}$ and wildtype C57BL/6J mice (obtained from Gendron-Maguire et al., 1993) were homogenized in 0.25 mL of ice-cold sucrose buffer [0.25 M sucrose, 15 mM Tris-HCl (pH 7.9), 60 mM KCl, 15 mM NaCl, 5 mM EDTA (pH 8.0), 1 mM DTT, 0.5 mM PMSF, and 1% protease inhibitor cocktail (Sigma)]. The volume of the homogenate was increased to 0.75 mL with sucrose buffer and centrifuged at 2,000 g for 10 min at 4^{o}C. Pellets were resuspended in 0.4 mL of wash buffer [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF, and 1% of protease inhibitor cocktail], pelleted at 4,000 g for 10 min (4^{o}C) and then incubated for 1 hr on ice in 20 mM HEPES (pH 7.9), 0.75 mM $MgCl_2$, 10 mM EDTA (pH 8.0), 0.5 mM KCl, 12.5% glycerol, 1 mM DTT, 0.5 mM PMSF, and 1% protease inhibitor cocktail. The supernatant was collected after centrifugation at 14,000 g for 30 min at 4^{o}C. All nuclear extracts were aliquoted and stored at –70^{o}C.

DNA probes for all of the Hoxa2 target sequences were produced by digestion of pBluescriptSKII^{+} containing target sequences with *Xho* I and *Xba* I and labeling with [α^{32}P]dATP using Klenow enzyme. Binding reactions were performed in 30 µl of binding buffer [10 mM HEPES (pH 7.9), 1 mM $MgCl_2$, 60 mM KCl, 0.5 mM EDTA, 1 mM DTT, 10% glycerol] with 2 µg of polydI-dC, 2 µg nuclear extract (unless indicated otherwise) and 50,000 cpm of probe at room temperature for 20 min. Reactions for supershift assays were performed as described above, with the addition of B579 antiserum. Control reactions were performed using an equal amount of non-immune rabbit antiserum. Nuclear extracts were incubated for 20 min (4^{o}C) with antisera prior to

addition of probe. All competition reactions involved the addition of 100 X concentration of unlabelled target sequence. Samples were electrophoresed on 6% to 8% TBE and Tris-Glycine gels, dried for 2 hr and exposed to Kodak X-OMAT film at –70^{o}C.

3.7 *DNase* I Footprinting

A modified protocol of Spiro and McMurray (1999) was utilized for thermal cycle sequencing of target sequences for footprinting assays. Forward and Reverse primers for pBluescript SKII^{+} were labeled with γ^{32}P using T4 kinase in sequencing reactions. Template DNA was sequenced using universal primer (5′ GTAAAACGACGGCCAGT 3′) and reverse primer (5′ GGAAACAGCTATGACCATG 3′), 2 U Taq DNA polymerase (Invitrogen) and dNTP/ddNTP mix (reaction mix **A**: 450 μM ddATP, 15 μM dATP, 50 μM each dCTP, dGTP,dTTP; reaction mix **C**: 125 μM ddCTP, 15 μM dATP, 18 μM dCTP, 50 μM each dGTP, dTTP; reaction mix **G**: 125 μM ddGTP, 15 μM dATP, 18 μM dGTP, 50 μM each dCTP, dGTP; reaction mix **T**: 300 μM ddTTP, 15 μM dATP, 18 μM dTTP, 50 μM each dCTP, dGTP). Reactions were incubated for 2 min at 95^{o}C followed by 20 cycles of 95^{o}C for 30 sec, 58^{o}C for 15 sec, and 72^{o}C for 40 sec. The procedure of Leblanc and Moss (2000) was used for *DNase* I footprinting of the target sequences. Varying amounts of NE taken from E12, E18 and P1 mice were used in the footprinting reactions. Protein was incubated with T4 kinase labeled γ^{32}P DNA probes (5 X 10^{4} cpm) in 50 μl binding buffer [20% glycerol, 0.2 mM EDTA, 1 mM DTT, 20 mM HEPES (pH 7.9), 60 mM KCl] with 2 μg of polydI-dC overnight at 4^{o}C or at room temperature for 2 hr. Samples were treated in the presence of 5 mM $MgCl_2$ and 0.25 mM $CaCl_2$ for 2 min

with 5 X 10^{-4} to 5 X 10^{-3} U of *DNase* I for control reactions and 2 X 10^{-3} to 8 X 10^{-2} U for DNA-protein reactions. DNA fragments were ethanol precipitated for several hours at –20^{o}C, and DNA pellets were dissolved in formamide loading buffer (90% v/v deionized formamide, 0.025% w/v xylene cyanol, 0.025% w/v bromophenol blue). Reactions were visualized by electrophoresis on 6% denaturing sequencing gels and exposed to Kodak X-OMAT film at –70^{o}C.

3.8 Southwestern Blot Analysis

A method outlined by Papavassiliou (2001), which entails performing a southwestern blot followed by nuclease footprinting of the sequences while bound to the membrane was used. In brief, approximately 20 μg of NE (per lane) from E18 mice was separated by electrophoresis on 12% SDS-PAGE gels. Proteins were transferred and immobilized onto PVDF membranes (NEN Dupont). The membrane was washed with SW renaturation buffer [25 mM HEPES (pH 7.5), 50 mM KCl, 6.25 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 0.1% Nonidet P-40, and 0.2 mM PMSF]. Blocking of the membrane was performed overnight in SW renaturation buffer containing 3% milk or 5% BSA. Blots were probed for 4 hr with γ^{32}P ATP labeled DNA at either the 5′ end on the sense or antisense strand in SW binding/wash buffer [12.5 mM HEPES (pH 7.5), 50 mM KCl, 6.25 mM $MgCl_2$, 0.5 mM DTT, 2% PVP, 10% glycerol, 0.05% Nonidet P-40, and 0.2 mM PMSF]. Excess probe was removed by multiple washes with SW wash buffer, and the blots were subsequently exposed to Kodak X-OMAT film for 3 hr.

3.9 Tail Genomic DNA Isolation and Mouse Genotyping

Tail tissue was excised from both embryonic (CD-1) and P1 (C57BL/6J) mice, and genomic DNA was extracted using a simplified protocol of Laird (1991). Briefly,

tails were digested in lysis buffer [10 mM Tris-HCl (pH 8.5), 0.05 M EDTA, 1%SDS, 0.5 M NaCl and 2 mg/ml Proteinase K] for 3 to 5 hr at 55^{o}C. Genomic DNA was precipitated and quantified using UV spectrophotometry, and approximately 1 μg of DNA used for subsequent PCR analysis. For identification of wildtype and *Hoxa2*$^{+/-}$ mice, PCR amplification of a 330 bp fragment of the *Hoxa2* gene was performed using primers wt-5 (5′ GTTGGAACTGACCTCCTCTTG 3′) and wt-6 (5′ GGGTCCGAGCAGGGTTATTCC 3′) (Gendron-Maguire et al., 1993). For identification of homozygous knockout *Hoxa2* mice, PCR was performed using DNA primers KO-1 (5′ GTTGGTGTACGCGGTTCTCAG 3′) and NEO-1 (5′ TCGCCTTCTATCGCCTTCTTG 3′) resulting in a 380 bp fragment amplification of the *Neomycin* sequence (Gendron-Maguire et al., 1993). PCR conditions were as follows: 94°C for 3 min followed by 30 cycles at 94°C for 45 sec, 59°C for 30 sec and 72°C for 1 min. Samples were incubated for 10 min at 72°C for elongation and terminated at 4^{o}C. DNA fragments were visualized by electrophoresis on a 1% agarose gel, and *Hoxa2*$^{-/-}$ mice were identified by the presence of a 380 bp band and the absence of a 330 bp fragment.

3.10 RNA Isolation and RT-PCR Analyses

Embryos at stages E10, 12, 14, 16, and 18 were removed and homogenized in TRI Reagent (Sigma) for RNA extraction according to the manufacturer's instructions. Also, hindbrain and spinal cord tissues were removed from P1 *Hoxa2*$^{+/+}$, *Hoxa2*$^{+/-}$, and *Hoxa2*$^{-/-}$ mice (C57BL/6J) for RNA extraction. Mouse genotyping was performed by PCR analysis as described above. RNA was stored in ethanol at –70^{o}C, and dissolved in water immediately before use. Quantification of RNA was performed by UV

spectrophotometry at 260 nm. DNA was digested in 1 μg of RNA sample with 1 U *DNase* I (Sigma) at room temperature for 15 min. The digestion was terminated by the addition of stop buffer (2.5 mM EDTA) and heating at 70°C for 10 min. Reverse transcription reactions were performed in a volume of 20 μl using 1 μg of *DNase* I treated RNA with 200 U of Superscript II *RNase H*⁻ Reverse Transcriptase (Invitrogen) and 2.5 μM of random nonamers (Sigma). Subsequent cDNA amplification was performed using 2 μl of RT product and 2 U Taq DNA polymerase (Invitrogen), unless otherwise specified. PCR conditions and primer sequences used for individual genes are described below.

In all RT-PCR analyses amplification of the mouse *β actin* gene, using primers 5′ GGCATCGGATGGACTCCG 3′ and 5′ GCTGGAAGGTGGACAGCGA 3′ (Remacle et al., 2002) for amplification of a 612 bp fragment, was utilized as an internal control. For multiplex PCR amplification of the RT reactions from the P1 *Hoxa2* wildtype and mutant hindbrain and spinal cord tissue, *β actin* primers were used at a concentration of 0.1 μM while the gene specific primer concentration was 0.5 μM.

Tasp fragment amplification from RT reactions was performed using touchdown PCR as follows: 10 cycles of 95°C for 45 sec, 68°C for 1 min, and 76°C for 1 min, followed by 20 cycles of 94°C for 45 sec, 62°C for 1 min, and 76°C for 1 min, and a final extension step at 78°C for 10 min. PCR amplification of the *Tasp* fragment from the RT reactions was quantitated through imaging by a gel doc system (BioRad), followed by analysis using the pixel density function of the Quantity 1 program (BioRad).

Dyrk4 was amplified by RT-PCR using 2 μl of RT samples with 0.2 μM *β actin* primers and 0.5 μM *Dryk4* primers for embryonic RNA samples. Amplification of

Dyrk4 for RT reactions on adult and P1 RNA extract was performed as described above using 4 μl of RT reactions. RNA samples were tested for contaminating DNA by PCR amplification of *DNase* I treated RNA. PCR samples were incubated as follows: 95°C for 5 min and 30 cycles of 95°C for 45 sec, 62°C for 1 min, 72°C for 45 sec and an elongation step at 72°C for 10 min.

3.11 Amplification of *Tasp* and *Dyrk4* Probes

All RT reactions used for the production of probes utilized RNA isolated from E18 embryos and P1 mice. A *Tasp* DNA probe was produced by PCR amplification of RT product using primers 5′ CCAACCCAGACTTTCCAGCG 3′ and 5′ AGCTGAAATTAAGGGTCA 3′ corresponding to position 1361 and 1770 of the 9530081K03RIKEN clone sequence (NCBI Accession# AK035194). PCR conditions consisted of an initial denaturation step at 95°C for 5 min, followed by 30 cycles of 95°C for 45 sec, 60°C for 1 min, 72°C for 45 sec and an elongation step at 72°C for 10 min. PCR products were purified by micron filters (Millipore) and subcloned into pBluescript SKII$^+$ at the *Xho* I and *Xba* I sites. Automated DNA sequence analysis at the Saskatoon Cancer Research Unit, University of Saskatchewan verified the nucleotide sequence.

PCR amplification of *Dyrk4* from 1 μg mouse tail genomic DNA with primers 5′ GGGCTCGAGAGATGAGGCTACCAA 3′ and 5′ GGGTCTAGACACGATGGGTGG TAAGATG 3′ was performed as described above, with the exception of the annealing step which was performed at 62°C for 1 min. The resulting 229 bp product was subcloned into pBluescript SKII$^+$ at the *Xho* I and *Xba* I sites. The insert was verified as corresponding to positions 1738-1967 within the mouse *Dyrk4* cDNA sequence

(Ensemble–EMI/Sanger Institute, Transcript ID: ENSMUST00000032495) by DNA sequencing.

3.12 Southern Hybridization

RT-PCR products were visualized by electrophoresis on 1% agarose gels, followed by capillary transfer using GeneScreen Plus (NEN Dupont) as described by the manufacturer. DNA probes were produced by digestion with *Xho* I and *Xba* I, followed by termini fill-in with the Large Fragment of *DNA Polymerase I* (Klenow Fragment) (Invitrogen). DNA fragments were labeled with 20 μCi of [α^{32}P]dCTP and 0.5 U of Klenow fragment for 30 min at 4^{o}C, and the reactions were terminated by phenol extraction. Membranes were hybridized with 2.5 x 10^6 cpm of α^{32}P Klenow labeled DNA probes in QuickHyb solution (Stratagene) as per the manufacturer's instructions. The membrane was washed 1 X in 2 X SSC/0.1% SDS at room temperature and 3 X in 0.1 X SSC/0.1% SDS at 65^{o}C. Blots were exposed to Kodak X-OMAT AR film overnight at -70^{o}C.

3.13 Cell Culture, Transient Transfection and Luciferase Assays

HeLa cells are a human cervical carcinomal cell line that is commonly used in transcient transfections. The HeLa cells were maintained in Glutamax Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (Invitrogen) in a 37^{o}C/5% CO_2 atmosphere. Cells were transfected at approximately 50-70% confluency using the calcium phosphate method of *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES)-buffered saline, as previously described by Greaves and O'Hare (1989).

Potential Hoxa2 target sequences, corresponding to the intronic regions of *Tasp* and *Dyrk4* previously isolated by immunoprecipitation were digested from pBluescript SKII^{+} at the *Bam*H I and *Sal* I sites and ligated into the pGL3-Promoter vector (Promega) downstream of the luciferase coding region. The resulting reporter plasmid, as well as the pGFP, pcDNA3, pRSV-Hoxa2, and pCMV-RL vectors were used to transform DH5α *E.coli* cells. All DNA for use in transfection was isolated using the Endofree Plasmid Maxi Kit (Qiagen). DNA concentrations were quantified by three methods: spectrophotometry at 260 nm, by ethidium bromide spot testing and agarose electrophoresis. Efficiency of transfection was assessed by cotransfection with 0.1 μg of pGFP.

DNA concentrations of all transfections were supplemented to a total of 5 μg with pcDNA3 vector, and performed in 6-well plates. Cells were incubated for 48 hr and then washed with PBS prior to harvesting for luciferase assays as per manufacturer's instructions (Promega). In brief, cells were incubated with 500 μl of Passive Lysis Buffer (Promega) for 15 min at room temperature, and then subjected to 3 rapid freeze thaw cycles. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega), where 20 μl of the cell lysate was added to 100 μl of Luciferase assay reagent AR II and luminescence was measured for the firefly luciferase. Activity of the *Renilla* luciferase reporter was subsequently measured by the addition of 100 μl of Stop and Glo reagent.

3.14 Transient Transfection of *Hoxa2* in Cell Culture and Expression Analysis of *Dryk4* and *Tasp*

HeLa cells were transfected in 100 mm plates with 0, 0.05, 0.10, 0.25, 0.50, 1, and 2 μg of pRSV-Hoxa2 supplemented with pcDNA3 to a total of 6 μg of DNA/plate using 50 μl of Polyfect Reagent (Qiagen). Cells were washed 1 X with PBS and transfection efficiency was assessed by cotransfection of cells with a Green Fluorescent Protein (GFP) encoding plasmid and visualized by fluorescence microscopy. Harvesting of cells for RNA and protein extraction was performed using 1 ml of TRI reagent (Sigma) as per the manufacturer's instructions. RNA was stored as a pellet in 75% *RNase* free ethanol at –20^{o}C. Subsequent to use in RT-PCR analysis, RNA was dissolved in water and quantified as previously described.

Analysis of *Dyrk4* and *Tasp* expression in HeLa cells was performed using two-step RT-PCR as described above. Protein was extracted simultaneously with RNA and stored as a pellet in 100% ethanol at –20^{o}C. Solubilization of the protein was performed by dissolving in 0.1% SDS followed by heating at 45^{o}C and sonication for 60 min (Bath Sonicator 5200; Branson Ultrasonics, Danbury, CT). After determination of protein concentrations (Lowry et al., 1951), samples were aliquoted and stored at –20^{o}C for western blot analysis.

3.15 Statistical Methods

Differences in the RT-PCR analysis of target gene expression in *Hoxa2* wildtype and mutant embryonic tissue was assessed using a one-tailed t-test. The transfection data was analyzed using a one-way ANOVA, and all analyses were performed using GraphPad Prism 4.0 software (GraphPad Software, Inc.).

4. RESULTS

4.1 Purification of Recombinant Hoxa2 in *E.coli* using the pFLAG system for Affinity Purification of Hoxa2 Specific Antibody from Polyclonal Antisera

Recombinant Hoxa2 protein (~ 41 kDa) was expressed in *E.coli* using the pFLAG-2 expression vector (Appendix I). The predicted molecular weight of Hoxa2 is 40,793 Da and when expressed in bacterial cultures it is observed as a protein migrating at approximately 39-41 kDa by SDS-PAGE analysis. Differences in the observed migration on SDS-PAGE may be in part due to variations in the different molecular weight markers. Also, the migration of Hoxa2 may have been altered due to its acidic nature (pI 5.53).

Optimization of Hoxa2 expression had been previously conducted for isolation of inclusion bodies. IPTG induction of protein expression was performed for 16 hr at 37^{o}C for the production of high levels of insoluble recombinant Hoxa2 protein. Upon sonication and treatment with lysozyme, the protein was extracted from the cellular debris by isolation of inclusion bodies with Guanidine-HCl [4 M final concentration] (Figure 4.1). Afterwards the protein was renatured by dialysis, at which point a significant amount of protein was lost due to precipitation. This protein precipitant was also used for extraction of protein by the addition of Triton X-100 and 0.1 M Guanidine-HCl. Both the dialyzed protein and the solublized protein precipitant were used for purification of Hoxa2 using affinity chromotagraphy with the anti-FLAG M2 antibody (Figure 4.2, *lane 8*). Although affinity chromatography with anti-FLAG antibodies resulted in a highly pure protein product, the yield for each elution from 1 ml of affinity

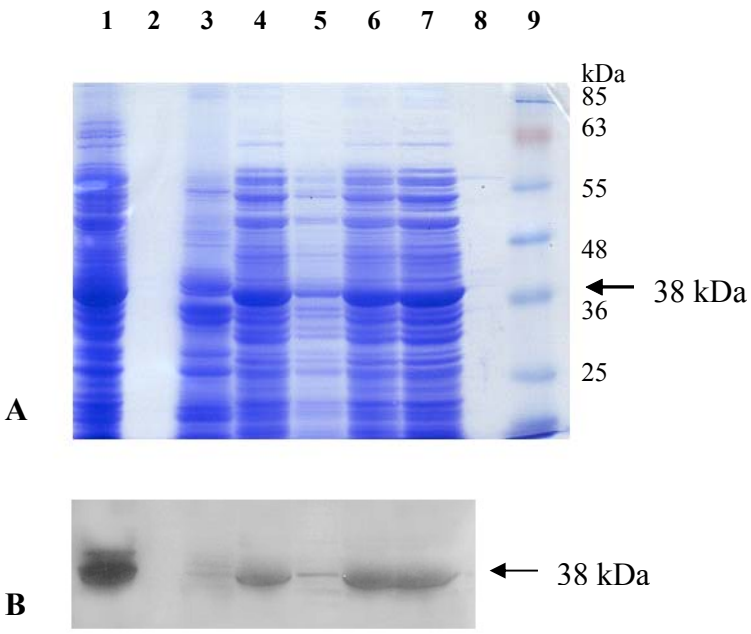


Figure 4.1 Purification of insoluble recombinant Hoxa2 in *E.coli*

Figure A: Protein samples from a 16 hr IPTG induction of *E.coli* cultures containing the pFLAG-Hoxa2 plasmid were analyzed by 12% SDS-PAGE, followed by coomassie blue staining. *Lane 1*: crude bacterial pellet from pFLAG-Hoxa2 *E.coli* cultures; *Lane 2*: empty; *Lane 3*: Supernatant of bacterial sonicate; *Lane 4*: Pellet after sonication; *Lane 5*: Bacterial pellet after extraction with 4 M guanidine-HCl; *Lane 6*: Supernatant after extraction with 4 guanidine-HCl; *Lane 7*: Protein precipitant after dialysis; *Lane 8*: empty; *Lane 9*: Gibco-BRL Pre-stained Ladder, the 38 kDa protein marker is indicated by the arrow.

Figure B: Western Blot analysis using anti-Hoxa2 polyclonal antibodies of the protein samples from a 16 hr IPTG induction of *E.coli* cultures containing the pFLAG-Hoxa2 cultures. Lanes correspond to those of the coomassie blue SDS-PAGE gel. Recombinant Hoxa2 is mainly present in the pellet after sonication, and is extracted with Guandine-HCl in subsequent steps.

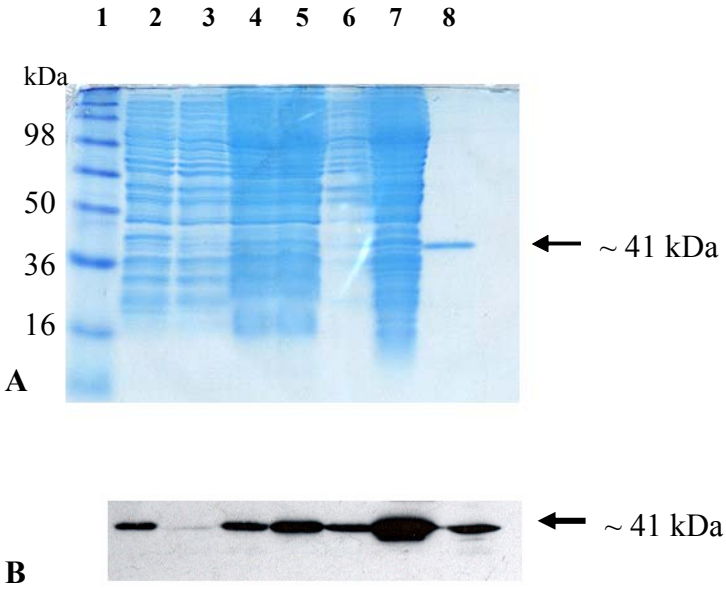


Figure 4.2 Purification of recombinant Hoxa2 from inclusion bodies

Figure A: Recombinant Hoxa2 extracted and purified from induced bacterial cultures resolved by 12% SDS-PAGE, followed by coomassie staining. *Lane 1*: Gibco-BRL Seeblue protein marker; *Lane 2*: crude bacterial pellet after IPTG induction; *Lane 3*: Supernatant after sonication of bacterial culture; *Lane 4* and *5*: Guanidine-HCl extraction of inclusion bodies from bacterial pellet; *Lane 6*; bacterial pellet after guanidine-HCl extraction; *Lane 7*: insoluble protein precipitant after dialysis; *Lane 8*: anti-FLAG affinity chromatography purification of recombinant FLAG-Hoxa2 protein which migrates as a 41 kDa protein.

Figure B: Western Blot analysis of the above protein samples using anti-FLAG M2 antibody corresponding to the lanes in the coomassie stained gel shown above.

column is low, at approximately 30-50 μg. A total yield of approximately 100 μg of purified Hoxa2 protein was obtained from 1 L of induced culture, as determined by BioRad Protein Assay.

Hoxa2 protein was partially purified by extraction of inclusion bodies, followed by ammonium sulphate precipitation. Recombinant Hoxa2 precipitates upon the addition of 30-80% ammonium sulphate. The minimal amount of coprecipitation of Hoxa2 with bacterial proteins is at 30% ammonium sulphate (Figure 4.3).

Extraction of recombinant FLAG-Hoxa2 protein in the form of soluble protein was performed by optimization of IPTG induction to 4 hr at 37^{o}C, followed by disruption of the bacterial cells using B-Per™ Reagent (Figure 4.4). Increasing the induction time results in the presence of Hoxa2 as insoluble protein, while a reduction in induction temperature yields very low amounts of recombinant protein. Hoxa2 protein was then partially purified by precipitation with ammonium sulphate and collection of the 0-30% ammonium sulphate protein fraction.

Both purified FLAG-Hoxa2 and ammonium sulphate precipitated proteins were dialyzed for renaturation and then combined. Protein samples were concentrated by centrifugation through a Centricon-30 filter (Figure 4.5). Protein samples were analyzed by Circular Dichroism for verification of the presence of alpha-helical structures (Figure 4.6). The partially purified protein sample consisted of approximately 30% alpha helical structure with the remaining 70% consisting of mainly beta-turn (type II) and random unordered structures, followed by the presence of some beta-sheets (Appendix II).

Approximately 20 mg of partially purified Hoxa2 protein was conjugated per ml of AffiGel (BioRad) matrix. Conjugation of protein to the matrix was determined by

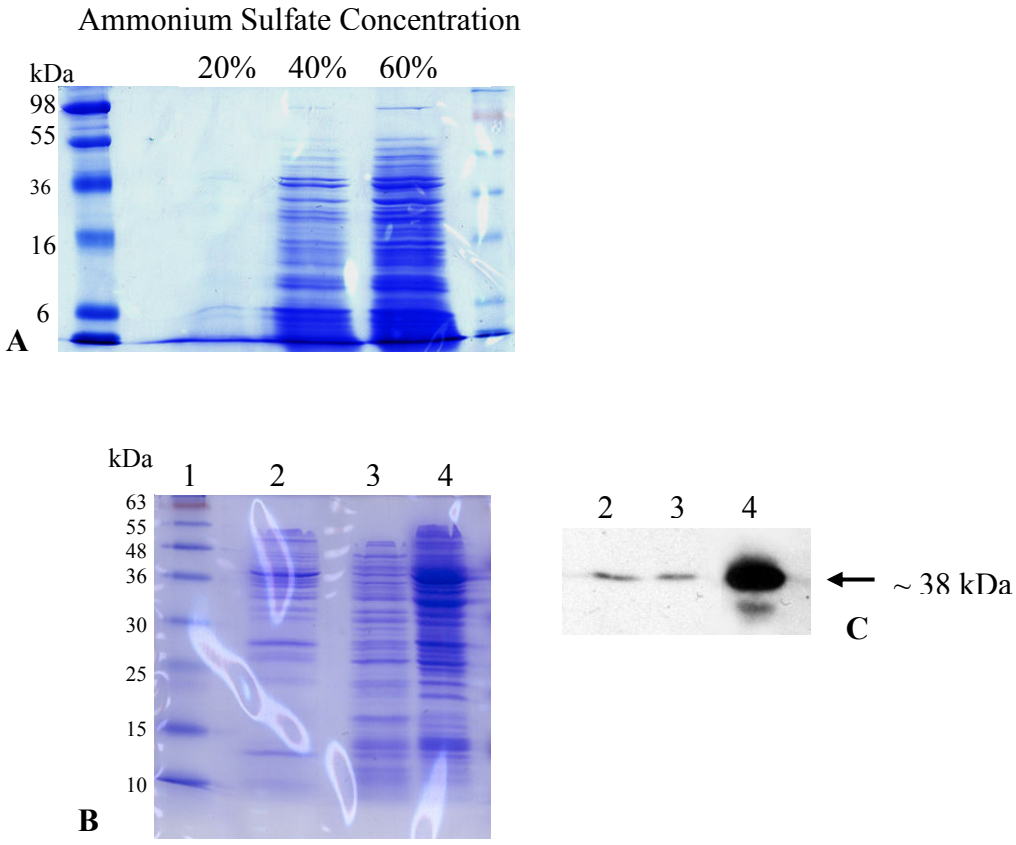


Figure 4.3 Ammonium sulphate precipitation of recombinant Hoxa2 protein from bacterial protein extract

Figure A: SDS-PAGE analysis of protein samples from *E.coli* induced for recombinant Hoxa2 expression precipitated with ammonium sulphate at a concentration of 20, 40 and 60%.

Figure B: SDS-PAGE analysis of 0-30% ammonium sulphate precipitant fraction of protein from bacterial cultures induced for recombinant Hoxa2 expression (*lane 2*), remaining protein in bacterial extract after precipitation (*lane 3*), and bacterial extract before precipitation (*lane 4*). Prestained protein ladder (Gibco-Brl) was used as a standard for estimation of molecular weight (*lane 1*). Western Blot using anti-FLAG M2 antibody showed that Hoxa2 (~ 40 kDa) coprecipitates with other proteins in 30% ammonium sulphate (Figure C).

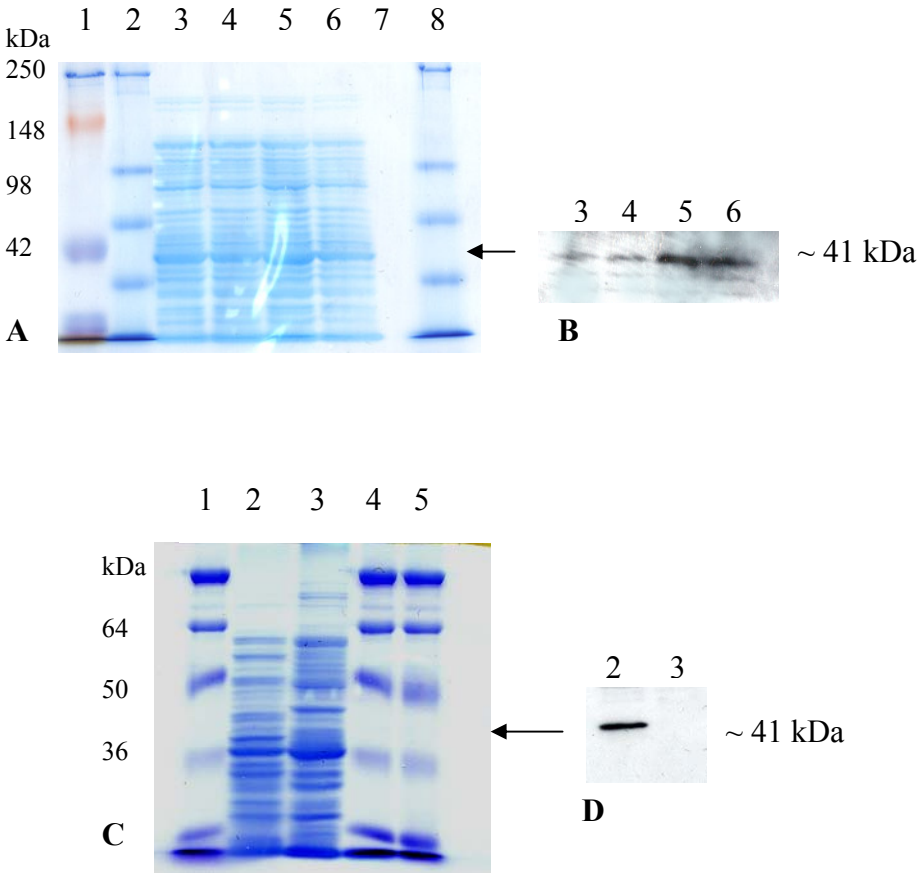


Figure 4.4 Expression of recombinant Hoxa2 in *E.coli* as soluble protein

Figures A and B, as well as C and D are true alignments according to migration of molecular weight markers.

Figure A: SDS-PAGE analysis of the supernatant from bacterial suspensions in B-Per™ reagent for recombinant Hoxa2 protein induced cultures at 37°C for 2 hr (*lane 3*), 3 hr (*lane 4*), 4 hr (*lane 5*), and 6 hr (*lane 6*). (Figure B) Western blot analysis with anti-Hoxa2 polyclonal antisera identify recombinant Hoxa2 protein (*lane 2*). Migration of Hoxa2 on the coomassie stained gel is shown by a black arrow.

Figure C: SDS-PAGE analysis of B-Per™ extracted recombinant Hoxa2 protein induced at 37°C for 4 hr. (Figure D) Western Blot analysis with anti-FLAG M2 antibody showed that Hoxa2 is present as soluble protein within the supernatant following disruption of bacterial cell by B-Per™ reagent (*lane 2*) and not as inclusion bodies within the cellular debris (*lane 3*).

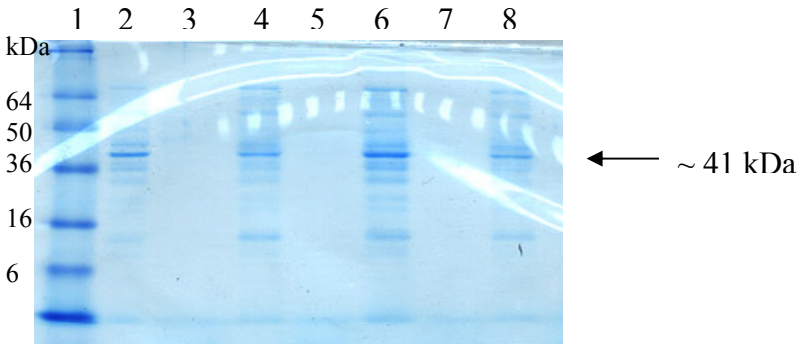


Figure 4.5 Concentration of partially purified recombinant Hoxa2 protein

Several batches of B-Per™ extracted soluble Hoxa2 protein fractions were partially purified with ammonium sulphate precipitation and then combined with FLAG affinity purified recombinant Hoxa2 protein extracted from *E.coli* as inclusion bodies. Fractions were combined, dialyzed to remove ammonium sulphate and concentrated using a Centricon-30 filter. Purity of the protein samples from separate batches of purification was assessed by 12% SDS-PAGE and Coomassie Blue Staining (*above, lanes 2, 4, 6, 8*). Recombinant Hoxa2 ran slightly above the 36 kDa band of the Gibco-BRL Seeblue Marker (*lane 1*).

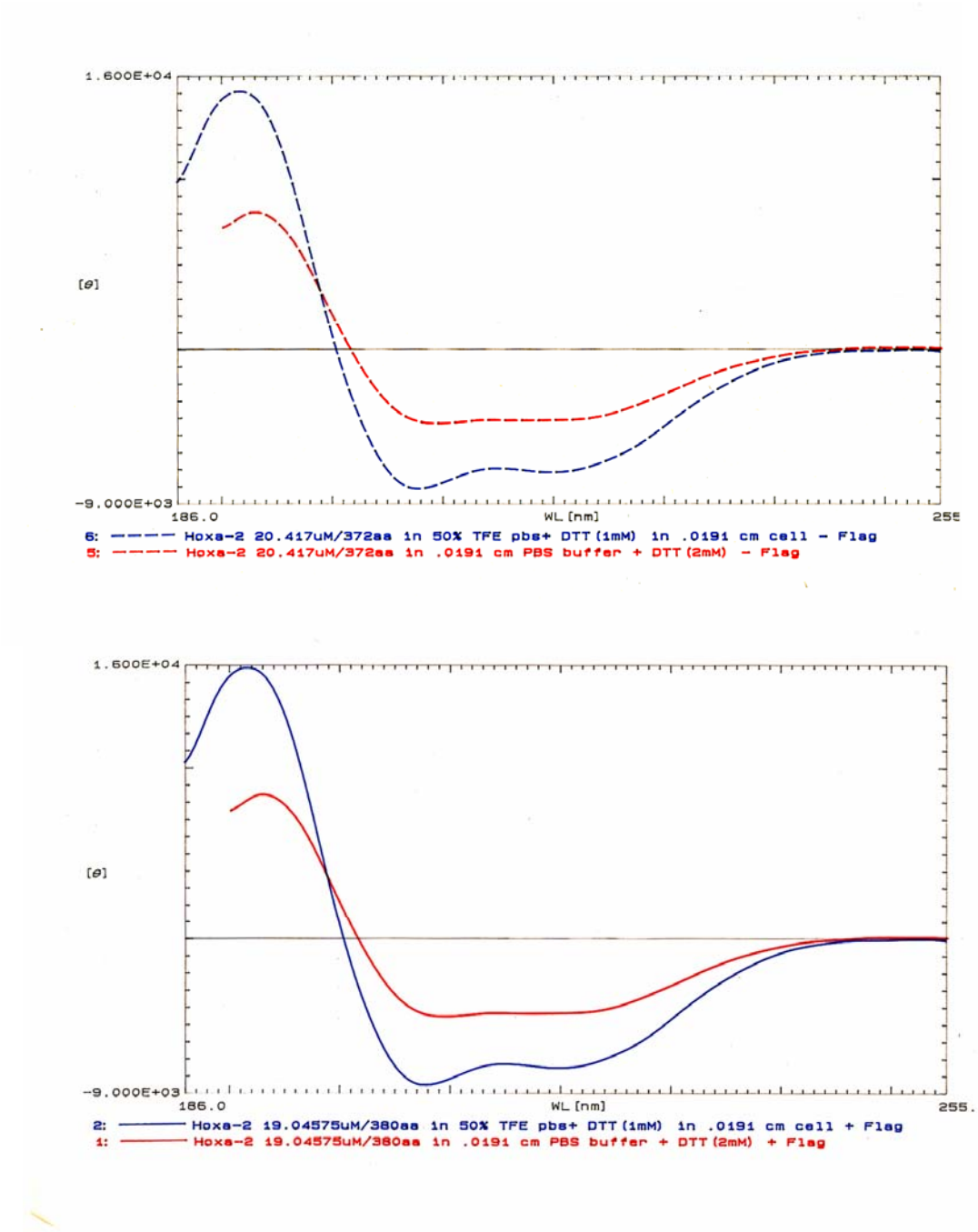


Figure 4.6 Circular Dichroism (CD) Scan of partially purified recombinant Hoxa2

CD Spectra of the FLAG affinity purified Hoxa2 combined with the 30% ammonium sulphate precipitated protein fraction. The samples were dialyzed in PBS buffer and then concentrated before analysis. Protein samples analyzed in PBS (*red line*) displayed a CD spectrum indicating the presence of alpha helical structures, which is improved by dissolution in 50% 2,2,2-trifluoroethanol (TFE). The presence of the FLAG epitope did not appear to dramatically affect folding of the recombinant Hoxa2 (*Bottom)*.

measuring absorbance at 280 nm and by western blot analysis. Affinity chromatography was used to purify anti-Hoxa2 specific antibodies from polyclonal Hoxa2 antiserum, and the specificity of purified antibody was determined by immunohistochemistry on mouse embryonic tissue, peptide inhibition studies and ELISA (Hao et al., 1999; Wolf et al., 2001).

Approximately 3 mg of Hoxa2 antibodies were conjugated to AffiGel (BioRad) matrix for isolation of chromatin preparations from the hindbrain and spinal cord tissue of E18 embryos. Hoxa2-DNA complexes were isolated by immunoprecipitation, and putative targets were subcloned into the *Sma I* site of pBluescript SKII+ (Appendix III).

4.2 Cloning and Identification of Putative Hoxa2 Target Sequences

Hoxa2 expression within the CNS begins as early as E10 and continues throughout development into adulthood, with the highest levels of expression observed at E12. Therefore, we attempted to isolate the downstream target gene(s) of Hoxa2 at the E12 stage utilizing an immunoprecipitation method and affinity purified IgG antibodies from Hoxa2 antiserum. The DNA isolated by this method was contaminated with EDTA precipitation and was therefore purified for ligation. The clones isolated from ligations were short DNA sequences (<100 bp), and hence too small for use in target gene identification. The experiment was repeated using chromatin preparations isolated from E18 embryos with the number of dialysis steps of the eluted DNA increased to ensure removal of 3 M NaSCN and thereby alleviate difficulties observed with precipitation. However, precipitation persisted and the resulting DNA did not yield transformants.

Although expression of Hoxa2 is highest at E12, due to availability of embryonic tissue the chromatin preparation was isolated from E18 CNS tissue. Also, use of older stage embryos was advantageous because it allows for the use of a more specific subset of CNS tissue for chromatin isolation. Therefore, target sequences of Hoxa2 were isolated from E18 spinal cord and hindbrain chromatin preparations utilizing a modification of the immunoprecipitation method utilized by Safaei (1997). Samples were digested with proteinase K in the presence of a decreased concentration of EDTA, and then isolated after column purification. The resulting DNA was devoid of precipitant. Thirteen clones were isolated using this method, of which seven were >50 bp in length. Identification of target genes by screening either Genomic libraries or DNA databases is less efficient using shorter sequences. The seven clones ranging in size from ~ 70-280 bp were sequenced (Figure 4.7).

All target sequences were analyzed by BLAST query against various GenBank databases (Altschul et al., 1990). Sequences for clones 2, 3, 4, 8 and 12 were found to have alignment with mouse trace sequences present in the Trace Archive database at the NIH NCBI GenBank website. Trace sequences (500–1000 bp in length) were used to screen various EST databases to identify potential target genes. The most significant alignment observed was with clone 8 and the *E.coli deoxyguanosine triphosphate triphosphohydrolase* (*dgt*) gene, having 98% identity. Clone 8 also aligned with several mouse trace sequences (500–1000 bp). Trace sequences exhibited 95–98% identity with the *dgt* gene but did not produce hits using BLAST for screening of the mouse or human genome, or any EST databases. Clone 4 also displayed high identity (94%) to *E.coli*

>clone 1 (222 bp)
GTCGTCGTGCCAGCGAAATCTGGCCCAGGTGGTCGCTCCACAGCCGGGCATT
GCAAGCTCATGAAGTCCCGCACCCAGCACGCGCGGGTCGGCCGGCAGCGGC
TTGCAGCAAGCGCCTGCGTGCGCGGGATGCGCAGGTTGAACATCGCCGCGA
TGATGTAGAGCAGCACCATGGCCGCGATCGCCGCCTCGGGCGGGGTGTCGA
TGCCGGTGTCGATGCCC

>clone 2 (103 bp)
CCAGTCTGCTTAGACTCAGCAGACACAGGCTGGCAAAGTCCCTGGAAACAG
CAGCCACAGCCATGCTTTCCCAAATACTATAGAACAAAACCAGGCATCATGG

>clone 3 (272 bp)
CGYGCACGCGTCGCCTGCGAGACCATGGTCAAGACCGGTGTTGCCATCGTGG
CCGGTGAAATCACCACCAGCGCCTGGATCGACCTGGAAGCACTGACCCGCA
AGGTCATCACGGACATCGGCTTACGACAGCTCGACGTCGGCTTGACGGCGGC
GCCACCTGCGGCGTGCTGAASACTGATCCGGCAAGCAGTCACCGTACATTGC
CCAGGGCGTGGATCGCAAGAAGCCGGAAGAAATGGGTGCCTGGCGACCCAG
GGCCTGATGTTCGG

>clone 4 (103 bp)
CCAGAGAAAACCGGTAATACGCCGGACGGTCGTCGCCGCCGGAACACCGTT
CGCGCCGGGCGCTAACCCCGATGCATGGTCTTTGACCGCAAAGGTGCCGTGG

>clone 5 (76 bp)
GTGCACGGCATGTCGGTGATCCAGTGGGGTGCACGGGAACGGCATCGGTAT
CGCCATCCAGGGCGACGAACACGCG

>clone 8 (142 bp)
CCTATGTCGAGGCTTCAGTAAATTACCGTCAGATTCTCCTGAGTTTCCGCTAT
GGGAATATTATTACCGTTGCCGCCTGCTGCAGGATTATATCAGCGGTATGAC
CGCCTCTATGCGTGGGATGAATACCGACGTCTGATGG

>clone 12 (218 bp)
CCATCGGCAGCCCCTTTGCCCTGCAGAACACCGTGACAACGGGTATTGTCAG
CACTGCCCAGCGGGATGGCAAGGAGCTGGGTCTCCGGGACTCAGACATGGA
CTATATCCAGACCGATGCCATCATCAATGTGAGTGCTGTGGGGAAGGCTGAC
CTCAGCAACTTTGGACCAGCTTGTGCCCTGTCCTATCATACAACACACCACC
ATCTAGGAAGG

Figure 4.7 Putative Hoxa2 Target Sequences isolated by immunoprecipitation

The potential Hoxa2 target sequences were isolated by immunoprecipitation using E18 hindbrain and spinal cord preparations. Clone 2 was identified as the mouse homologue of the human *DYRK4*. Clone12 showed 99% identity with the *Toll-associated serine protease* (*Tasp*) gene, which is the mouse homolog of the human *HTRA3* gene.

sequences, but not within a coding region. Trace sequences aligning with clone 12 exhibited significant identity (99%) with a mouse RIKEN cDNA gene referred to as *toll-associated serine protease* (*Tasp*). However, the full-length gene sequence of *Tasp* was not available at that time.

BLAST queries were later performed using the Ensemble Mouse Genome Database (EBI/Sanger Institute) rather than the NCBI megaBLAST search. Clone 12 displayed alignment with *Tasp* in the area spanning the latter (100 bp) half of exon 4 and the first ~ 200 bp of the fifth intronic sequence and is flanked by *Hae* III enzyme recognition sites. *Tasp* is a serine protease belonging to the HTRA family of genes, whose transcript is 1380 bp in length and consists of 9 exons (Figure 4.8).

Clone 2 was identified by BLAST alignment as the murine homolog of the human *Dual specificity tyrosine kinase 4 (Dyrk4)* gene located on chromosome 6. The *Dyrk4* cDNA is 1794 bp in length and consists of 15 exonic sequences (Figure 4.8). Clone 2 aligns within 3' end intronic sequences and is also flanked by *Hae* III restriction sites within the intron sequence.

4.3 *In Vitro* DNA Binding of Hoxa2 to the Target DNA

Initial attempts to study *in vitro* DNA binding were performed using recombinant FLAG-Hoxa2 protein isolated as soluble protein from *E.coli*. The use of bacterial protein extracts resulted in weak binding of Hoxa2 to clone 2 (Figure 4.9), but shift bands for the remaining target sequences aggregated in the wells and did not separate during electrophoresis (clones 1, 3-5, 8 and 12). The shift complex observed in *lane 2* formed a faint supershift band (SS) upon addition of the anti-Hoxa2 antibodies (*lane 4*), although most of the shift complex (S) appeared to remain unbound by the antibodies.

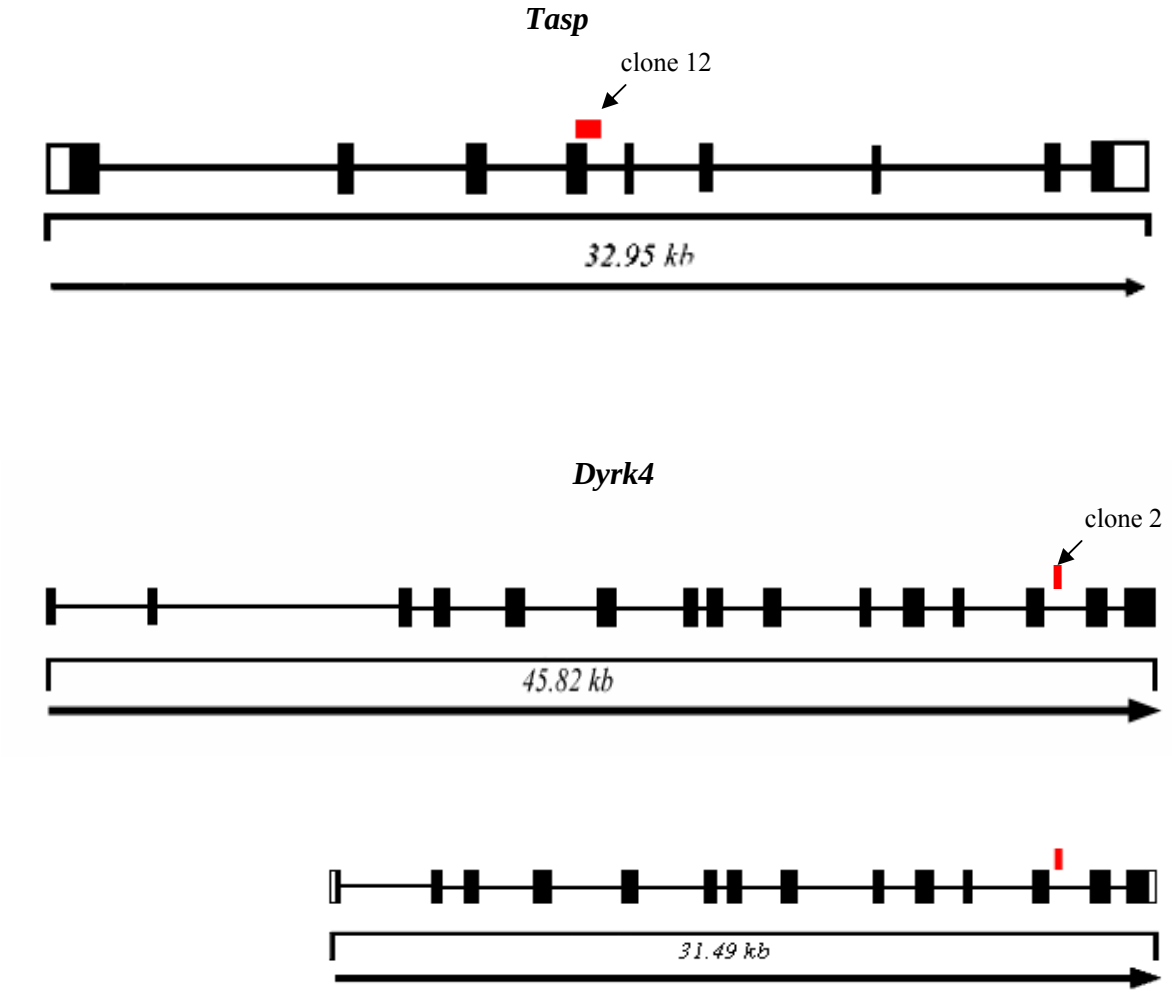


Figure 4.8 Schematic representation of the murine *Tasp* and *Dyrk4* genes

Exonic sequences are indicated by closed black boxes, intronic sequences are represented by the black line running between the closed boxes, and the UTRs by open boxes. Schematic representation of the 32.95 kb *Tasp* (*Top*) genomic sequence present on mouse chromosome 5, direction of transcription is indicated by the black arrow. Clone 12 displayed 99% identity with the 4th exonic and 5th intronic sequence (indicated by red box). Schematic representation of the two potential transcripts for the murine homolog of the *DYRK4* gene (*Bottom*) present on mouse chromosome 6, direction of transcription is indicated by the black arrow. Clone 2 displayed 100% identity with the intronic sequences present in both *Dyrk4* transcripts (indicated by red boxes).

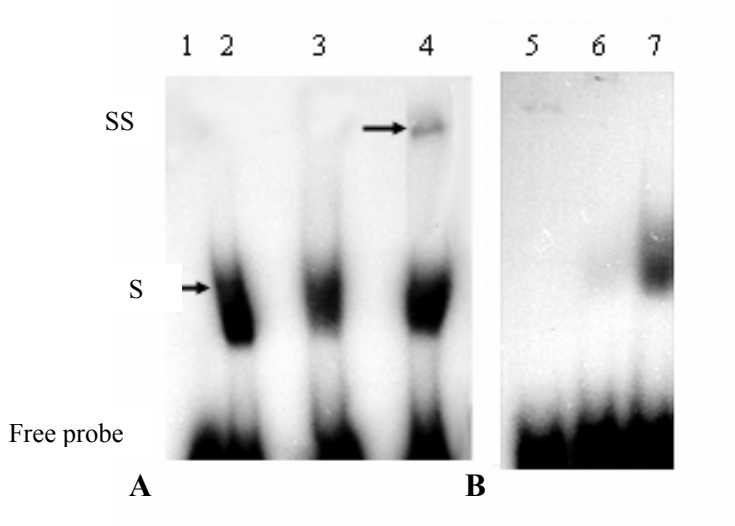


Figure 4.9 EMSA analysis of clone 2 using recombinant Hoxa2 protein

Figure A: The clone 2 target sequence was incubated with 10 μg of bacterial protein extract from FLAG-Hoxa2 expressing *E.coli* (*lane 2-4*). The addition of anti-Hoxd1 antibody (Wolf et al, 2001) did not affect shift band formation (S) (*lane 3*), while the addition of anti-Hoxa2 antibodies resulted in a small supershift (SS) (*lane 4*).

Figure B: The band shift formed by incubation of clone 2 with crude recombinant FLAG-Hoxa2 protein extract (*lane 7*), is inhibited by the addition of 100 X unlabelled clone 2 sequence to the binding reaction (*lane 6*). Migration of the labeled unbound probe is shown in *lanes 1* and *5*.

This may have resulted from an insufficient amount of antibody present in the SS reactions for binding to high levels of recombinant Hoxa2 protein in the bacterial extract. Alternatively, some of the shift complex (S) may have been formed by bacterial factors (*lane 2*) that are unaffected by Hoxa2 antibodies. Addition of anti-Hoxd1 polyclonal antiserum (Wolf et al., 2001) demonstrated that SS was not due to binding of clone 2 by serum factors (*lane 3*).

In vitro binding of Hoxa2 with the isolated target sequences was further studied by electrophoretic mobility shift assays (EMSA) using nuclear extracts (NE). Binding reactions using nuclear protein from E12 whole embryos resulted in the formation of multiple shift complexes with clones 4, 5, 3, 12 and 2 (Figure 4.10 A, B, C, D, and E respectively). For clones 4 and 5 (Figure 4.10 A and B) nuclear complexes formed three shift bands (Sa, Sb, and Sc). Supershift assays using anti-Hoxa2 antibodies demonstrated an absence in the third shift band (Sc), resulting in the formation of a supershift (SS). EMSAs for clones 3 and 12 (Figure 4.10 C and D) displayed two shift bands upon addition of NE (*lane 2,* Sa and Sb). Formation of the high molecular weight shift band (Sa) was inhibited by incubation with anti-Hoxa2 antibodies and a supershift complex (SS) was formed by binding of anti-Hoxa2 antibodies to the low molecular weight shift complex (Sb) (*lane 3*). It is possible that more shift bands were present for clone 12 (Figure 4.10 D, *lane 2*) that were not resolved by electrophoresis due to an excess of nuclear protein present in the binding reaction. Binding of nuclear protein with target 2 resulted in the formation of one shift complex (S) (Figure 4.10 E, *lane 2*). The binding complex did not form a supershift band upon the addition of anti-Hoxa2 antibodies; instead it displayed the formation of a lower molecular weight shift band

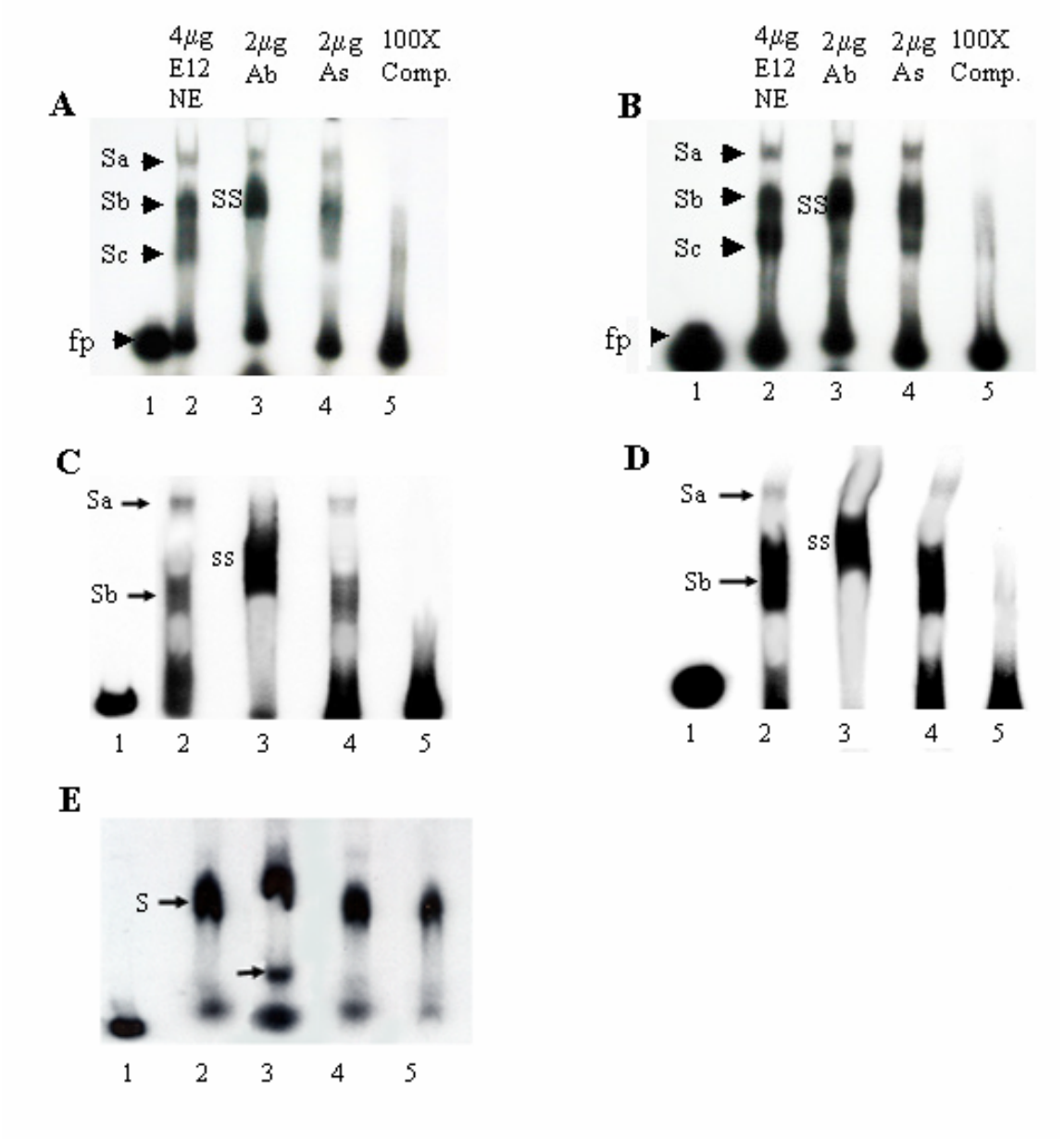


Figure 4.10 EMSAs for the Hoxa2 target sequences using E12 NE

EMSA analysis of target sequences from clone 4 (**A**), clone 5 (**B**), clone 3 (**C**), clone 12 (**D**) and clone 2 (**E**). Binding reactions for each target sequence were performed with 10 μg of E12 NE (*lanes 2-5*, **A-E**). Migration of unbound target sequences can be observed in *lane 1* of **A-E**. The addition of anti-Hoxa2 polyclonal antiserum resulted in the formation of a supershift band (*lane 3*, **A-D**). Incubation of binding reactions with non-immune rabbit serum did not affect shift formation (*lane 4*, **A-E**). Competition reactions using 100 X unlabelled probe resulted in inhibition of shift band formation (*lane 5*, **A-D**). In figure **E,** addition of anti-Hoxa2 antiserum resulted in the formation of a lower molecular weight shift complex rather than a supershift (*lane 3, black arrow*) that was not observed in the presence of non-immune rabbit serum (*lane 4*). Abbreviations: Nuclear Extract (NE), Shift band (S), Supershift band (SS), anti-Hoxa2 antiserum (Ab), rabbit antiserum (As), Competition (Comp), free probe (fp).

(Figure 4.10 E, *lane 3*, *black arrow*). Also, clone 2 displayed less competition at 100 X unlabelled probe than the other target sequences (Figure 4.10 A-E, *lane 5*). Clone 1 was not analyzed for binding with E12 NE, and clone 8 did not show binding with any Hoxa2 containing nuclear complexes with E12 NE.

Factors present in NE isolated from E18 hindbrain and spinal cord tissue also demonstrated binding to target sequences (Figure 4.11). Clone 8 exhibited two shift bands upon binding with E18 NE (Figure 4.11 A, *lanes 1-3*) that migrated in close proximity to one another. Addition of anti-Hoxa2 resulted in the appearance of two shift bands (Figure 4.11 A, *lane 4*) that were likely due to DNA binding by factors present within the antisera, since they are also observed in the presence of control antisera (Figure 4.11 A, *lane 5*). The high molecular weight shift complex seen in *lanes 1-3* was unaffected by the control antiserum (*lane 5*), but was not observed in the presence of Hoxa2 antibodies (*lane 4*). Due to poorly resolved shift bands, in *lane 5* it is difficult to assess whether the lower molecular weight shift band from *lane 1* was affected by the addition of the Hoxa2 antibodies. However, this band was clearly seen in the control antisera and did not migrate at a much larger molecular weight in *lane 5*, making it unlikely to be formed by a supershift. It is possible that anti-Hoxa2 antibody mediated inhibition of DNA binding by the higher molecular weight complex resulted in an increase in binding by the lower molecular weight complex and therefore a larger shift band was observed in *lane 5*. Although competition assays showed that both shift bands were specific for the target sequence, it is likely that Hoxa2 was only involved in DNA binding of the top shift band. As seen for clone 8, binding by E18 nuclear factors to clone 5 resulted in the formation of similar shift bands (Figure 4.11 B). The high

Figure 4.11 EMSA analysis of potential Hoxa2 target sequences using E18 hindbrain and spinal cord NE
In vitro binding studies using E18 NE was performed on: clone 8 (**A**), clone 5 (**B**), clone 2 (**C**), clone 4 (**D**), clone 12 (**E**), and clone 1 (**F**). Shift bands are indicated by black arrows.

Figure A: Clone 8 was incubated with decreasing amounts of NE at 15 μg (*lane 1*), 10 μg (*lane 2*), and 5 μg (*lane 3*); 15 μg of NE was incubated with 4 μg of anti-Hoxa2 polyclonal antiserum (*lane 4*), and non-immune rabbit serum (*lane 5*), as well as 100 X unlabelled probe for competition assay (*lane 6*).

Figure B: Clone 5 was incubated with decreasing amounts of NE as well, at a concentration of 10 μg (*lane 1*) and 5 μg (*lane 2*). Supershift assays were performed by incubating 10 μg of NE with 2 μg (*lane 3*) and 4 μg (*lane 4*) of anti-Hoxa2 antiserum. Corresponding amounts of non-immune serum were also used (*lane 5* and *lane 6*, respectively). Competition was performed at 100 X (*lane 7*).

Figure C: Clone 2 was incubated with NE at 15 μg (*lane 1*), 10 μg (*lane 2*), and 5 μg (*lane 3*); 15 μg of NE was incubated with 4 μg (*lane 4*) and 2 μg (*lane 5*) of anti-Hoxa2 polyclonal antiserum, 4 μg of control serum (*lane 6*), and 100 X unlabelled probe (*lane 7*).

Figure D: Shift bands appeared upon binding of clone 4 to 2 μg and 4 μg of NE (*lane 1* and *2*, respectively). Supershift assays were performed on 4 μg of NE by incubation with 2 and 4 μg of anti-Hoxa2 antiserum (*lanes 3* and *4* respectively), or corresponding amounts of non-immune serum (*lanes 5* and *6*). Competition of the 4 μg NE binding reaction with 100 X unlabelled probe is shown in *lane 7*.

Figure E: Binding reations for clone 12 were performed with 0, 2 and 5 μg of NE (*lanes 1*, *2*, and *3* respectively). Protein DNA complexes were unaffected by the addition of rabbit antiserum (*lane 4*), but shift bands were inhibited upon incubation with 2 and 4 μg of anti-Hoxa2 antiserum (*lanes 5* and *6*); competition was performed at 25 X and 50 X unlabelled probe concentrations (*lanes 7* and *8*).

Figure F: Clone 1 binding reactions with 0, 2, 4 and 8 μg of NE protein (*lanes 1*, *2*, *3* and *4* respectively) resulted in large unresolved shift bands. The addition of 2 and 4 μg of anti-Hoxa2 antiserum resulted in removal of some of the protein complexes involved in binding, thereby increasing resolution of the remaining shift bands (*lanes 5* and *6*). Incubation with corresponding amounts of non-immune rabbit serum resulted in an overload of protein in the wells at the top of the gel (*lanes 7* and *8*). Competition using 25 X and 50 X unlabelled probe resulted in a reduction in the intensity of the bands in *lane 1* but is not sufficient in blocking binding entirely (*lane 9* and *10*).

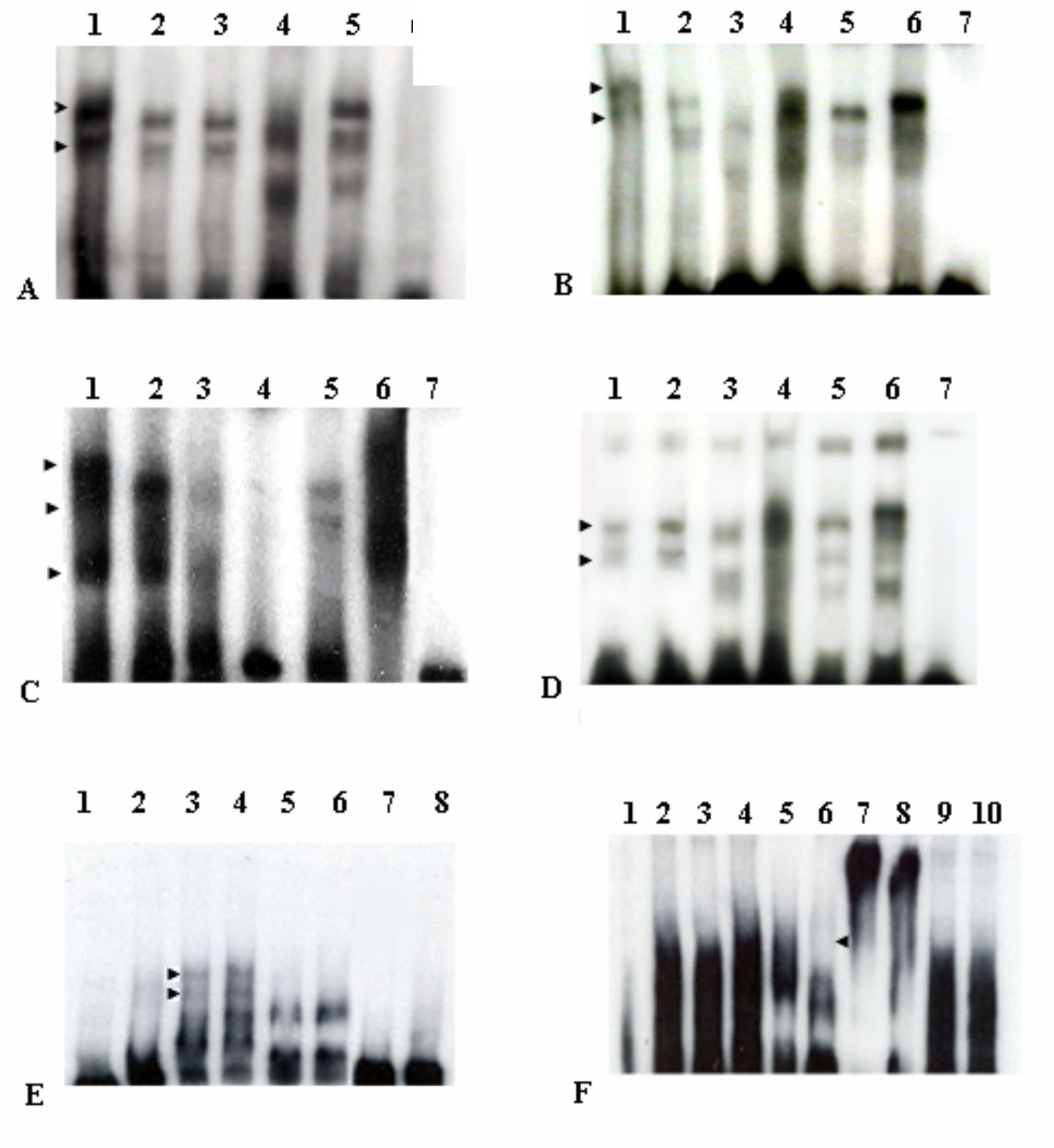


Figure 4.11 EMSAs for potential Hoxa2 target sequences using E18 hindbrain and spinal cord NE

molecular weight shift band from *lane 1* was not seen in the presence of anti-Hoxa2 antibodies, while binding by the lower molecular weight protein complex was unaffected (*lane 3* and *4*).

Binding reactions performed for the clone 2 target sequence resulted in the formation of three shift bands (Figure 4.11 C, *lanes 1* and *2*). The top two shift bands migrated in close proximity to one another and were poorly resolved, but could be observed upon addition of Hoxa2 antibodies (*lane 5*). However, formation of these shift bands was inhibited by the addition of a higher amount of Hoxa2 antibodies (*lane 4*). The lower molecular weight shift complex was absent in the presence of both antibody concentrations (*lanes 4* and *5*). Smearing of the bands in *lanes 1* and *2*, which is also observed upon the addition of rabbit serum (*lane 6*), was likely due to protein degradation of the nuclear extract.

Although three different protein complexes exhibited binding to clone 4 (Figure 4.11 D, *lanes 1* and *2*), competition assays demonstrated that the highest shift band was due to non-specific DNA binding by NE factors (*lane 7*). Supershift assays indicated that the lowest molecular weight complex involved Hoxa2 (*lanes 3* and *4*), but the larger molecular weight shift band was unaffected by the addition of anti-Hoxa2 antibodies.

Nuclear factors produced three band shifts in the presence of clone 12 (Figure 4.11 E, *lanes 3* and *4*), but incubation of binding reactions with Hoxa2 antibodies inhibited only the top two shift bands (*lanes 5* and *6*). Competition of all protein complexes bound to clone 12 occured with the addition of 25 and 50 X unlabelled probe. The same binding reactions performed with clone 1 (Figure 4.11 F, *lanes 2-4*) resulted in large smeared shift bands. This may have been due to: an overload of nuclear protein

required for binding to this particular target sequence, aggregation of multiple protein complexes on the target sequence, the presence of multiple binding sites in close proximity resulting in bands with very similar migration rates, and the use of an excess amount of radiolabelled probe. Upon the addition of increasing amounts of Hoxa2 antibody (*lanes 5* and *6*), the upper portion of the shift band from *lanes 2-4* was removed and a sharper, lower molecular weight shift band remained. Incubation of the binding reactions with control antiserum (*lanes 7* and *8*) resulted in protein overload at the top of the gel, indicating large aggregates of serum protein combined with NE factor binding to the probe. Although there is a slight decrease in intensity of the shift band, competition at 25 and 50 X concentrations of unlabelled probe did not completely block binding to the target sequence (*lanes 9* and *10*).

In vitro binding analyses were also performed by EMSAs using P1 hindbrain and spinal cord NE of both *Hoxa2*$^{-/-}$ and *Hoxa2*$^{+/+}$ mice. These revealed a difference in the binding complexes observed in the presence and absence of Hoxa2 protein for clone 1 (Figure 4.12 A and B). In wildtype mice, three shift bands were observed (Sa, Sb, and Sc) with clone 1 (Figure 4.12 A, *lanes 1-3*), while only one prominent shift was observed in the binding reactions with the mutant NE (Figure 4.12 B, *lane 2*). Supershift assays suggested that the lower molecular weight complexes (Sb and Sc) involved Hoxa2 activity (Figure 4.12 A, *lane 4*).

Hoxa2 protein synthesized *in vitro* with wheat germ extract (Figure 4.12 C and D) was also used for DNA binding analysis (Figure 4.12 A, *lane 5*). Expression of Hoxa2 *in vitro* resulted in the production of two protein bands, at 42 kDa and 48 kDa (Figure 4.12 C). The migration of the *in vitro* translated protein differed from that of the

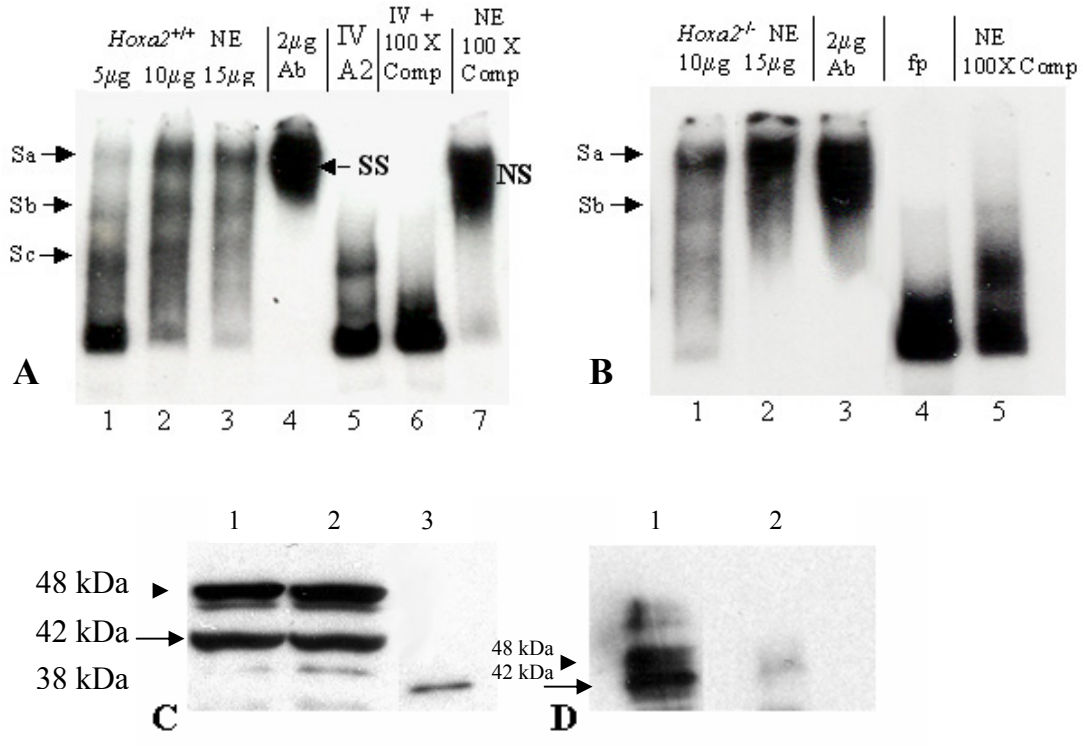


Figure 4.12 EMSA analysis of clone 1 using P1 NE and *in vitro* translated Hoxa2

EMSA analysis for clone 1 was performed using NE isolated from P1 hindbrain and spinal cord tissue of *Hoxa2*$^{+/+}$ (Figure A) and -/- mice (Figure B). Incubation with 5, 10, and 15 μg of wildtype NE resulted in shift bands (Sa, Sb, and Sc) (Figure A, *lanes 1*, *2* and *3* respectively). Supershift of Sb and Sc occured with addition of Hoxa2 antisera to the binding reactions containing 10 μg of NE (*lane 4*). *In vitro* translated Hoxa2 protein bound to the target sequence and displayed faster mobility than complexes formed by NE (*lane 5*, Figure A). Competition resulted in the removal of the shift band for *in vitro* translated Hoxa2 (*lane 6*) and for NE shift bands Sb and Sc (Figure A, *lane 7*). Binding of nuclear factors to the target sequence also occurred in mutant NE (Figure B, *lanes 1* and *2*,) and was unaffected by the addition of Hoxa2 antibodies (Figure B, *lane 3*). Western Blot was performed using anti-Hoxa2 polyclonal antisera and Hoxa2 protein translated *in vitro* by wheat germ extract (Promega) (Figure C and D). *In vitro* translated Hoxa2 protein resolved by 12% SDS-PAGE (Figure C, *lanes 1* and *2*) appeared to migrate as a 42 kDa (black arrow) and a 48 kDa (arrowhead) protein, in comparison with Hoxa2 protein present in E12 nuclear extract which appeared to be ~ 38 kDa (*lane 3*). (Figure D) Analysis using 10% SDS-PAGE showed that the Hoxa2 protein bands (*lane 1*) did not appear in the wheat germ extract containing no *Hoxa2* cDNA (*lane 2*).

Abbreviations: Nuclear Extract (NE), anti-Hoxa2 Antibodies (Ab), free probe (fp), *in vitro* translated Hoxa2 (IV), nonspecific binding (NS), Supershift (SS).

Hoxa2 present in NE (Figure 4.12 C), which appeared to migrate as a 38 kDa protein. However, electrophoresis using gels with an increasing percentage of acrylamide and in the presence of multiple protein markers showed that Hoxa2 protein appeared to be 38-48 kDa. The appearance of a reduced mobility of *in vitro* translated Hoxa2 may have occured as a result of its high acidity (pI 5.53), which is known to alter migration of proteins in 1D electrophoresis (Hames, 1990). It may also have been due to pH differences between the nuclear extraction buffer and the wheat germ extract, or the use of prestained markers that sometimes migrated slower than their estimated molecular weight due to the presence of chromophores. The appearance of a doublet for *in vitro* translated Hoxa2 may have been due to proteolytic cleavage of the protein product *in vitro* or post-translational modifications such as phosphorylation.

A single shift band was observed for clone 12 (Figure 4.13, *lanes 5* and *6*) that was distinct from complexes formed by binding with mutant NE (Figure 4.13, *lane 8*). Although anti-Hoxa2 antibodies did not remove the shift band entirely, the intensity of binding was reduced indicating inhibition of Hoxa2 binding (Figure 4.13, *lane 7*). Binding analyses using null mutant NE and *in vitro* translated Hoxa2 protein was only performed with clones 1 and 12.

DNase I footprinting was performed to isolate the specific Hoxa2 DNA binding site within the target sequences and to confirm Hoxa2 recognition of the target sequences. Protein from P1 and E18 hindbrain/spinal cord NE were not stable in binding DNA, resulting in a lack of protection. Therefore, NE isolated from E12 whole embryos was used in footprinting analysis due to a high level of Hoxa2 expression at this time in development (Figure 4.14). Although some protection in the presence of NE was

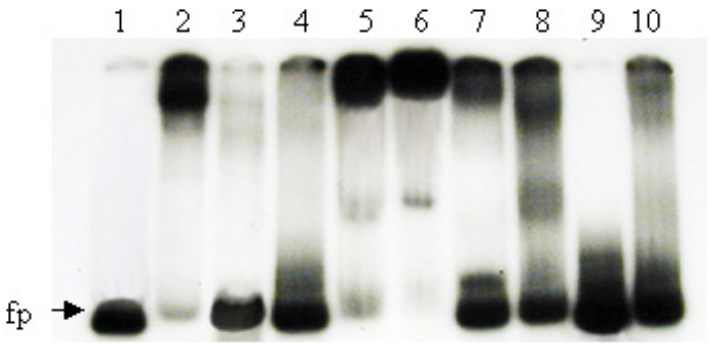


Figure 4.13 EMSA analysis of clone 12 using P1 Hoxa2 wildtype and mutant NE

In vitro translated Hoxa2 protein (3 μl) bound the target sequence (*lane 2*) and was inactivated by anti-Hoxa2 antibodies (Ab) (*lane 3*). Migration of the unbound probe (fp) is shown in *lane 1*. Competition reactions for *in vitro* translated Hoxa2 protein (*lane 9*), and NE (*lane 10*) using 100 X unlabelled clone 12. Increasing concentrations of wildtype NE at 5, 10, 15 μg appeared to produce shift bands with the target sequence (*lanes 4*, *5*, and *6* respectively), and binding of these complexes was reduced upon addition of Hoxa2 antisera (*lane 7*). *Hoxa2* mutant NE also displayed shift bands that appeared to migrate at a different rate than those present in the wildtype NE (*lane 8*). All supershift assays were performed using 10 μg of NE and 2 μg of Hoxa2 antibodies.

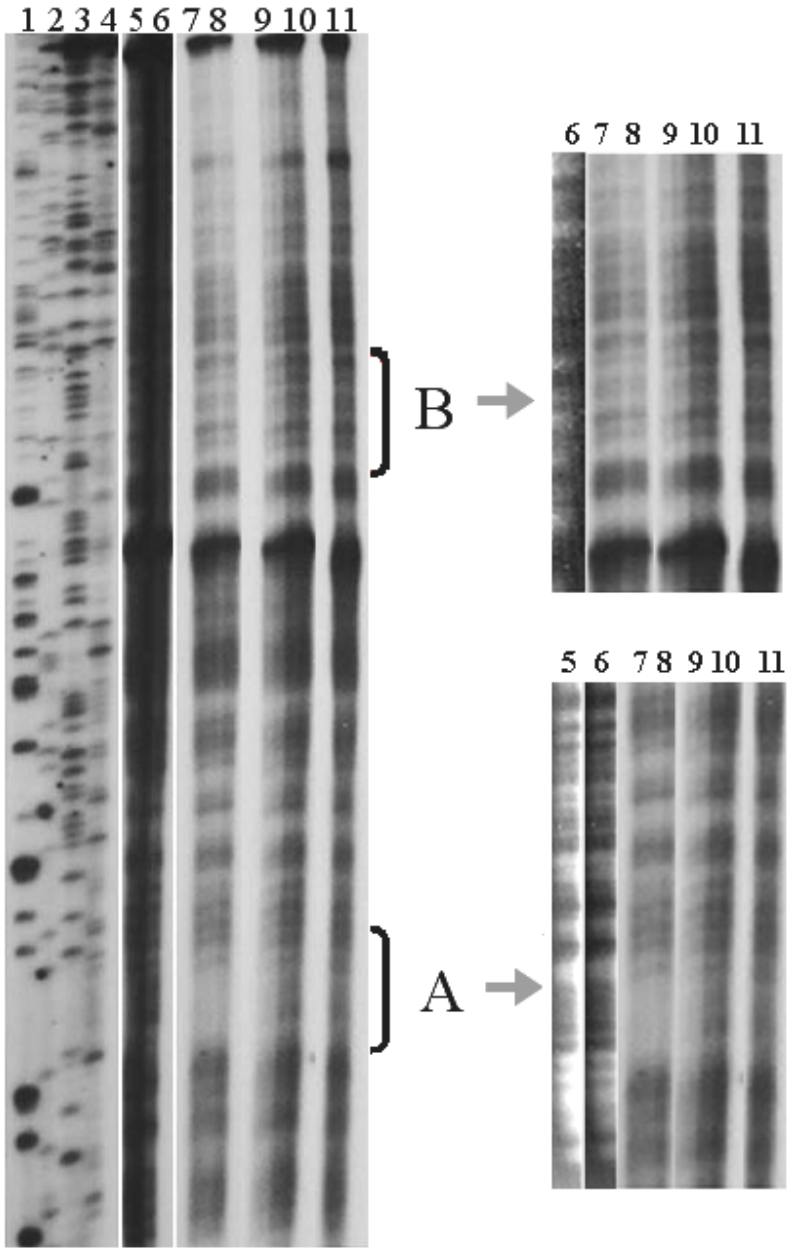


Figure 4.14 *DNase* I footprinting analysis of clone 2

Left: Thermocycle sequencing was used as a DNA ladder for footprinting reactions (*lanes 1-4*, GATC respectively). The probe was digested with increasing concentration of *DNase* I in the absence of protein (*lanes 5* and *6*). Binding reactions using 20 μg of E12 NE were digested with increasing concentrations of *DNase* I (0.002-0.08 U) in *lanes 7 –11*, respectively.
Right: Enlargement of footprinting sites A: 5′ TACCGTAGTACC 3′, and B: 5′ GACCGTTTCAGGGA 3′

observed using clone 2 (sites A and B), the footprints did not display a clear consensus sequence. Nor can any firm conclusions be drawn as to a specific Hoxa2 binding site until purified recombinant Hoxa2 protein is used in footprinting reations. A database search for potential transcription factor binding sites using TESS (Transcriptional Element Search System; www.cbil.upenn.edu/tess/) did not reveal any known transcription binding sites similar to site A or B (Figure 4.15). The only characterized Hoxa2 binding site (Lampe et al., 2004), a Pbx bipartite consensus sequence, 5′ T/AGATT/GA/GAT/AG/CG/T/A 3′, could not be found within the clone 2 or 12 sequence by ClustalW alignment. TESS analysis demonstrated the presence of multiple binding sites within clone 12 for the *Drosophila* factor Zeste (Figure 4.16 A), and a Zeste binding site flanking site B in clone 2 (Table 4.1 and 4.2). A search for homeobox binding sites of Clone 12 revealed the presence of a Thyroid Transcription Factor (TTF-1) binding site (Figure 4.16 B), but no previously characterized homeobox binding sites were found within the clone 2 sequence. Although both clone 2 and 12 exhibited multiple binding sites for the Glucocorticoid Receptor (GR), ClustalW alignment of the two clones revealed few similarities between their sequences.

To identify the specific Hoxa2 binding site a method utilizing *DNase* I footprinting coupled with Southwestern Blotting (SW) was performed (Figure 4.17). In this approach *DNase* I footprinting reactions were performed on probes using protein that was immobilized on a membrane, with the position of Hoxa2 determined by concurrent immunoblotting. Although Hoxa2 demonstrated binding to target sequences 2 and 12 (Figures 4.17 A and B, respectively), low binding capacity of the probe to the immobilized protein made it difficult to isolate DNA subsequent to *DNAse* I treatment.

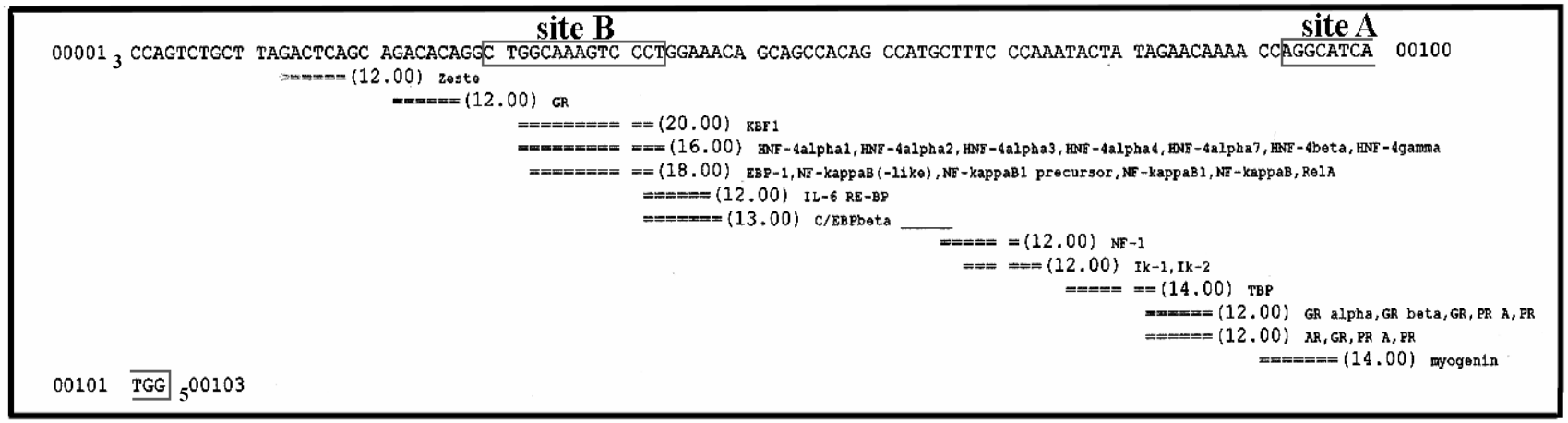
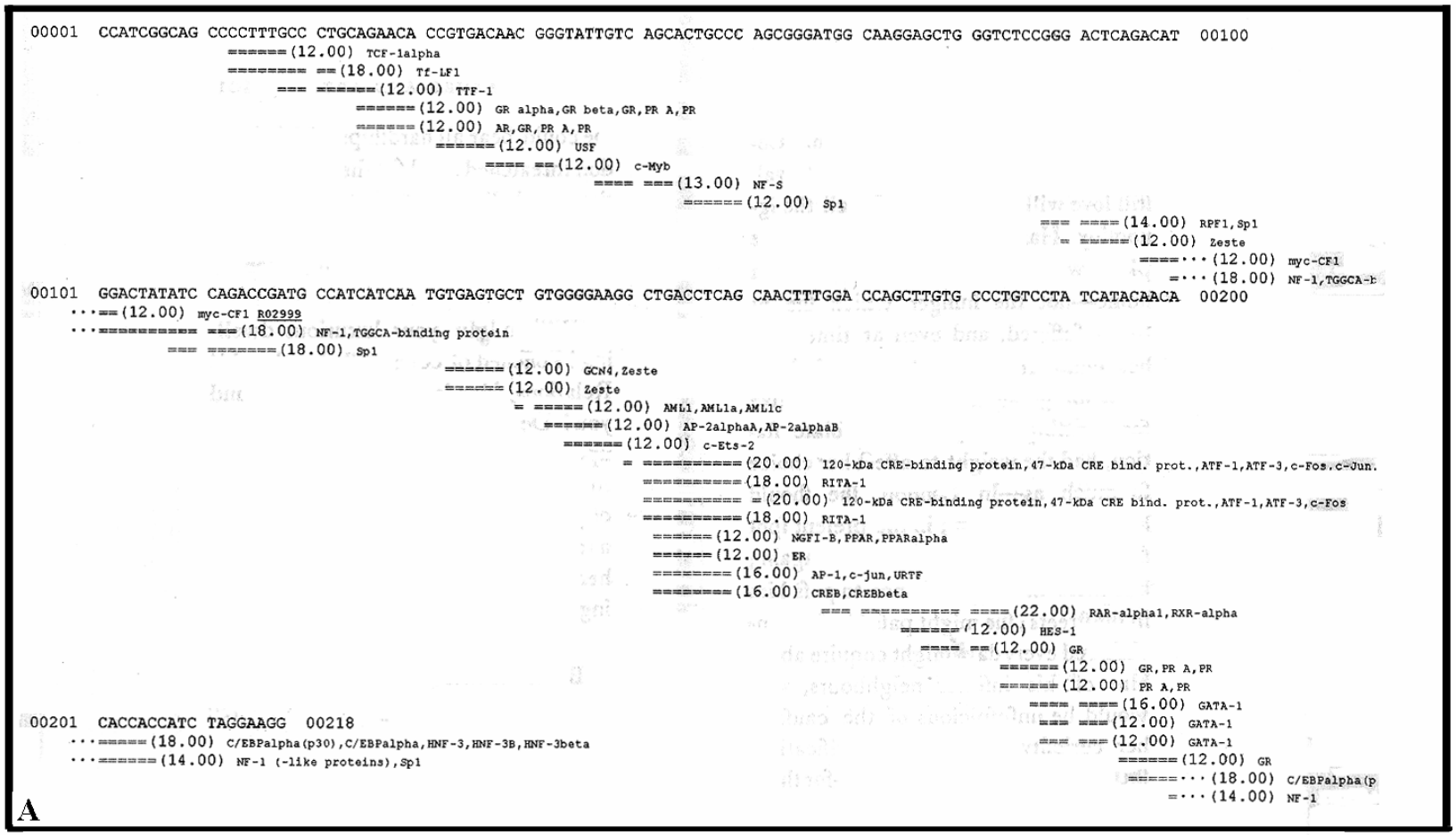


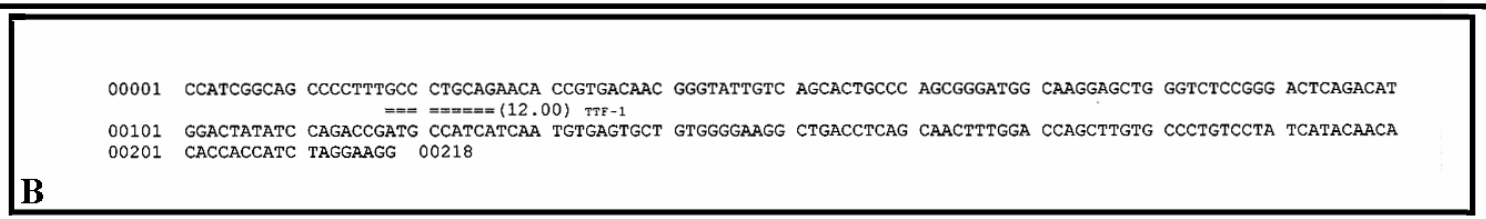
Figure 4.15 TESS analysis of clone 2 target sequence

TESS analysis of potential transcription factor binding sites for clone 2 did not reveal any known homeobox binding sites. Sites of protection from the *DNase* I footprinting for clone 2 are shown boxed. DNA binding sites are shown by dotted lines, followed by the transcription factor name. The GR and Zeste binding sites flank site B, while site A overlaps with a myogenin binding site. Tabular results of the TESS analysis are shown in Table 4.1.

Figure 4.16 TESS analysis of clone 12 target sequence

Figure A: TESS analysis of the clone 12 sequence for potential binding sites for the vertebrate class of transcription factors. Similarities between the TESS analyses of clone 12 and clone 2 was the presence of GR and Zeste binding sites. A search for the presence of homeobox binding sites revealed the presence of a potential TTF-1 binding site (Figure B). The TTF-1 transcription factor belongs to the NK2 class of homeodomain proteins, and its binding site has been characterized as 5′ CAAG 3′.


00001 CCATCGGCAG CCCCTTTGCC CTGCAGAACA CCGTGACAAC GGGTATTGTC AGCACTGCCC AGCGGGATGG CAAGGAGCTG GGTCTCCGGG ACTCAGACAT 00100
(12.00) TCF-1alpha
(18.00) Tf-LF1
(12.00) TTF-1
(12.00) GR alpha,GR beta,GR,PR A,PR
(12.00) AR,GR,PR A,PR
(12.00) USF
(12.00) c-Myb
(13.00) NF-S
(12.00) Sp1
(14.00) RPF1,Sp1
(12.00) Zeste
(12.00) myc-CF1
(18.00) NF-1,TGGCA-b
00101 GGACTATATC CAGACCGATG CCATCATCAA TGTGAGTGCT GTGGGGAAGG CTGACCTCAG CAACTTTGGA CCAGCTTGTG CCCTGTCCTA TCATACAACA 00200
(12.00) myc-CF1 R02999
(18.00) NF-1,TGGCA-binding protein
(18.00) Sp1
(12.00) GCN4,Zeste
(12.00) Zeste
(12.00) AML1,AML1a,AML1c
(12.00) AP-2alphaA,AP-2alphaB
(12.00) c-Ets-2
(20.00) 120-kDa CRE-binding protein,47-kDa CRE bind. prot.,ATF-1,ATF-3,c-Fos,c-Jun.
(18.00) RITA-1
(20.00) 120-kDa CRE-binding protein,47-kDa CRE bind. prot.,ATF-1,ATF-3,c-Fos
(18.00) RITA-1
(12.00) NGFI-B,PPAR,PPARalpha
(12.00) ER
(16.00) AP-1,c-jun,URTF
(16.00) CREB,CREBbeta
(22.00) RAR-alpha1,RXR-alpha
(12.00) HES-1
(12.00) GR
(12.00) GR,PR A,PR
(12.00) PR A,PR
(16.00) GATA-1
(12.00) GATA-1
(12.00) GATA-1
(12.00) GR
(18.00) C/EBPalpha(p
(14.00) NF-1
00201 CACCACCATC TAGGAAGG 00218
(18.00) C/EBPalpha(p30),C/EBPalpha,HNF-3,HNF-3B,HNF-3beta
(14.00) NF-1 (-like proteins),Sp1
A


00001 CCATCGGCAG CCCCTTTGCC CTGCAGAACA CCGTGACAAC GGGTATTGTC AGCACTGCCC AGCGGGATGG CAAGGAGCTG GGTCTCCGGG ACTCAGACAT
(12.00) TTF-1
00101 GGACTATATC CAGACCGATG CCATCATCAA TGTGAGTGCT GTGGGGAAGG CTGACCTCAG CAACTTTGGA CCAGCTTGTG CCCTGTCCTA TCATACAACA
00201 CACCACCATC TAGGAAGG 00218
B

Factor	Classification	Beginning	Length	Sequence
Zeste	Invertebrate	13	6	GACTCA
GR	Vertebrate	22	6	GACACA
KBF1	Vertebrate	32	11	GGgAAAGTCCC
HNF-4	Vertebrate	32	12	KGCWARGKYCaY
NF-kappa B	Vertebrate	33	10	GgAAAGTCCC
IL-6 RE-BP	Vertebrate	42	6	CTGGAA
C/EBPbeta	Vertebrate	42	7	CTGGRAA
NF-1	Vertebrate	66	6	CTTTCC
Ik-1, Ik-2	Vertebrate	68	6	TTCCCA
TBP	Vertebrate	76	7	TACTATA
GR	Vertebrate	82	6	AGAACA
myogenin	Vertebrate	91	7	CCAGGCA

Table 4.1 Tabular results for analysis of clone 2 by TESS

Tabular results of TESS using the TRANSFAC 5.0 database for clone 2, corresponding with Figure 4.16. The transcription factors have been classified as either vertebrate or invertebrate. The binding site for Zeste, a *Drosophila* transcription factor, is also present in clone 12. Glucocorticoid Receptor binding sites are also present in both clones 2 and 12. The start site of the binding site is shown under **Beginning** and **Length** refers to the length of the transcription factor binding site. For the transcription factor binding site sequence: W represents nucleotides A and T, R represents A and G, K represents G and T, and Y represents C and T.

Factor	Classification	Beg	Length	Sequence
TCF-1alpha	Vertebrate	13	6	CCTTTG
Tf-LF1	Vertebrate	13	10	CCTTTGaCCT
TTF-1	Vertebrate	18	9	GCNCTNNAG
GR	Vertebrate	25	6	AGAACA
USF	Vertebrate	32	6	CGTGAC
c-Myb	Vertebrate	37	6	CAACGG
NF-S	Vertebrate	47	7	YGTCAGC
Sp-1	Vertebrate	55	6	CTGCCC
RPF1	Vertebrate	88	7	GGGACTC
Zeste	Invertebrate	90	6	GACTCA
myc-CF1	Vertebrate	97	6	ACATGG
NF-1	Vertebrate	100	14	TGGANNNNATCCAa
Sp1	Vertebrate	108	10	ATCCAGcCCG
Zeste	Invertebrate	133	6	TGAGTG
AML1	Vertebrate	140	6	TGTGGG
AP-2alphaA	Vertebrate	142	6	TGGGGA
c-Ets-2	Vertebrate	144	6	GGGAAG
120-kDa CRE-binding protein	Vertebrate	150	11	GCTGACgTCAG
RITA-1	Invertebrate	151	10	CTGACgTCAG
CREM tau alpha	Vertebrate	151	11	CTGACgTCAGC
PPAR	Vertebrate	152	6	TGACCT
ER	Vertebrate	152	6	TGACCT
URTF, CREB	Vertebrate	152	8	TGACCTCA
RAR-alpha1	Vertebrate	168	17	GGACCANNNNTGaCCT
HES-1	Vertebrate	175	6	CTTGTG
GR	Vertebrate	177	6	TGTGCC
GR	Vertebrate	184	6	TGTCCT
GATA-1	Vertebrate	187	8	CCTATCAT
GR	Vertebrate	195	6	ACAACA
C/EBPalph	Vertebrate	196	10	CAAaACACCA
NF-1 (-like proteins)	Vertebrate	200	7	ACACCAC

Table 4.2 Tabular results for analysis of clone 12 by TESS

The results of the analysis of clone 12 by TESS in Figure 4.17 A and B have been arranged by position within the sequence, name of the transcription factor and classification. **Beg** indicates the start site of the binding site and **Length** refers to the length of the transcription factor binding site that overlaps with the in clone 12 sequence. For the sequences of the transcription factor binding site Y represents C and T.

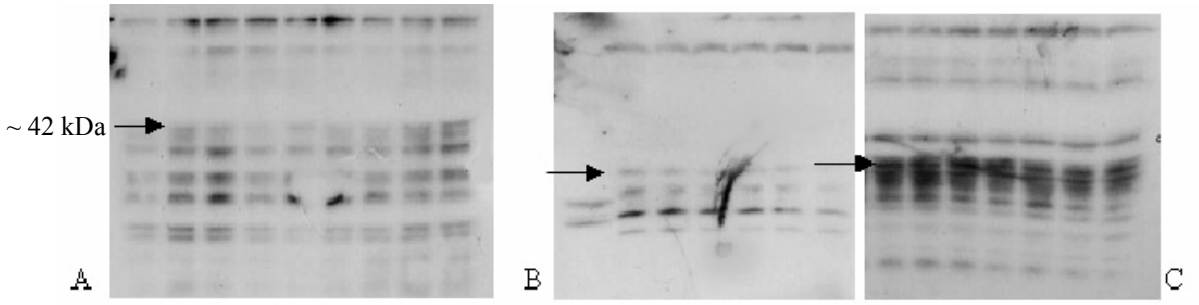


Figure 4.17 Analysis of DNA binding by P1 NE to clone 2 and 12 using Southwestern Blot technique

Southwestern Blots (SW) of probe 12 (Figure A) and 2 (Figure B and C) using NE isolated from hindbrains and spinal cord of P1 mice. All lanes contained NE at 20 μg protein/lane. Two SWs were performed for probe 2, the first blot was performed using milk as a blocking agent (Figure B) and the second SW was blocked with Bovine Serum Albumin (Figure C). Hoxa2 was identified by concurrent immunoblotting with anti-Hoxa2 polylconal antiserum. In addition to other nuclear factors, Hoxa2 protein (~ 42 kDa) bound to both target sequences. However, its binding activity for probe 2 appeared to be increased when BSA was used as a blocking agent rather than milk. Bands corresponding to the position of Hoxa2 were excised for *DNase* I footprinting but were too weak for visualization by autoradiography.

It is apparent however that multiple proteins in addition to Hoxa2 were capable of recognizing the target sequences. The activity of some of these factors may have been affected by phosphorylation (Figure 4.17 C). Although recognition of the potential target sequences by Hoxa2 has been demonstrated, the specific binding site and the cofactors by which this is mediated were not evident. Transcription factor database analyses of the remaining clones revealed multiple binding sites such as *GATA-2*, *GATA-3*, *Oct1*, and *CdxA*. *GATA-2*, *GATA-3*, and *Oct1* all have been implicated in neural development, and *CdxA* is known to be involved in spinal cord development.

4.4 Luciferase Assays of Hoxa2 mediated Regulatory Activity on the Target Sequences from Clones 2 (*Dyrk4*) and 12 (*Tasp*) in HeLa cells.

The putative Hoxa2 target sequences in clones 2 and 12 are present within intronic sequences rather than a promoter region. Hence, the target sequences were subcloned into the pGL3 Promoter vector (Promega) (Appendix IV), expression from which is regulated by an SV40 minimal promoter. HeLa cells were found to endogenously express the human HOXA2 (see section 4.5), which displays 94.44% identity with the mouse Hoxa2 protein. Cotransfection with a Hoxa2 expressing plasmid under the control of the RSV promoter was performed to assess whether overexpression of Hoxa2 affected the regulation of the target sequences.

Although the initial transfection efficiency assessed by cotransfection with GFP was found to be ~ 60-80%, the luciferase activity in pGL3 transfected cells was considerably low. Hence, transfections with 1, 2 and 3 μg amounts of pGL3 plasmid were assayed with the internal control vector at the recommended ratio of 50:1. Increasing reporter plasmid concentrations did not substantially increase the basal luciferase activity of the pGL3 vector. Therefore, transfections of the pGL3 Promoter

vector containing the *Tasp* intronic element from clone 12 (pGL3-c12, Figure 4.18) and the reporter vector with the *Dyrk4* intronic element from clone 2 (pGL3-c2, Figure 4.19) were performed using 2 μg:40 ng amounts of pGL3 to pCMV-RL (*Renilla* luciferase).

The use of an internal control at the recommended amount of 50:1 for the reporter to control vector (Figure 4.18 D and 4.19 D) resulted in the appearance of 'trans' effects between the reporter plasmid and pCMV-RL, where *Renilla* luciferase activity increased from 1,000-3,000 relative light units in pGL3 transfections, to ≥10,000 units in pGL3-c2 transfections and 5,000–9,000 units in pGL3-c12. 'Trans' effects are sometimes observed when either the control or reporter vector contains very strong promoter/enhancer activity. The activity of a strong promoter affects the ability of the promoter/enhancer of the cotransfected vector to direct transcription (Promega, Dual-Luciferase Reporter Assay System Technical Manual). This increase in activity of the internal control aberrantly reduced the ratio of firefly/*Renilla* luciferase activity, resulting in the alteration of transfection data. Therefore, varying amounts of reporter to internal control vector were assayed for both pGL3-c2 and pGL3-c12 for optimization of the assay (Figure A, B, C in 4.18 and 4.19). However, less than 4 ng of internal control vector could not be used since *Renilla* luciferase activity became too low for measurement. An increase in the ratio of reporter:control from 125:1 to 1000:1 alleviated the problem of inflated *Renilla* luciferase values. Although transfection using a ratio of 4 μg reporter plasmid to 4 ng of control plasmid decreased the appearance of 'trans' effects, it resulted in a high degree of variability in firefly luciferase activity of the control when cotransfected with pRSV-Hoxa2. The inhibition in luciferase activity

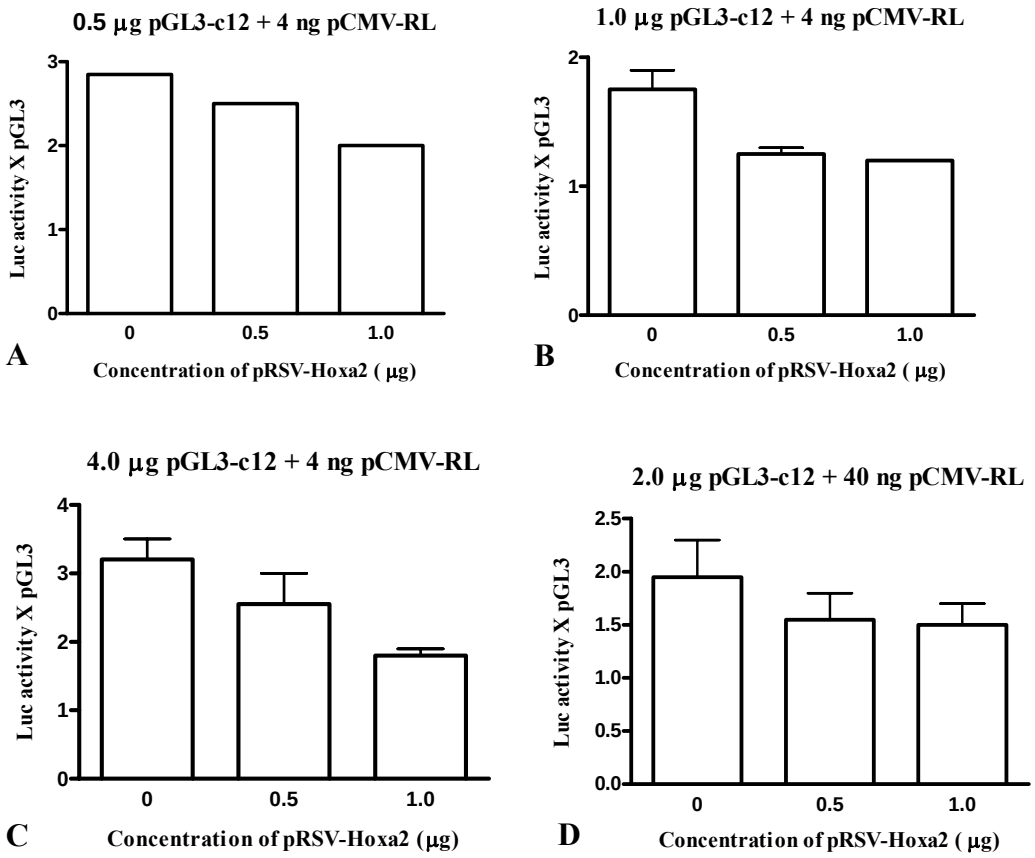


Figure 4.18 Luciferase assay of transfections using pGL3-c12

Transfection of the pGL3-c12 representing the intronic element of the murine *Tasp*. Firefly luciferase activity was normalized with *Renilla* luciferase activity (pGL3-12/pCMV-RL = Luc Activity), and is shown as a ratio to the control pGL3 luciferase activity (Luc Activity/pGL3 activity). Ratios of internal control to reporter vector are 1:125 (Figure A), 1:250 (Figure B), 1:1000 (Figure C), 1:50 (Figure D). Cotransfection with increasing concentrations of pRSV-Hoxa2 showed a trend of decreasing firefly luciferase activity for the pGL3-c12. Error bars represent the range, with *n*=2.

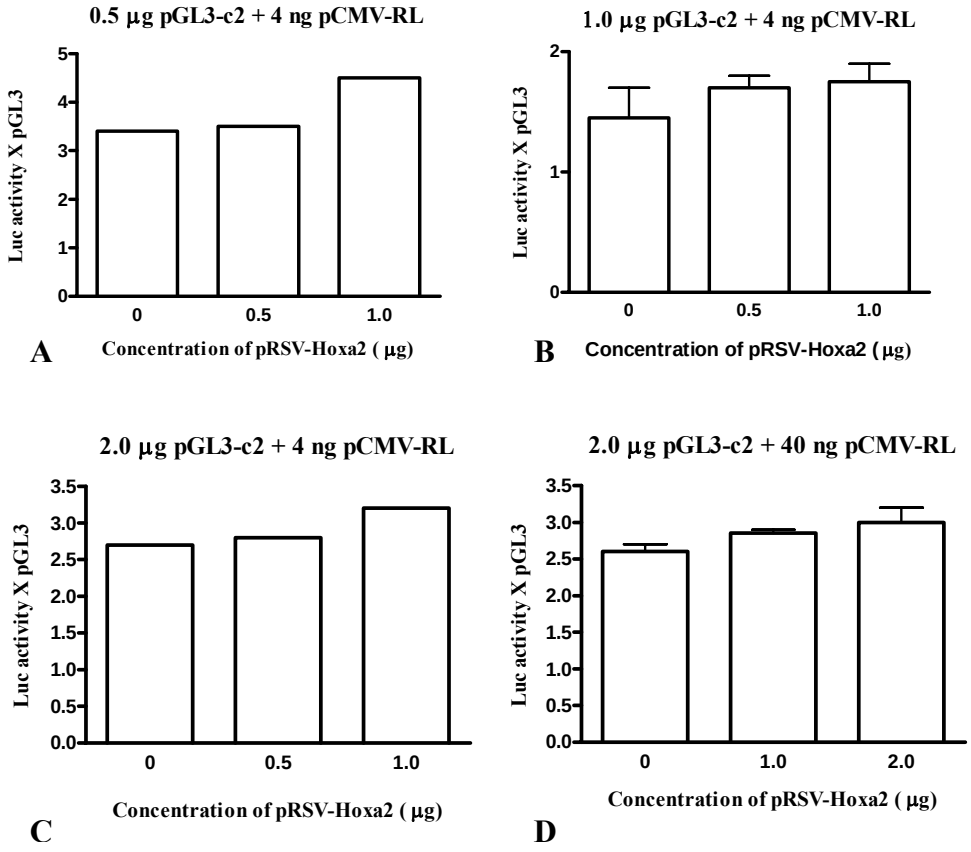


Figure 4.19 Luciferase assay of transfections with various concentrations of pGL3-c2: pCMV-RL and pRSV-Hoxa2

Transfection of the pGL3-c2, containing the target element from the intron of the murine *Dyrk4* homolog. Firefly luciferase activity was normalized with *Renilla* luciferase activity, and is shown as a ratio to the control pGL3 luciferase activity. Transfection of the pCMV-Renilla (RL) internal control vector to reporter plasmid at dilutions of 1:125 (Figure A), 1:250 (Figure B), 1:500 (Figure C), and 1:50 (Figure D). Cotransfection with increasing concentrations of pRSV-Hoxa2 showed a trend of increasing luciferase activity. Error bars represents the range, with n=2.

of the pGL3-c12 vector was not statistically significant as calculated by a one-way ANOVA test.

The *p* values for the luciferase assays of transfections using 1, 2 and 4 μg of pGL3-c12 were p= 0.104, 0.087 and 0.059, respectively (Appendix V, VI and VII) and therefore were not statistically significant. However, cotransfection of the pGL3 Promoter vector containing the *Tasp* intronic element from clone 12 (pGL3-c12) at various reporter:internal control concentrations all displayed a trend in decreasing firefly luciferase activity with increasing amounts of Hoxa2 (Figure 4.18 A, B, C, and D).

For the mouse *Dyrk4* intronic sequence, transfection data with pGL3-c2 indicated a trend in increased firefly luciferase activity after overexpression of Hoxa2. However, the *p* value as calculated by one-way ANOVA for transfections with 1 and 2 μg of pGL3-c2 (Figure 4.19 B and D) were 0.18 and 0.12, respectively (Appendix VIII and IX) and therefore did not demonstrate a statistically significant difference.

4.5 *Dryk4* and *Tasp* Expression in the Developing Mouse

Although Expression sequence tags (ESTs) have been isolated for *Dyrk4* and *Tasp* from various mouse tissues, their specific developmental expression patterns have not been studied. RT-PCR analyses of both genes showed that they are expressed from E10–18 as well in the adult and P1 hindbrain and spinal cord. Hoxa2 has previously been demonstrated to be present at these stages of development, and in the adult CNS (Hao et al., 1999).

The expression of the murine homolog of *DYRK4* during embryonic development at stages E10, 12, 14, 16 and 18 (Figure 4.20 A, *lanes 1, 2, 3, 4* and *5* respectively) was analyzed by RT-PCR amplification of a 229 bp fragment from within

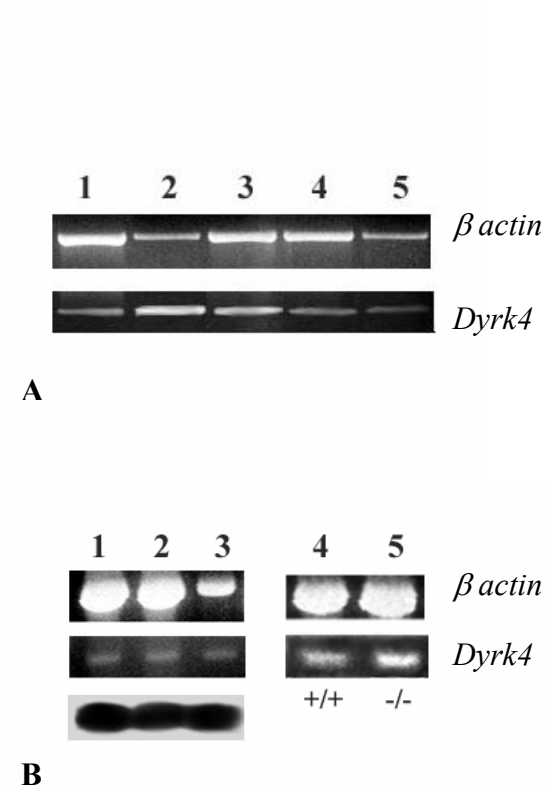


Figure 4.20 Expression of the mouse *Dyrk4* in embryonic and adult mouse tissue

Figure A: RT-PCR analysis of embryonic *Dyrk4* expression at stages E10 (*lane 1*), E12 (*lane 2*), E14 (*lane 3*), E16 (*lane 4*), E18 (*lane 5*). Figure B: A 229 bp *Dyrk4* fragment was amplified by PCR using RNA samples from (*middle*) the adult mouse forebrain (*lane 1*), hindbrain (*lane 2*), and spinal cord (*lane 3*), and the sequence verified by Southern blot analysis (*bottom*). Expression of *Dyrk4* was observed in both *Hoxa2* wildtype (*lane 4*) and mutant (*lane 5*) hindbrain and spinal cord tissue.

the 15th exon. Levels of the mRNA for the *Dyrk4* homolog appear to be abundant at E12, coinciding with one of the developmental stages (E10-12) at which *Hoxa2* has the highest levels of expression during embryogenesis (Tan et al., 1992; Wolf et al., 2001). Low levels of *Dyrk4* mRNA were also present within the mouse adult forebrain, hindbrain and spinal cord (Figure 4.20 B, *lanes 1, 2* and *3* respectively). Analysis of *Dyrk4* expression by RT-PCR amplification using RNA isolated from P1 hindbrain and spinal cord tissue of *Hoxa2* wildtype and mutant mice showed the presence of the *Dyrk4* in both genotypes (Figure 4.20 B, *lanes 4* and *5*). It was difficult to determine the change in levels of mRNA for the *Hoxa2* mutant, therefore further quantitative analysis of multiple litters is required.

Analysis of *Tasp* expression was performed by RT-PCR amplification of a 429 bp fragment spanning the 3′ end of exon 8, into exon 9, including a portion of the 3′ UTR. Due to the presence of a serine protease and an IGFBP (Insulin Growth Factor Binding Protein) encoding sequence, this region is the only area of the *Tasp* sequence that does not bear significant overlap with any other serine protease or IGFBP. *Tasp* mRNA is detected at embryonic stages E10-E18 (Figure 4.21 A and B), as well as in the adult mouse forebrain, hindbrain and spinal cord (Figure 4.21 C). Expression of *Tasp* is also present in the hindbrain and spinal cord of P1 mice (Figure 4.21 C). The absence of *Hoxa2* expression does not appear to have an all or none effect on *Tasp* mRNA levels in that it is gene expression is not entirely turned on or off. However, *Tasp* expression within the hindbrain and spinal cord of *Hoxa2* mutant mice appears to be slightly higher than in wildtype mice (Figure 4.21 C and 4.22 A, B, C).

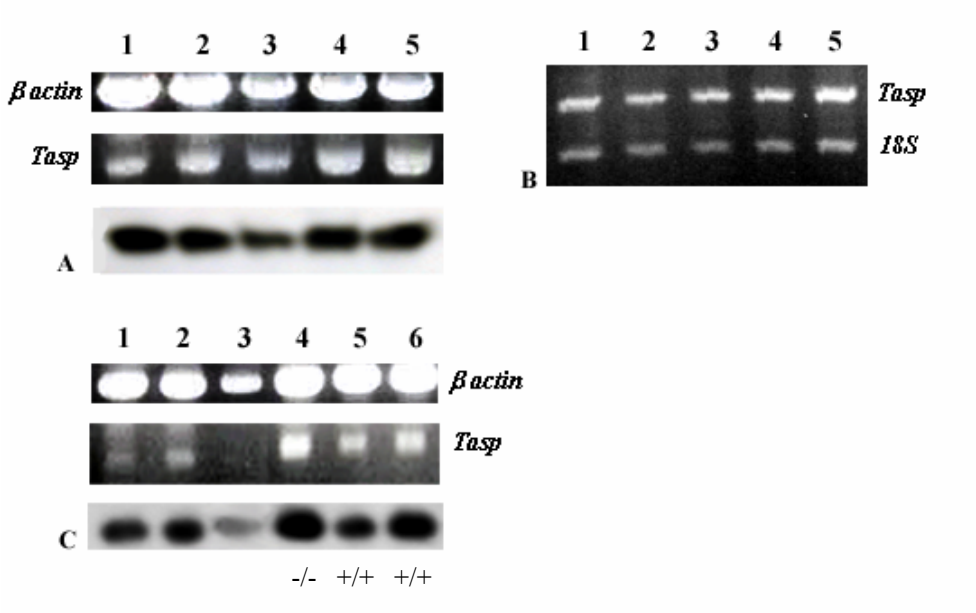


Figure 4.21 RT-PCR analysis of *Tasp* expression in embryonic and adult mouse

Figure A: RT-PCR amplification of RNA extracted from whole embryonic mouse tissue from stages E10 (*lane 1*), E12 (*lane 2*), E14 (*lane 3*), E16 (*lane 4*) and E18 (*lane 5*). *β actin* (*top*) was coamplified with the 429 bp *Tasp* sequence (*middle*). Southern blot hybridization was subsequently performed with the *Tasp* probe (*bottom*) for validation of the RT-PCR product.
Figure B: PCR amplification using *Tasp* primers and an *18S* primer:competimer mix for mouse E10 (*lane 1*), E12 (*lane 2*), E14 (*lane 3*), E16 (*lane 4*) and E18 (*lane 5*) RNA samples.
Figure C: RT-PCR analysis of *Tasp* expression in the adult mouse forebrain (*lane 1*), hindbrain (*lane 2*), and spinal cord (*lane 3*). *Tasp* was also expressed in P1 mouse hindbrain and spinal cord tissue (*middle, lanes 4-6*), in both *Hoxa2* mutant (*lane 4*) and wildtype mice (*lanes 5* and *6*). Analyses of *Tasp* by RT-PCR showed expression in both the adult mouse forebrain and hindbrain (*middle*, *lanes 1* and *2*) but not in the spinal cord. However, southern blot analysis showed the presence of low levels of *Tasp* in the spinal cord as well (*bottom*, *lane 3*).

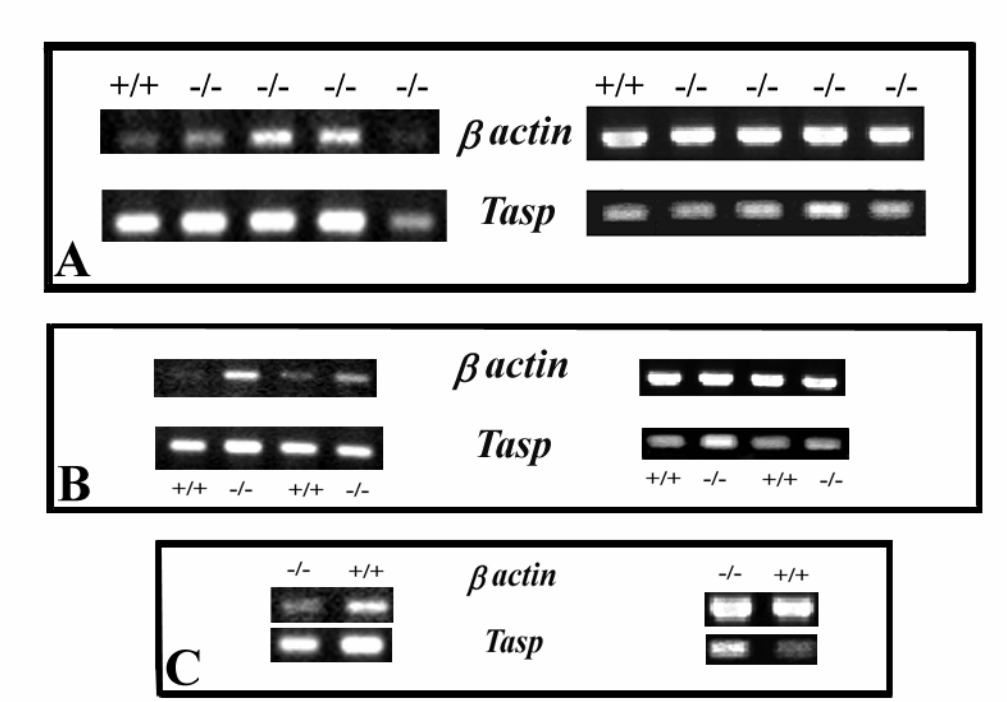


Figure 4.22 *Tasp* expression in P1 *Hoxa2* mutant and wildtype mice

Tasp expression was analyzed by RT-PCR using RNA from both *Hoxa2* mutant and wildtype hindbrain and spinal cord tissue. Amplification of *Tasp* by RT-PCR is shown for litters 4 (Figure A), 7 (Figure B), and 2 (Figure C). *Tasp* was coamplificated with *β actin* as an internal control. PCR amplification of the RT reactions was performed using two different concentrations of *β actin* primers, 0.1 μM (*left*, Figure A, B and C) and 0.2 μM (*right*, Figure A, B, and C). Agarose gels were subsequently used for density measurements for normalization of *Tasp* expression values with *β actin* (Figure 4.23).

Tasp expression in *Hoxa2* mutant and wildtype mice was analyzed by comparing density measurements of RT-PCR products separated by gel electrophoresis (Figure4.23). RT-PCRs performed using 0.2 μM concentration of *β actin* primer were analyzed with a densitometer (Figure 4.23). Three litters were assessed for *Tasp* expression in *Hoxa2* mutant mice and their wildtype littermates. Normalized density measurements for *Tasp* were plotted for individual litters (Figure 4.23 B, C and D) and also as total mutant versus wildtype (Figure 4.23 A). Overall, there appeared to be a trend of increasing *Tasp* expression in the absence of *Hoxa2* (Figure 4.23 A). However, the degree of change in *Tasp* expression was variable between litters.

A normality test for equal variance indicates that the data follow a Gaussian distribution. Statistical analysis using a one-tailed unpaired t-test (assuming equal variance) shows that the differences in *Tasp* expression between *Hoxa2* mutant to wildtype mice are significantly different (p=0.03).

4.6 RT-PCR Analyses of *Tasp* Expression during Overexpression of Hoxa2 in HeLa cells

The human HOXA2 displays over 94% identity with the murine Hoxa2 protein, and has within its C-terminal sequence the J3 peptide that was used for the production of anti-Hoxa2 polyclonal antiserum (Hao et al., 1999). HeLa cells, which endogenously express HOXA2, were used for overexpression of murine Hoxa2 by transfection with pRSV-Hoxa2 (Figure 4.24 A and B). Western Blot analysis of protein extracted from HeLa cell culture showed Hoxa2 migration at the 62 kDa molecular weight as opposed to 42 kDa in nuclear cell extract from murine P1 tissues (Figure 4.24 C, *lanes 2-5*) and whole cell mouse embryonic protein (Hao et al., 1999). However, protein

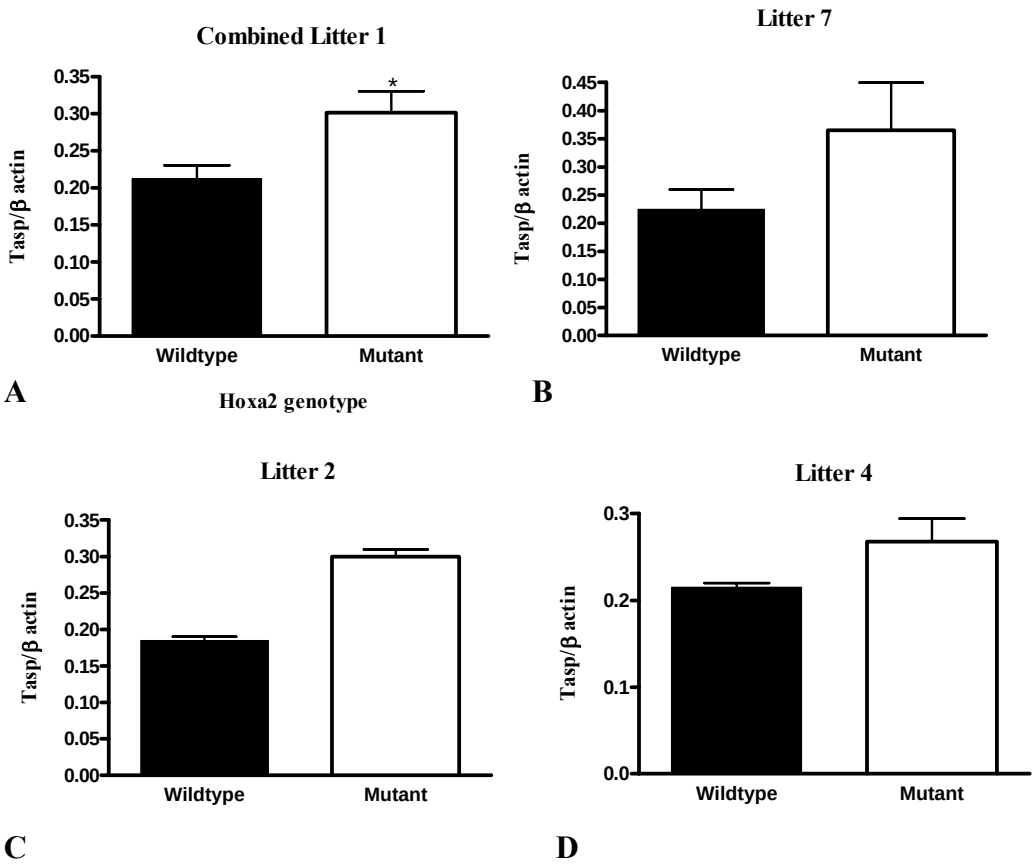


Figure 4.23 Analysis of normalized *Tasp* expression in *Hoxa2* wildtype and mutant mice

Density measurements of *Tasp* RT-PCR were normalized to *β actin* amplified with 0.2 μM *β actin* primer, corresponding to samples observed in Figure 4.22 A, B and C (*right*). In total, three litters were analyzed for *Tasp* expression changes in the hindbrain and spinal cord tissue of mutants as compared with their wildtype littermates (Figure B, C, and D). In figure A, changes in *Tasp* expression for mutants compared with wildtype are shown by comparing the total values for all litters. All PCR reactions were performed in duplicate. A one-tailed t-test shows that expression of *Tasp* in *Hoxa2* mutant mice is significantly higher (**p*<0.05) than in *Hoxa2* wildtype mice. Error bars represent SD, with an *n*=4 for wildtype and *n*=7 for mutant for the combined litter.

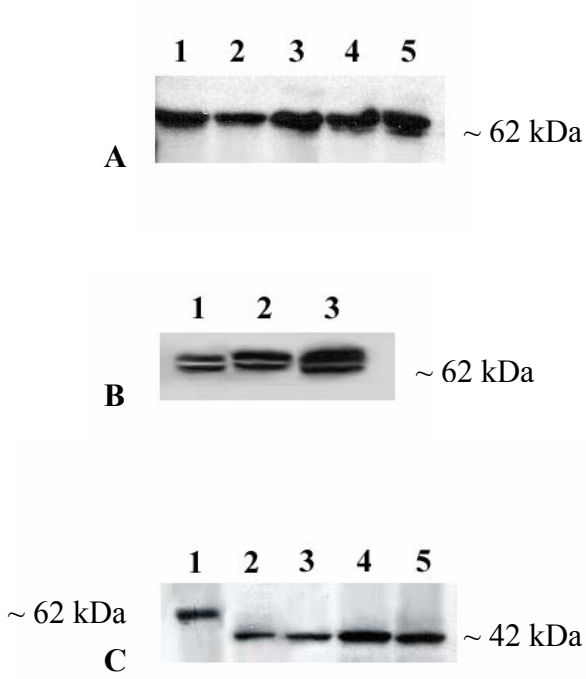


Figure 4.24 Hoxa2 expression in HeLa cells transfected with pRSV-Hoxa2

Figure A: Western Blot analysis using anti-Hoxa2 antibodies on 20 μg protein extract from HeLa cells transfected with pRSV-Hoxa2 at concentrations of 0, 0.05, 0.25, 0.5 μg (*lanes 1*, *2*, *3*, *4* and *5* respectively). Figure B: Western Blot analysis using 10 μg of protein extracted from HeLa cells transfected with 0, 1, 2 μg of pRSV-Hoxa2 (*lanes 1*, *2*, and *3* respectively). Expression of Hoxa2 in a mammalian system displayed the presence of a doublet of protein bands with very similar molecular weights that were difficult to distinguish with higher amounts of Hoxa2. Western Blot analysis of Hoxa2 protein present in 20 μg of nuclear extract from the hindbrain, spinal cord, heart and lung tissue of P1 mice (Figure C, *lanes 2*, *3*, *4* and *5* respectively) showed Hoxa2 at the expected 42 kDa size. However, whole cell protein extracted from the P1 eye appeared to be 62 kDa (Figure C, *lane 1*).

extract from murine P1 eyes also displayed migration of Hoxa2 at 62 kDa (Figure 4.24 C, *lane 1*). Also, at lower concentrations two protein bands with very similar molecular weights were observed when Hoxa2 was expressed in mammalian cells (Figure 4.24 B, *lane 1*).

The predicted human ortholog of the mouse *Tasp* gene is *HTRA3* (Accession number, gi:24475740 NCBI/Entrez), which displays over 87% identity with the coding sequence of the mouse *Tasp* gene. The gene specific primers for the mouse *Tasp* gene anneal at positions 1328-1747 of the human *HTRA3*, resulting in amplification of a 419 bp fragment (Figure 4.25). HeLa cells endogenously expressed *HTRA3*, and overexpression of Hoxa2 in HeLa cells with transfections of 0, 0.05, 0.25, and 0.5 μg of pRSV-Hoxa2 did not have any effect on *HTRA3* expression when normalized to *β actin*. Transfection with 1 and 2 μg of pRSV-Hoxa2 showed a slight decrease in *HTRA3* expression (Figure 4.26), although differences in expression when assessed by one-way ANOVA test are not significant (p=0.32).

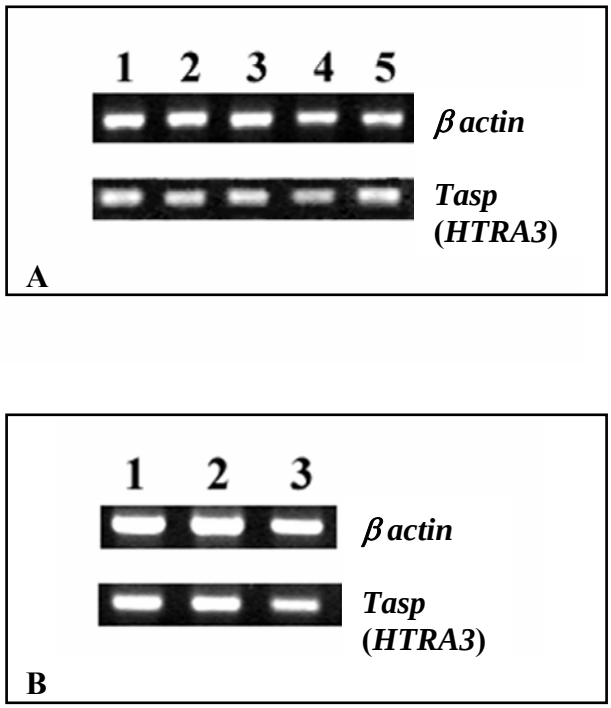
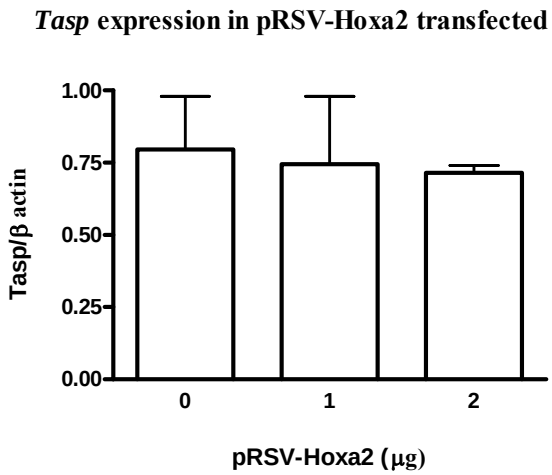


Figure 4.25 RT-PCR analysis of *Tasp* expression in pRSV-Hoxa2 transfected HeLa cells

An analysis of *Tasp* expression using RT-PCR was performed on RNA from HeLa cells transfected with pRSV-Hoxa2. PCR amplification of the human *HTRA3* using mouse *Tasp* specific primers results in a 419 bp fragment. Figure A: Overexpression of Hoxa2 in HeLa cells did not show a significant change in human *HTRA3* (*Tasp*) expression in cells transfected with 0, 0.05, 0.25, 0.5 μg (Figure A, *lanes 1*, *2*, *3*, *4* and *5*, respectively). Figure B: Transfections using higher concentrations of pRSV-Hoxa2 at 0, 1, 2 μg (*lanes 1*, *2*, and *3*, respectively), also did not significantly affect *Tasp* expression.



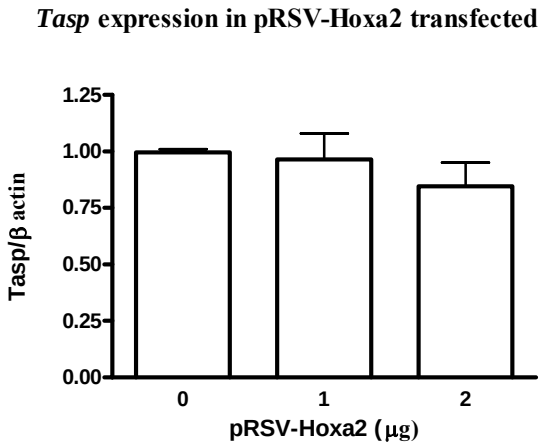


Figure 4.26 Effects of Hoxa2 overexpression in HeLa on *Tasp* mRNA levels

Expression of *Tasp* from RT-PCR was normalized with *β actin* for HeLa cells transfected with 0, 1, 2 μg of pRSV-Hoxa2. Analyses were performed for two independent transfections in triplicate using 0.2 and 0.1 μM of *β actin* primer (top and bottom respectively). Analysis using a one-way ANOVA showed that overexpression of Hoxa2 did not significantly alter *Tasp* expression in HeLa cells, $p>0.05$ for all three groups. Error bars represent SD.

5. DISCUSSION

Hoxa2 is expressed throughout development from E10 into adulthood and may play a role in dorsoventral patterning (Hao et al., 1999). Isolation of *in vivo* downstream targets of Hoxa2 by immunoprecipitation from chromatin preparations of E18 spinal cord and hindbrain tissue would be helpful in identifying putative target genes through which Hoxa2 may specify dorsoventral patterning.

5.1 Limitations of Study

Chromatin immunopreciptiation (ChIP) has become a more popular method of studing DNA binding factors *in vivo*. The advantage of using this system is the ability to study previously unknown genes that are targets of transcription factors. The targets are isolated in the presence of regulatory cofactors and thereby reflecting their true *in vivo* environment. Also, this approach allows for identification of direct downstream targets in previously uncharacterized pathways. It is of importance to note that several improvements of this method have been established since its use in this study. It has been found that optimization of protein-DNA crosslinking is dependent on the anatomical region of the tissue, the developmental stage, cell number and the protein factor that is being analyzed (Kurdistani and Grunstein, 2003; Wells and Farnham, 2002; Zhou et al., 2004). Also, digestion of chromatin DNA is now mostly performed using sonication since *DNase* I digestion is more difficult to control. Before immunoprecipitation is performed, sonication is optimized for the production of genomic fragments that are 200-1000 bp in length. Also, chromatin preparations are

precleared with Protein A, BSA or sonicated DNA from calf thymus or salmon sperm to reduce non-specific binding. Controls are sometimes performed that consist of either immunoprecipitation using no antibody, or chromatin extracted from tissues where the transcription factor is not present.

The efficiency of target gene isolation is also dependent on a variety of factors, namely the use of high affinity antibodies. In some instances researchers have purified polyclonal antibodies multiple times to ensure high affinity (Weinmann and Farnham, 2002). Also, the use of antibodies produced against whole protein rather than a peptide region may be advantageous in that some protein-protein interactions within the transcription complex may mask the antigenic region thereby preventing immunoprecipitation. Therefore, antibodies recognizing multiple epitopes may increase the number of protein-DNA complexes recognized. Chromatin preparations are sometimes enriched by performing multiple consecutive immunoprecipitations. Specificity can also be increased by using excess antibody with decreasing amounts of chromatin preparation so that non-specific binding by antibodies is not caused by an excess of protein. The use of cultured cells for chromatin extract rather than embryonic tissue may also simplify interpretation of target gene function, although these targets would be *in vitro* and not *in vivo*.

5.2 Hoxa2 Protein Expression and Purification of Hoxa2 antibodies

Expression of recombinant protein in bacterial cultures commonly results in the formation of inclusion bodies due to misfolding of the recombinant protein within the bacterial cell. The advantage of extracting recombinant protein in the form of inclusion bodies is that high yields of the expressed protein can be separated from most bacterial

cytoplasmic proteins by centrifugation. Recombinant FLAG-Hoxa2 protein was isolated from bacterial cell extract in the form of inclusion bodies by solubilization with guanidine-HCl. One disadvantage of inclusion body purification is that the expressed protein is denatured and may therefore be biologically inactive. As the B579 antibody was produced by an immune reaction to the J3 peptide present within the C-terminal end of the homeodomain (Hao et al., 1999), it was not necessary for the recombinant Hoxa2 to be properly folded for affinity purification of Hoxa2 specific antibodies. However, dialysis prior to affinity purification of the protein was necessary due to incompatibility of the anti-FLAG M2 antibody with guanidine-HCl.

Although purification of recombinant Hoxa2 protein using the FLAG system resulted in very pure protein, the yield of protein was insufficient for use in purification of Hoxa2 specific antibodies. Therefore ammonium sulphate precipitation was utilized to increase the amount of recombinant protein isolated. Salting out of protein is a common procedure for concentrating protein in fractions or as a partial purification step. The 0-30% ammonium sulphate protein fraction was used to partially purify the recombinant Hoxa2 protein. As the concentration of salt in solution increases more proteins become insoluble, therefore partial purification of Hoxa2 was performed at a lower salt concentration.

The final protein mixture of ammonium sulphate precipitated and FLAG purified Hoxa2 was further purified by centrifugation with a Centricon-30, to exclude proteins with a molecular weight lower than 30 kDa. Analysis of the Hoxa2 protein mixture by a CD scan indicated that ~ 30% of the proteins in this solution exhibited an alpha-helical structure. Hoxa2 consists of a helix-turn-helix motif within the homeodomain sequence

and as well as unordered structures within the N- and C-terminal sequences. Therefore the partially purified recombinant Hoxa2 should exhibit mostly alpha helical, type I turn and unordered structures. However, alpha helical proteins are present in many bacterial proteins and therefore CD analysis does not accurately reflect the percentage of Hoxa2 present within the solution. Also, addition of the helical inducer TFE increased the percentage of alpha-helical structures indicating that a lack of alpha helical structures present was due to improper renaturation after purification rather than contamination.

To minimize the amount of contaminating serum proteins present in the Hoxa2 polyclonal antiserum, Protein A sepharose was used to isolate the IgG antibodies. Cross-reactivity of the B579 antiserum to bacterial proteins was not observed when tested against Hoxd1 expressing *E.coli* cultures in western blot analysis (Appendix X). Also, immunohistochemistry using mouse embryonic tissue sections indicated that the subsequent purification of the IgG specific Hoxa2 polyclonal serum using partially purified recombinant Hoxa2 resulted in isolation of Hoxa2 specific antibodies (Wolf et al., 2001).

5.3 Chromatin Immunoprecipitation of Hoxa2-DNA Target Sequences and Identification of potential Hoxa2 Target Genes

Immunoprecipitation of E12 chromatin for the identification of Hoxa2 targets would be advantageous since *Hoxa2* embryonic expression is particularly high at this time point. Also, specification of the rhombomeric segments in the hindbrain occurs early on in development (~ E10) and *Hoxa2* is the most anteriorly expressed Hox gene within these rhombomeres at this stage (see section 2.6). Immunoprecipitation of Hoxa2-DNA targets from chromatin using the method described by Safaei (1997)

resulted in contamination with a precipitant and poor yield in transformants after cloning. Although target sequences were isolated, all clones were less than 100 bp in length. Contamination of the DNA was likely a result of high concentrations of EDTA used for proteinase K removal of protein from the target sequences, described by Safaei (1997) as a working concentration of 0.1 M EDTA. Several washes with 70% ethanol did not remove the precipitant. It is unlikely that contamination was due to a carry over of salts as increased dialysis following immunoprecipitation did not alleviate the contamination, and any salts present in the sample would have been removed by washing the DNA pellet in 70% ethanol. To remove contaminants, samples were dissolved in water and purifed by buffer exchange using Sephadex G-25. However, the subsequent DNA sample contained EDTA after precipitation with ethanol, albeit at lower levels. High levels of EDTA do not affect proteinase K activity (Invitrogen); but instead protect DNA against nuclease activity. However, EDTA can chelate Mg^{2+} which is required for subsequent enzyme reactions such as ligation for cloning. This resulted in isolation of only a few clones from the immunoprecipitation. Subsequent immunoprecipitation with E18 hindbrain and spinal cord chromatin preparations, performed with reduced amounts of EDTA (10 mM), resulted in the isolation of seven potential target sequences. Although anteroposterior specification of hindbrain and spinal cord segmentation is complete at E18, expression and mutant analysis of *Hoxa2* indicates that Hoxa2 may specify dorsoventral patterning at E18-P1 (Davenne et al., 1999; Hao et al., 1999). More specifically, in later stages of development Hoxa2 is likely involved in the specification of neuronal phenotypes within the CNS (Hao et al., 1999; Ohnemus et al., 2001). Therefore, potential target sequences isolated at this

developmental stage may reflect more of a cell specific role for Hoxa2 in differentiation rather than functioning as a global selector gene controlling morphology as is observed with earlier stages of development.

Clone 8 was found to display an identity of 99% with the *E.coli dgt* gene but this information did not provide insight into identification of a potential murine target gene. The *dgt* gene encodes a dGTPase enzyme involved in the DNA repair pathway, where it is responsible for dephosphorylating dGTP to deoxyguanosine and tripolyphosphate (Nakai et al, 1990). Some human and rodent dGTPases have been isolated that are homologs of the MutT and MutY enzymes; however they do not bear any significant identity with the *dgt* gene. Conservation of this sequence from bacteria to mice is suggestive of an evolutionarily conserved element so it is possible that this sequence represents a mitochondrial sequence. However, screening of human mitochondrial DNA databases, as well as other species, yielded no results. Also, clone 4 aligns with several *E.coli* genomic sequences but did not produce high identity (98%) with any vertebrate sequences. Although both clones 4 and 8 were shown to align with various Trace Archive Sequences, these did not appear to be mapped within the present build (v.32) of the mouse genome. It is possible that clones 4 and 8 are a result of contamination of the immunoprecipitation with bacterial DNA. Hoxa2 protein may have promiscuously bound these *E.coli* sequences which both consist of the *in vitro* Antp homeodomain 5' TAAT 3' site, in particular clone 8 has several of these sites present. Carry-over of bacterial sequences may have occured by accidental elution of Hoxa2 protein with Hoxa2 specific antibodies from the AffiGel matrix when purifying the B579 antiserum. The presence of these sequences within the Trace Archive may be due to partial

sequencing of the ends of BAC clones used for Whole Genome Shotgun sequencing of the mouse genome.

Clones 1, 3 and 5 showed no alignments to *E.coli* sequences and therefore were not due to contamination. However, using BLAST alignment only low identitiy (<70%) was observed with multiple mouse sequences in the Ensembl Genome database. These sequences may represent areas of the mouse genome that have not yet been assembled, and may be identifiable in subsequent builds of the mouse genome. Hence, screening a genomic library with the immunoprecipitated target sequences may have been advantageous because it would allow for the isolation of larger genomic fragments that may facilitate identification of the target genes and is not reliant on the amount of information available through the Mouse Genome Browser.

BLAST queries of the Ensemble database identified two of the isolated clones as partial intronic sequences of the murine homolog of the human *DYRK4* gene and the murine homolog of the human *HTRA3*, also known as *Tasp*. Both target genes identified for clone 2 and clone 12 belong to superfamilies that are known to be involved in CNS development. It was expected that as a transcription factor Hoxa2 would bind to promoter sequences for regulation of transcription and therefore sequences isolated by this method would identify putative promoters. There are however many examples of homeodomain proteins acting on regulatory elements within intronic sequences to specify expression within tissues particularly in the CNS (Cvekl et al., 1995; Haerry and Gehring, 1997; Lonigro et al., 2001; Lou et al., 1995; Zhu et al., 2004).

The novel gene that was identified from clone 2 is dual specificity tyrosine phosphorylation regulated kinase 4 (Dyrk4) (as predicted by Ensemble pipeline

analysis), which belongs to a protein family consisting of 6 murine members (Becker et al., 1998). These proteins are so named for their activity in autophosphorylation of tyrosine residues, resulting in their activation and subsequent phosphorylation of other target proteins. The pipeline analysis involves the use of a GeneWise or GenScan prediction followed by confirmation of the exons by comparison to protein, cDNA and EST databases. The Dyrk protein family, including several homeodomain interacting protein kinases referred to as HIPK proteins was identified as a group of co-repressors for homeodomain transcription factors. Using Nkx1.2 homeoprotein as bait, a yeast two-hybrid screen isolated HIPK2 as a potential cofactor that differentially interacted with homeoproteins, and in the case of Nkx1.2 repressed its transcriptional activity (Kim et al, 1998). These cofactors were identified as proteins that affect the function of homeodomain proteins by phosphorylation. The human *DYRK4* has been implicated in neuronal differentiation in retinoic acid induced postmitotic neurons, although the mechanism through which this occurs has not been studied (Leypoldt et al, 2001). The *Dyrk4* gene is the least characterized kinase of the family and its expression and function has not been widely studied. However, recently a large body of research on the function of kinase activity for Dyrk family members has emerged. *Dyrk1a* is expressed in the embryonic and adult cerebellum, brainstem motor nuclei and spinal cord and its mutation is associated with impaired motor dysfunction (Becker et al., 1998; Martinez de Lagran et al., 2004; Guimera et al., 1996, 1999; Marti et al., 2003; Song et al., 1996). Also, the expression analysis of *Dyrk1a* coupled with its position within the so called Down Syndrome Critical Region of human chromosome 21 has implicated it as playing a role in Down Syndrome (reviewed in Hammerle et al., 2003). Dyrk1b has three

different isoforms which are expressed in a variety of mouse tissues and in several carcinomas (Leder et al., 2003). Phosphorylation by Dyrk1a and 1b affects a variety of functions through activation of transcription (Lim et al., 2002 a, b), and was implicated in the regulation of epithelial cell migration (Zhu et al., 2003). They are also involved in the post-translational turnover of cell-cycle regulators (Ewton et al., 2003; de Graaf et al., 2004). DYRK2 was shown to phosphorylate factors involved in transcription and is overexpressed in several adenocarcinomas (Campbell and Proud, 2002; Miller et al., 2003). Apoptosis is modulated in part by kinase activity on the CRE (cAMP Response Element) and CREB (CRE binding protein) pathways by DYRK3 during erythropoiesis (Li et al., 2002). Although both DYRK2 and DYRK3 are localized within the cytoplasm, DYRK1 and its isoforms function within the nucleus. The murine Dyrk4 protein consists of a bipartite nuclear localization signal and is therefore likely a nuclear protein as well (Ensemble Mouse Genome Database; EBI/Sanger Institute).

All of the DYRK family members are implicated in serine and tyrosine phophorylation. Dyrk4 also has the conserved serine/threonine and protein kinase domain present within all family members, and therefore likely functions in a similar manner in phosphorylation of its targets. The substrate specificities for several of the DYRK family members have been determined. Although there were some differences in specificities for various family members, those investigated required the presence of an arginine amino acid on the N-terminal side of their target residue (Campbell and Proud, 2002). Also, several DYRK family members are capable of targeting the same substrate (Campbell and Proud, 2002).

The RIKEN institute, which first identified the *Tasp* cDNA sequence, has designated the name of the murine homolog of *HTRA3* as *Tasp* but no experimental evidence to date suggests that it is related to any of the *Drosophila* proteins involved in cleavage of the Toll ligand Spätzle. Also, the HTRA family of proteins is highly conserved in humans, rat and mice, but there is no evidence of their homology to *Drosophila* serine proteases. The *Drosophila* Toll transmembrane receptor is well known for its important role in dorsoventral patterning of the *Drosophila* embryo. Its function is dependent on binding with its ligand Spätzle, which is a secreted protein that requires activation by a serine protease cascade requiring several protease factors. Activation of Toll results in the initiation of a signaling pathway, which in turn transports the factor Dorsal into the nucleus and hence induces polarization of the embryo (reviewed in: Stathopoulos and Levine, 2002; Armstrong et al., 1998).

The serine protease superfamily consists of a large number of different proteins that function in varying roles. *HtrA2*, or *Omi*, is a mitochondrial specific protease related to cellular stress response, and was shown in mice to cause the neuromuscular disorder of motor neuron degeneration 2 (Gray et al., 2000; Jones et al., 2003). The human *HTRA1* has been studied in cartilage cells where it was found to potentially play a role in the regulation of cell growth, and its overexpression in various cell types caused inhibition of cell growth and proliferation (Hu et al., 1998; Baldi et al., 2002). *Tasp* and *HtrA1* have been found to be upregulated in the mouse uterus during placentation and pregnancy, and low levels of a potential alternative transcript of *Tasp* were also observed (Nie et al., 2003; de Luca et al., 2004). The probable human counterpart of *Tasp*

displayed high expression in human tissues such as the adult brain and heart, with low expression found in numerous other tissues (Nie et al., 2003).

The Tasp protein does have several proteolytic domains and there are multiple Toll-like receptors identified in the murine system, however no evidence of Toll receptor involvement in murine embryogenesis has been observed. Also, the activity of Tasp is further complicated by the presence of several different overlapping protein domains such as: HtrA/DegQ protease, a kazal-type serine protease inhibitor, serine protease V8, a trypsin-family serine protease, PDZ/DHR/GLGF domain, as well as a bipartite nuclear localization signal (for a list of abbreviations see pg xiv-xviii). Recently the murine Tasp, or HtrA3, was found to bind to similar TGFβ signaling protein targets as those previously identified for HtrA1, and also inhibited signaling of BMP-4,-2, and TGFβ1 (Tocharus et al., 2004). The specific role of Tasp regulated TGF signaling in embryonic development has not been identified, and whether these signaling pathways are controlled by Tasp *in vivo* has not been shown. Although pathways downstream of TGF signaling have been shown to regulate the actions of Dorsal in *Xenopus* and contribute to dorsoventral patterning during early development of *Xenopus* and zebrafish, this has not been demonstrated in mice (Shimizu et al., 2000; Takebayashi-Suzuki et al., 2003). However, TGF signaling along with homeobox genes mediate vertebrate asymmetric gene expression along the left-right axis during murine embryogenesis (reviewed in Hackett, 2002). It is important to note that although HtrA1 and HtrA3/Tasp have very similar protein domains and appear to recognize similar signaling targets, only HtrA3/Tasp has a nuclear localization signal. Therefore, HtrA3/Tasp may have a more specific nuclear function *in vivo* that is different from the secreted HtrA1 and perhaps is

more similar to HtrA2, which has been shown to aggregate in the nucleus during cellular response, where it mediates apoptosis (Gray et al., 2000; Fahrenkog et al., 2004).

Both the *Dyrk4* and *Tasp* genes are quite long (>30 kb) and the isolated target sequences are present within introns that are closer to the 3' end of the gene. It is possible that these intronic sequences are not involved in regulation of the gene they are present in but instead regulate genes positioned up or downstream of *Tasp* and *Dyrk4*. This is unlikely for the *Tasp* gene since the closest adjacent gene is >30 kb in distance. Although there have been examples of very long range regulatory sequences that act >50 kb away (Crisponi et al., 2004; Carter et al., 2002), and there are no examples of vertebrate homeodomain proteins acting over such a long-range in regulation of transcription. Also, most examples to date of distal enhancer-promoter interactions describe regulation of genes that reside within gene clusters or are linked, and most consist of highly conserved sequences present in multiple species (Calhoun and Levine, 2003). Interestingly, the specific genes flanking *Dyrk4* and *Tasp* are conserved in various species (chicken, rat and human). Also, in some cases the interchromosomal distance between adjacent genes is also conserved. However, BLAST and ClustalW analysis show that neither target sequence from the introns of either gene is present within the human or rat homologs. *Dyrk4* is flanked by two genes encoding protein kinase A anchoring protein 3 and RAD51 associated protein, which are situated ~16 and 34 kb away respectively from the *Dyrk4* intronic sequence recognized by Hoxa2. If no regulatory effect is observed with Hoxa2 on these target genes, then methods such as FISH and RNA TRAP could be utilized to further study possible long-range activity of these sequences (reviewed in: Dekker, 2003; Carter et al., 2002).

There have also been examples of intronic enhancers regulating other genes by acting as a distal promoter for downstream genes and for alternative transcripts within a gene (Molina et al., 1993; Nakayama et al., 2001; Schausi et al., 2003; Tiffoche et al., 2001; Wijesuriya et al., 1999). Promoter analysis of both sequences using ProScan Version 1.7 (BMIS, CIT, NIH: http://bimas.cit.nih.gov/molbio/proscan/) did not reveal putative eukaryotic Pol II promoter sequences. Exonic sequences have also been shown to influence regulation of transcription (Dirksen et al., 2003; Yanicostas and Lepesant, 1990; Wang et al., 2002 a, b), and part of the chromatin immunoprecipitated sequence for *Tasp* included the exonic region. Therefore, it may be possible that elements within the adjacent exonic sequences are involved in transcriptional regulation as well.

Several Dyrk family members encode alternative transcripts, and the human *DYRK4* has two potential transcripts of 12 and 13 exons (Ensemble Mouse Genome Database; EBI/Sanger Institute). Also, *Tasp* has been shown to encode an alternative transcript present in the uterus. Therefore, the possibility that Hoxa2 is involved in regulation of alternative transcripts should be examined. Although the occurrence of intronic enhancer regulated alternative splicing has been well documented (Dirksen et al., 2003; Scamborova et al., 2004 and referenced therein), homeobox genes have not been implicated in this type of transcriptional regulation. It would be interesting to investigate whether Hoxa2 plays a role in differential regulation of cell or tissue specific transcripts.

5.4 *In Vitro* DNA Binding Analyses of the Immunoprecipitated Target Sequences

It should be noted that *in vitro* binding analyses of the immunoprecipitated clones was performed prior to determining the identity of the target sequences since the mouse genome database was incomplete at that time. Only clone 12 had been identified as a potential RIKEN clone by partial overlap with its sequence and the cDNA, however the genomic sequence was not available at that time. In addition, classification of the target gene was difficult to determine due to the high homology between the serine protease family members. Hence, analysis of *in vitro* binding of Hoxa2 to the potential target sequences was performed on all isolated clones. Both clones 4 and 8 showed binding by Hoxa2 *in vitro*, this is likely due to the presence of several TAAT sites within these sequences resulting in promiscuous binding of Hoxa2 (Ekker et al., 1994).

Analyses of *in vitro* DNA binding of the target sequences indicated that various nuclear factors present in E12 and E18 tissues are able to recognize and bind to these sequences. Supershift assays indicated that Hoxa2 was present in protein complexes binding to the target sequences. E12 NE supershift assays and *in vitro* binding with recombinant Hoxa2 demonstrated binding of Hoxa2 with clone 2 (*Dyrk4*), albeit with low affinity (Figure 4.9 and 4.10 E). Southwestern blot analyses indicated that the phosphorylation state of Hoxa2 may be important in its ability to recognize the *Dyrk4* intronic element, and therefore EMSAs using *in vitro* translated Hoxa2 protein may have been more appropriate. DNA binding analysis using recombinant Hoxa2 protein may also indicate that in the context of the *Dyrk4* target sequence, Hoxa2 requires the presence of stabilizing nuclear cofactors for high affinity DNA binding. Also, the

addition of B579 antiserum resulted in a downshift rather than a supershift with E12 NE (Figure 4.10 E). It is possible that removal of Hoxa2 from the binding complex does not affect the ability of other transcription factors to recognize the *Dyrk4* element and therefore Hoxa2 itself may be a cofactor rather than the primary nuclear factor binding to the DNA. Binding using E18 NE was also observed for clone 2, although resolution of the shift band makes it difficult to determine the number of transcription factors involved.

Clone 12 was bound by nuclear factors present in E12 NE, and supershift assays also indicated that Hoxa2 was involved in recognition of this sequence (Figure 4.10 D). It is possible that multiple shift complexes with similar molecular weights were bound to clone 12. It would therefore be optimal to perform EMSAs with electrophoresis on a larger gel apparatus so that increased separation can occur. Alternatively, decreasing amounts of NE could be used to determine which complex has the highest affinity for this target. Binding with varying amounts of E18 NE showed that three shift bands were present, and supershift assays demonstrated that two of these complexes involved Hoxa2 activity (Figure 4.11 E). It is possible that some of the complexes present for E12 and E18 NE were the same. However, it has been hypothesized by researchers that homeodomain protein activity is regulated by the presence of cell and tissue restricted expression of various cofactors, which in turn increase the specificity of their target sequences (see Section 2.3; Chariot et al., 1999a; Vigano et al., 1998). Binding by E18 NE would more accurately reflect the activity of Hoxa2 on *Tasp* since ChIP was performed on CNS tissue from this developmental stage. The use of *in vitro* translated Hoxa2 in EMSAs with clone 12 demonstrated that Hoxa2 is capable of binding to this

target in the absence of cofactors (Figure 4.13). It would be interesting to determine whether one or both the 42 or 48 kDa *in vitro* translated Hoxa2 protein were active in binding to the target sequence. Binding analyses of *Hoxa2*$^{-/-}$ NE indicated that although Hoxa2 is required for binding of some protein complexes to clone 12, it is neither present nor required in all complexes involved in binding these DNA sequences. It is possible that one of the protein complexes observed for the mutant NE involved activity of Hoxb2 or another homeodomain that is capable of functionally compensating for Hoxa2. Also, expression of *Tasp* is observed in a variety of tissues throughout development and into adulthood, and expression of Hoxa2 has not been observed in several of these tissues. It is likely that *Tasp* is co-regulated by multiple transcription pathways and plays an important role in the cell.

In vitro binding analysis of clones 1, 3 and 5 was also performed. Clone 1 demonstrated binding with nuclear factors present in E18 NE, but it was difficult to assess how many shift complexes Hoxa2 was involved in (Figure 4.11 F). Analysis using P1 NE from *Hoxa2*$^{+/+}$ and $^{-/-}$ mice showed two shift complexes that involved Hoxa2 recognition of clone 1 (Figure 4.12). However, there are larger molecular weight complexes present in mutant NE that were capable of binding to clone 1 in the absence of Hoxa2. Binding with *in vitro* translated Hoxa2 indicated that recognition of clone 1 by Hoxa2 was direct rather than through protein-protein interactions (Figure 4.12). As both clones 1 and 12 are longer sequences (>200 bp) it is possible that multiple protein complexes recognized these targets due to the presence of several transcription binding sites. It is therefore not surprising that Hoxa2 was not involved in recognition of these targets for all of shift complexes that were observed by EMSA. Binding analysis of

clone 3 was performed using E12 NE (Figure 4.11 C), where two shift complexes containing Hoxa2 were observed. EMSA performed on clone 3 with E18 NE resulted in unresolved shift bands retained at the top of the gel (Appendix XI). Clone 3, which is the longest sequence isolated by ChIP, is 272 bp in length. Hence, the presence of a large number of binding sites within this sequence may have resulted in an aggregation of large amounts of nuclear protein with the probe making separation of shift bands difficult by electrophoresis. *In vitro* binding analysis of clone 1 may yield better results with *in vitro* translated Hoxa2 rather than NE. Clone 5 demonstrated binding by both E12 and E18 NE (Figure 4.10 B and Figure 4.11 B). Although multiple shift complexes were formed by nuclear factors with clone 5, Hoxa2 was involved in only one shift complex for both E12 and E18 NE.

EMSA is generally performed utilizing probes that range from 30-100 bp for formation of distinct shift bands (Laniel et al., 2001). DNA binding analyses of these target sequences, in particular those targets >100 bp, with NE would be optimal using several shorter (~50 bp) overlapping probes for each target. Binding by nuclear factors and resolution of shift bands for these shorter sequences may enhance their stability during electrophoresis and the use of shorter sequences would also identify the putative Hoxa2 binding element. DNA binding analyses using *in vitro* translated Hoxa2 protein could determine whether these targets are recognized by Hoxa2 through direct recognition of binding site or through protein-protein interactions. Most examples of homeodomain recognition involve direct interactions with a DNA binding element. However, there have been examples where DNA recognition by a homeodomain is not necessary and the regulatory effect of the Hox protein is solely mediated via protein

interactions (Catron et al., 1995; Hussain and Habener, 1999; Suzuki et al., 2003). However, full-length homeobox proteins, particularly those within the 3' end of the cluster, have been shown to display low stability and specificity when binding DNA *in vitro* (Shen et al., 1996). Hence, EMSAs using recombinant and *in vitro* translated Hoxa2 may not be feasible for different target sequences, as was observed when attempting to perform EMSA with recombinant Hoxa2 with the majority of isolated target sequences. Difficulty in using recombinant Hoxa2 for *in vitro* binding analysis is likely due to protein aggregation. This is a common problem during expression of proteins resulting in decreased solubility and stability of the protein. Previous expression of recombinant Hoxa2 has shown this to be a problem, therefore utilizing recombinant Hoxa2 for *in vitro* binding experiments would require optimization of conditions to maximize solubility and stability after purification (Bondos and Bicknell, 2003). Also, purification of the recombinant Hoxa2 may be necessary to remove prokaryotic homeodomain factors that may compete by recognition of similar DNA binding sites or through conserved protein-protein interactions (Kant et al., 2002). Recent attempts by collegues in our research group at purifying soluble forms of GST-Hoxa2 show strong interactions with bacterial factors, which could potentially result in decreased DNA binding activity.

To help determine the specific binding element recognized by Hoxa2, *DNaseI* footprinting of clone 2 (*Dyrk4*) was performed. Although DNA footprinting analysis was attempted with clone 12 (*Tasp*) (Appendix XII), protection of sites greater than 4 bp was not observed. This is likely due to an insufficient amount of nuclear factors bound to the probe or unstable DNA binding. DNA binding analysis using EMSA is generally

more sensitive for detecting DNA-protein interactions unlike DNA footprinting which requires binding of all or most of the probe for protection to be evident. P1 and E18 NE were unable to provide protection for DNA footprinting of clone 2. Although Hoxa2 expression is present at these stages within the CNS, levels of protein are not as high as E12. Therefore, E12 NE was utilized because levels of Hoxa2 expression are highest at this development stage in several different tissues. Footprinting analysis using E12 NE revealed some protection at two sites within clone 2 (Figure 4.14). These sites do not resemble the typical homeobox TAAT site, however they may represent a half binding site for Hoxa2 with various cofactors. It is also possible that binding of Hoxa2 was too unstable for protection and these sites represent other factors involved in regulating *Dyrk4*. Problems associated with a lack of protection against *DNase I* may be alleviated by: using purified Hoxa2 protein rather than NE, or isolation of NE from cell cultures overexpressing Hoxa2.

Analysis of clone 2 using TESS did not reveal previously characterized homeodomain binding sites (Figure 4.15). It should be noted that almost all of the characterized *in vivo* binding sites for Hox proteins have been determined from auto and crossregulatory target genes involving the Pbx and Meis family of cofactors. These mechanisms and sequences appear to be highly conserved in early development, and may not reflect the DNA binding ability of Hox proteins with other types of cofactors at later stages of development. Site B from DNA footprinting is adjacent to both a Zeste and Glucocorticoid Receptor (GR) binding site. It partially overlaps with Hepatocyte Nuclear Factor-4 (HNF4) binding sites as well as NFκB and KBF1 sites. Members of the HNF steroid/thyroid receptor superfamily have important functions in liver and gut

development, but they are not known to play a role in the CNS (Taraviras et al., 1994). NFκB (Nuclear Factor kappa B) is a ubiquitously expressed transcription factor also found within the murine brain that dimerizes with various factors such as KBF1 to regulate transcription in response to various signaling pathways (Bakalkin et al., 1993). The only cooperative interaction documented for homeobox genes and NFκB is with the intestine specific homeodomain Cdx1, which is not a member of the *Hox* gene cluster (Kim et al., 2004). Both clone 2 and 12 have Zeste and GR binding sites present, and clone 12 has a binding site for the nonclustered TTF1 homeodomain (Figure 4.16). It is interesting that a TTF1 binding site is present within the *Tasp* intronic sequence in that it may represent possible regulatory interactions between homeobox factors on the same target sequence.

Zeste is a self-aggregating *Drosophila* transcription factor that is involved in transvection (reviewed in Duncan, 2002). Transvection is a term coined by E.B. Lewis in 1954 to describe the actions of an enhancer acting in *trans* between homologous chromosomes (referenced in Duncan, 2002). This phenomenon is common in *Drosophila* and has been observed for many genes within the homeotic complex. At present there are no proteins identified in vertebrates that exhibit an analogous function to that of the Zeste factor, and transvection has only been implicated in regulation of the β-globin locus (Ashe et al., 1997). Analysis of the structural and functional properties of the Zeste DNA binding domain indicates that it is most related to the Myb and homeodomain structures (Mohrmann et al., 2002). Although Zeste binding sites have been previously identified in the murine *Pax8* promoter, the activity of this site has not been determined (Okladnova et al., 1997).

Site A from clone 2 overlaps with a binding site for Myogenin, which is an early cell differentiation marker. Myogenin is a member of the myogenic bHLH family of transcription factors that are involved in skeletal muscle cell specification (reviewed in Kablar and Rudnicki, 2000). Some family members are expressed during development in the CNS where they function to inhibit neuronal differentiation (Delfini and Duprez, 2004; Kablar, 2002). All myogenic bHLH factors consisting of an E2a domain display a highly conserved N-terminal motif through which cooperative binding to DNA with the homeodomain heterodimers Pbx-Meis2/Prep1 may occur (Knoepfler et al., 1999). *In vitro* binding experiments have shown that Pbx-Meis/Prep1 sites flank the Myogenin binding site within E-box elements (Funk and Wright, 1992), and these sites are bound by Pbx-Meis cooperatively with all four Myogenin family members (Knoepfler et al., 1999). It is possible that Hoxa2 interacts with Myogenin in a manner similar to that of Pbx, or it is possible that Hoxa2 competes with Myogenin for binding to Pbx and Meis.

An interesting example of variability in the DNA binding specificities for homeodomain proteins, with regard to cell or tissue specific expression of a target gene, is the TTF1 homeodomain. This transcription factor is essential for thyroid and lung development, and is well characterized with respect to its structure and DNA binding specificity to the consensus sequence 5' CAAG 3' (Damante et al., 1994; Tell et al., 1998). More recently its role in specification of the rostral brain regions during early development has been shown, and TTF1 is required for CNS specific transcriptional activation of a neuroepithelial marker gene *nestin* (Lonigro et al., 2001). The CNS specific regulation of *nestin* is mediated by an intronic element where TTF1 does not recognize its canonical consensus sequence. Instead, transcription is activated through a

binding site resembling a nuclear hormone/cAMP response element that is very different from the typical TTF1 binding site. This sequence is essential for forebrain specific expression of *nestin* mediated by DNA binding of TTF1 *in vivo*, even though it is flanked by two 5' CAAG 3' motifs. Also, analysis of Hoxb2 DNA binding in the regulation of *Otx2* demonstrates that it recognized a 5' ACTT 3' repetitive sequence that does not resemble the typical Hox consensus sequence (Guazzi et al., 1998). Therefore, homeodomain proteins are capable of recognizing entirely different sequences in specific cell types, even in the presence of a typical binding site.

The Glucocorticoid Receptors have been known to mediate a transcriptional response by either binding to the Glucocorticoid Response Element (GRE), or through protein-protein interactions with transcription factors bound to DNA (reviewed in Almawi and Melemedjian, 2002). The only known regulatory interaction between GR and homeodomain proteins is between the ubiqitiously expressed Oct1, Pbx and Pit1 (Nalda et al., 1997; Subramaniam et al., 1997, 1998). The mechanism of this regulation involves competition for DNA binding of the same target element within a promoter sequence. Expression of the DNA binding domain of the GR is able to block the activity of Pbx-Oct1, or Pit1 on the target sequence resulting in repression of transcription.

The *in vitro* Hoxa3 half site present within clone 12 (Figure 4.16) that was identified by Patch analysis using *TRANSFAC5.0* may not be of significance. This site was found to display intermediate affinity for Hoxa3 and was identified by *in vitro* binding selection to random oligomers rather than an actual *in vivo* target gene (Catron et al., 1993).

ClustalW alignment (service at European Bioinformatics Institute: http://www.ebi.ac.uk/clustalW) was used to identify if a Hoxa2 consensus binding site was present in all targets. Alignment of all seven clones did not reveal any clear consensus sites, and alignment using only clone 2 and 12 also did not yield any consensus sites greater than 4 bp in length. However, a comparison of the Hox-Pbx consensus site identified by Lampe et al. (2004) with each clone reveals the presence of 5 sites displaying some similarity with the bipartite sequence (Figure 5.1). Although a clear 10 bp consensus sequence is cannot be found, there is a core sequence present in all five sites consisting of 5′ CCATCT/A 3′ that may represent a novel Hoxa2 *in vivo* binding site. It is interesting to note that the core CCATC sequence is also present in clone 2 adjacent to the myogenin binding site within site A (Figure 4.15). It is possible that this is a bipartite sequence recognized by Hoxa2 and myogenin. The recognition of clone 2 and 12 is likely mediated through different DNA binding sites due to cooperative binding with specific cofactors for each target gene, this would then potentially involve distinct binding elements. It is common for the core binding sequence to be conserved in DNA recognition by homeodomain proteins *in vitro*, with variability of the 3 nucleotides flanking the core (Liberzon et al., 2004).

5.5 Regulatory Activity of the *Dyrk4* and *Tasp* Intronic Sequences in Cell Culture

Luciferase assays for the activity of clone 2 and clone 12 in transient transfection of HeLa cells indicated that there is a trend of inhibition of transcription by Hoxa2 for clone 12 (*Tasp*) (Figure 4.18). For clone 2 (*Dyrk4*), Hoxa2 functions as an enhancer of transcription (Figure 4.19). The presence of the *Tasp* intronic element increased CMV

Hoxa2-Pbx bipartite consensus sequence (Lampe et al., 2004):

T/A/C G/C A/T T A/C A/C ATC A/T

Alignment of potential Hox-Pbx sites in clone 12:

GGTGGCCATCG
CCTTGCCATCC
CGATGCCATCA
CTGTCCTATCA
CACCACCATCT

Figure 5.1 Alignment of sites present in clone 12 that display some similarity with the Hoxa2-Pbx bipartite sequence.

All sites are shown in a 5′ to 3′ orientation. Five sites with some similarity to the Hox-Pbx sequence are aligned above. Grey boxes indicate the presence of a consensus nucleotide from the Hox-Pbx binding sequence within the clone 12 site. Overlap of the five sites found within clone 12 show that although the exact Hoxa2-Pbx site is not found, there are multiple sites that are similar with the bipartite consensus sequence. Alignment of these sites shows a 6 bp consensus site of CCATC A/T.

regulated expression of luciferase, but increasing concentrations of Hoxa2 resulted in a decrease in luciferase activity. Hoxa2 appeared to mediate an opposing effect on the *Dryk4* intronic sequence, where cotransfection with Hoxa2 resulted in increased luciferase activity. Although changes in luciferase activity were not significant according to one-way ANOVA testing, the same decreasing trend of luciferase activity at several different reporter:control vector concentrations is apparent. Non-significant *p* values may become significant by transfection with higher amounts of Hoxa2 (ie. 5 µg of pRSV-Hoxa2) than those used in our studies. Also, the number of transfections performed at a particular reporter concentration may also increase the statistical significance of the changes. A lack in significant differences may be due to the lack of cell specific cofactors required by Hoxa2 for transcriptional activity. A previous analysis of homeodomain intronic enhancers has shown their requirement *in vivo* for tissue specific expression while transfection studies were unable to demonstrate this requirement in cell culture (Dusing et al., 2001). Although HeLa cells are commonly used for transient transfection with a variety of transcription factors many of which are homeodomain proteins, it may not be an optimal cell culture system for Hoxa2. Western blot analysis of Hoxa2 expressed in HeLa cells demonstrated that a post-translational modification of Hoxa2 occured in this cell type, which is not observed in NE from mouse P1 CNS tissues (Figure 4.24). If this modification is involved in proteolytic targeting of Hoxa2, overexpression of Hoxa2 in this cell type may not result in increased levels of active Hoxa2 protein. In addition, this modification may alter the ability of Hoxa2 to interact with other nuclear factors and therefore may inhibit its ability to act on the target sequences. Hoxa2 transfection into cell culture studying its autoregulatory

effects have been performed in COS7 and P19 cells (Lampe et al., 2004). The activity of HOXB2 on the *Otx2* promoter was analyzed using the NT2/D1 embryonal carcinoma cell line (Guazzi et al., 1998). When similar transfection experiments were also conducted in HeLa and NIH3T3 cells, no effect was observed suggesting that the cellular environment plays a critical role in the transactivating activity of HOXB2 (Guazzi et al., 1998). Transfection studies may be optimized by using NT2/D1 cells or P19 cells, which also is a mouse embryonic carcinoma cell line capable of differentiation into many cell types (McBurney, 1993). Treatment with RA at low concentrations causes differentiation of embryonal cells into endodermal and mesodermal derivatives, while high concentrations results in the formation of neurons and glia (Jones-Villeneuve et al., 1982, 1983; Bain et al., 1994). RA is well characterized for its important role in inducing *Hox* gene expression, and treatment of these cells at high concentrations of RA resulted in an upregulation of various *Hox* genes (Pratt et al., 1993). However, due to functional redundancy of *Hox* genes it may also be valuable to assess transcriptional activity of Hoxa2 by underexpression using antisense oligonucleotides or siRNA.

5.6 Analyses of Hoxa2 mediated expression of *Tasp* and *Dryk4 In Vivo*

Analyses of RNA by RT-PCR demonstrated expression of *Dyrk4* and *Tasp* throughout murine embryonic development (E10-18), coinciding with the developmental stages at which Hoxa2 is expressed. Expression of both genes was observed in adult and newborn mouse CNS tissues, and analyses of *Hoxa2*$^{+/+}$ and $^{-/-}$ newborn mice also showed expression of *Dyrk4* and *Tasp* within the hindbrain and spinal cord tissue (Figure 4.21 and Figure 4.22). Although *Dyrk4* and *Tasp* were isolated at the E18 stage of embryonic development, expression of the putative target genes was studied using P1

CNS tissue from mutant and wildtype mice. The gestation period for mice is generally 19-21 days (Kaufman et al., 1992), however *Hoxa2*$^{+/-}$ pregnant mice appear to deliver pups at day 18 or 19. Hence, variation in Hoxa2 regulation of its target genes due to differences in development stages should be minimal.

Band intensity measurements of RT-PCR amplified *Tasp* in *Hoxa2*$^{-/-}$ mice showed a significant increase in expression compared with their wildtype littermates. Changes in *Tasp* expression in the absence of *Hoxa2* appeared to be more severe in some litters, and may reflect the presence of functional redundancy by other *Hox* genes or differences in mutation penetrance (Figure 4.23). This increase in expression of *Tasp* in the absence of Hoxa2 corresponds with the negative regulatory effects of Hoxa2 on the *Tasp* intronic sequence (Figure 4.18).

Overexpression of Hoxa2 in HeLa cells did not produce a reproducible effect on *Tasp* expression as determined by RT-PCR analysis (Figure 4.26). Only a slight decrease in *Tasp* expression was observed after overexpression of Hoxa2. This may be a result of the presence of a negative regulatory feedback loop controlling expression of *Hoxa2* or *Tasp*. As previously discussed above, HeLa cells may lack the necessary cofactors required by Hoxa2 for strong regulatory control, and overexpression of Hoxa2 in this cell type may not represent levels of functionally active Hoxa2. The transfection efficiency was assessed by cotransfection with a GFP expressing vector followed by visualization by fluorescence microscopy (Appendix XIII). Although the number of GFP expressing cells was fairly consistent for each plate (~ 30%), this is only an approximate assessment of transfection efficiency. It may be necessary to normalize the *Tasp*/*β actin* values further with measurement of an internal control for transfection

efficiency such as *Renilla* luciferase or β galactosidase. However, it has been shown that expression of *β actin* is altered in cell cultures between treated samples and can therefore be unreliable under certain conditions as an internal control (Selvey et al., 2001).

Levels of detected *Dyrk4* expression in P1 CNS tissues were low and analysis of the *Hoxa2*$^{-/-}$ and *Hoxa2*$^{+/+}$ mice did not produce reliable results. Although BLAST analysis of the primers designed for RT-PCR did not show significant cross-reactivity with other mouse EST sequences, it is still possible that an uncharacterized transcript is competing with *Dyrk4* for one of the primers used in our study. RT-PCR analysis may require designing alternative primers to various areas within the *Dyrk4* cDNA sequence. Also, one-step RT-PCR using gene specific primers rather than the two-step method using random primers may be more specific and therefore more sensitive in amplification of *Dyrk4*. RT inhibition of PCR amplification has been shown to occur for low copy number mRNAs and in some instances may be overcome by the addition of stabilizing factors such as the T4 gene 32 protein (Chandler et al., 1998).

Although density readings for RT-PCR provide some measure of quantitation, highly sensitive and accurate detection of expression changes by RT-PCR requires Quantitive Real Time PCR (reviewed in Bustin, 2000). It is also possible that levels of target gene expression do not significantly change, but instead their boundaries of expression are modified in *Hoxa2*$^{-/-}$ mice. Therefore, gene expression analysis using *in situ* hybridization histochemistry of mouse tissues may provide more information of Hoxa2 regulation of *Tasp* and *Dyrk4*.

5.7 Potential Post-translational modifications of Hoxa2

Translation of Hoxa2 mRNA in an *in vitro* system resulted in the appearance of a doublet protein, which was also observed when expressed in mammalian HeLa cell culture. Also, Hoxa2 expressed *in vitro* and in mammalian systems displayed an increase in molecular weight, 48 and 62 kDa respectively (Figure 4.12 C, D and Figure 4.24). It is unlikely that multiple products of Hoxa2 in the *in vitro* translated system were due to proteolytic degradation, since the observed transcripts are above the expected size of 41 kDa.

Although the murine Hoxa2 protein has been isolated by other research groups in mammalian cell culture systems, no biochemical analysis of the protein was presented nor was any data presented on the presence of potential post-translational modifications of the protein (Lampe et al., 2004; Matis et al., 2001). Expression of the murine Hoxa2 cDNA as a transgene under the control of a *heat-shock promoter* in *Drosophila* also produced Hoxa2 as a 62 kDa protein as demonstrated by western blot analysis (Percival-Smith and Laing Bondy, 1999). However, Hoxa2 expressed using a weaker promoter in *Drosophila* was observed by these researchers as migrating at a lower molecular weight (~ 42 kDa), the only difference between the two types of expression was high levels of transient expression (62 kDa) as opposed to lower levels of sustained expression (42 kDa) (Percival-Smith and Laing Bondy, 1999). The western blot detection of Hoxa2 was performed using commercially produced rabbit polyclonal Hoxa2 antibodies raised against a Hoxa2 peptide (Percival-Smith and Laing Bondy, 1999). High levels of transient Hoxa2 expression could in cell culture or *in vivo* activate a mechanism in which regulation of Hoxa2 occurs by post-translational modifications of the protein.

Regulation of many transcription factors occurs at the post-translational level; more specifically regulation of several homeodomain proteins is in part controlled through ubiquitin mediated proteolysis (Kurtzman et al., 2000; Gabellini et al., 2003; Zhang et al., 2003).

Ubiquitination with subsequent proteolytic degradation of some proteins has been associated with the presence of PEST sequences, which are sequences rich in the amino acids proline (P), glutamic acid (E), serine (S) and threonine (T) targeted to the 26S proteasome (Yaglom et al., 1995; Won and Reed, 1996; Chen et al., 1998b; Marchal et al., 1998). The PEST motif is important in degradation of the zebrafish Vsx-1 homeodomain protein and may be involved in modulating neuronal differentiation, as well as restricting its expression to specific cell types (Kurtzman et al., 2000). For the *Drosophila* Bicoid (Bcd) protein the PEST sequence determines whether Bcd functions as a translational repressor or as a transcriptional activator (Niessing et al., 1999). Analysis of the 372 aa Hoxa2 sequence with the program PESTfind (EMBnet Austria, Vienna Biocenter: http://vienna.at.embnet.org/htbin/embnet/PESTfind; Rechsteiner and Rogers, 1996) showed the presence of several sequences with poor PEST probability within the N- and C-terminal ends of the proteins. The putative PEST sequences within the N-terminus present with a poor score of -5.30 and -1.05 (at positions 7-38 and 44-64), while the C-terminal PEST sequences have a more significant score of +1.09 and +0.84 (at positions 242-271 and 285-369). Although scores above +5 are considered to be of great importance, scores above 0 are considered to be possible PEST regions (Rechsteiner and Rogers, 1996). Also, in several cases phosphorylation plays a role in activating latent PEST signals, converting a weak PEST into one that is recognized

(Marchal et al., 1998). All six of the putative casein kinase II phosphorylation sites of the Hoxa2 sequence occur within the potential PEST sequences, one within the N-terminal sequence and the remaining five are restricted to the C-terminal end of the protein (Tan et al., 1992). Casein kinase II has been implicated in regulating ubiquitination of several proteins and PEST mediated proteolysis (Pando and Verma, 2000; Penrose et al., 2004), and therefore may play a role in post-translational modification of Hoxa2.

Alternatively, the increased molecular weight of Hoxa2 could be the result of a modification where the covalent attachment of a protein or peptide to cytoplasmic Hoxa2 is cleaved on entry into the nucleus. Hence, it would be expected that in the nucleus Hoxa2 would be present as a 42 kDa protein, which is observed in NE from various murine tissues as opposed to the 62 kDa protein found in whole cell protein extracts of the murine P1 eye (Figure 4.24). Proteolytic cleavage of proteins has been shown to be essential in the nuclear translocation of various membrane associated proteins and cell signaling molecules (Ryo et al., 2003; reviewed in Hoppe et al., 2001). However, this has not been demonstrated for any homeodomain proteins. Also, this mechanism generally involves cleavage of multi-subunit proteins or of precursor molecules into their active form. Therefore it is highly unlikely that post-translational modification of Hoxa2 is required for transport, and then removed upon translocation to the nucleus. In fact, most identified post-translational modifications of homeodomain proteins thus far occur within the nucleus or are required for nuclear import and/or transcriptional activity and are therefore predominantly found to be a higher molecular

weight protein in the nucleus versus in the cytoplasm (Kishi et al., 2003; Lefebvre et al., 2002).

HeLa cells as well as pRSV-Hoxa2 expressing HeLa cells display migration of Hoxa2 at 62 kDa, indicating that it is unlikely for the discrepancy in molecular weight of Hoxa2 to be solely a result of overexpression, at least in the context of this particular cell type. It is possible that levels of the 62 kDa cytoplasmic Hoxa2 are much higher than the nuclear 42 kDa Hoxa2 protein, which would then require longer exposure with chemiluminescence for visualization of the 42 kDa band. It is more likely that this difference in molecular weight is a result of cell-specific expression, which has been observed for several post-translationally modified homeodomains (Heimberg et al., 2000; Kurtzman et al., 2000; Wall et al., 1992).

The homeodomain Pdx1 requires SUMOylation (SUMO is small ubiquitin-related modifier) for its nuclear localization, as well as stabilization by inhibiting its degradation by proteasomes (Kishi et al., 2003). Although Hoxa2 only displays motifs that have a low probability of SUMOylation as determined by computational sequence analysis (SUMOplot^{TM} by ABGENT, San Diego CA, USA: www.abgent.com/sumoplot.html), perhaps a similar ubiquitin like molecule is involved in transportation of Hoxa2 to the nucleus, proteolytic degradation, or increased protein stability in specific cell types.

Many other post-translation modifications have been observed for homeodomain proteins such as acetylation (Chariot et al., 1999b; Li et al., 2000; Scaloni et al., 1999; Yi et al., 2002; Iioka et al., 2003), glycosylation (Lefebvre et al., 2002; Gao et al., 2003), and phosphorylation (Adachi and Lewis, 2002; Jaffe et al., 1997; Yi et al., 2002; Melamed et al., 2002). It is unlikely that the differential molecular weight is due to

acetylation in part because most documented cases of homeodomain acetylation occur in the nucleus by binding with p300 acetyltransferase on a specific regulatory DNA sequence. Also, the increase in molecular weight would be very low as acetyl groups added to lysine residues are small. However, acetylation of several homeodomains occurs at conserved lysine residues and therefore is a potential regulatory mechanism for homeodomains in general (Li et al., 2000; Iioka et al., 2003). Acetylation has been shown for homeodomain proteins to regulate cofactor interactions and thereby affect DNA binding in the regulation of gene transcription (Chariot et al., 1999b; Iioka et al., 2003).

Many eukaryotic proteins are modified by covalent attachment of *O*-linked *N*-acetylglucosamine on their serine and threonine side chain hydroxyls or *N*-linked glycosylation on the amino residues of their asparagine side chains. Cytosolic and nuclear proteins are *O*-linked glycosylated and many of these nuclear proteins are transcription factors (reviewed in Comer and Hart, 1999). An analysis of the Hoxa2 sequence using the YingOYang 1.2 Server and NetOGlyc 3.1 (Center for Biological Sequence Analysis, Technical University of Denmark: http://www.cbs.dtu.dk/services/) indicated the presence of multiple putative glycosylation sites, and hyperglycosylation of proteins can result in a marked increase in their molecular weight. None of the putative glycosylation sites identified for Hoxa2 overlap with its potential phosphorylation sites, and most reside within the N-terminal region of the protein. Thus far only *O*-linked *N*-acetylglycosylation of homeodomians has been documented for the non-clustered homeodomain proteins Pdx1 and Pax6, although the specific effect of this glycosylation on their activity is not known (Gao et al., 2003; Lefebvre et al., 2002). The only

clustered homeobox genes analyzed for glycosylation are *HOXB7*, *HOXC6*, and *HOXD4* (Corsetti et al., 1992). Although these proteins were found to be phosphorylated, no glycosylation was observed. However, this could be a result of the expression of these human homeobox genes using an insect system in that glycosylated forms of protein are often cell or tissue specific, or occurs at specific stages in development (Kane et al., 2002; Hiromura et al., 2003; Shaufele et al., 1990). It is interesting to note that all *O*-linked glycosylated proteins are also phosphoproteins, and that there is a reciprocal relationship between both modifications with regard to regulation of transcription factor function (reviewed in Kamemura and Hart, 2003). Glycosylation of transcription factors has been shown to affect protein-protein interactions by alteration of tertiary structures, as well as function to increase protein stability, and regulation of nuclear import (Comer and Hart, 1999; Kamemura and Hart, 2003). Therefore, the possibility that Hoxa2 is glycosylated merits further study to help understand its function.

Several putative phosphorylation sites have been identified within the Hoxa2 sequence (Tan et al., 1992), and many homeodomain proteins have previously been shown to be phosphorylated *in vivo* (discussed in Nepveu, 2001; Fienberg et al., 1999; Kasahara et al., 1998; Bourbon et al., 1995; Li and Manley, 1999). Hoxa2 displays four potential phosphorylation sites for protein kinase C, six for casein kinase II, and one for cAMP-dependent protein kinase A (Tan et al., 1992). Several of these putative phosphorylation sites are within the homeodomain of Hoxa2. Phosphorylation of the homeodomain often has been shown to affect the DNA binding ability of various homeodomain proteins. In many instances the result of phosphorylation is decreased DNA binding activity (Berry and Gehring, 2000; Hjerrild et al., 2004; Jaffe et al., 1997;

Vijapurkar et al., 2004; discussed in Nepveu et al., 2001), increased DNA binding (Kasahara and Izumo, 1999), or in some cases both increased and decreased binding activity has been shown depending on the position of the phosphorylated target site (Bourbon et al., 1995; Hjerrild et al., 2004). Other aspects of homeoprotein function are also affected by phoshorylation such as cellular secretion (Maizel et al., 2002), nuclear localization (Elrick and Docherty, 2001), and cellular stability (Zhu and Kirschner, 2002; Yaron et al., 2001). Results from *in vitro* translation and Hoxa2 expression within HeLa cells indicates that there are two forms of Hoxa2 present at very similar molecular weights (Figure 4.12 C, D and 4.24). This information, coupled with sequence analysis of phosphorylation sites and the importance of phosphorylation for other homeodomain proteins, indicates that Hoxa2 is likely phosphorylated. Southwestern blot analysis suggests that DNA binding by Hoxa2 in the context of clone 2 (*Dyrk4* homolog) is affected by its phosphorylation state, as indicated by differential DNA binding in the presence of BSA as opposed to milk (Figure 4.18). This is due to the presence of protein kinase/phosphatase activites in commercially available nonfat dried milk. Therefore, blocking with lipid-free BSA, which contains only trace amounts of these activities, is sometimes preferred (Papavassiliou, et al., 1992; Polycarpou-Schwarz and Papavissiliou, 1995).

5.8 Conclusions

Hoxa2 is a member of the conserved clustered *Hox* gene family and is known to be expressed within murine CNS tissues throughout development and into adulthood. During early development *Hox* genes are required for proper specification of hindbrain and spinal cord morphology. Most Hox targets isolated to date are involved in auto and

crossregulatory mechanisms. The mechanism through which *Hox* genes control development is poorly understood and characterization of their downstream targets will reveal their function in embryonic pathways. Hoxa2 has no known direct downstream targets except for its involvement in rhombomere specific autoregulation. Its function as a morphological regulator in early hindbrain development has been shown through mutation analyses, however its function as a transcription factor expressed in the spinal cord and at later stages of CNS development is poorly understood.

Isolation of downstream Hoxa2 target genes using ChIP resulted in the identification of two potential target genes, the murine homolog of the human *dual specificity tyrosine kinase 4* (*Dyrk4*) gene and the *Toll-associated serine protease* (*Tasp*) gene. In total seven sequences were cloned from immunoprecipitation of E18 hindbrain and spinal cord chromatin. Two sequences resulted from promiscuous Hoxa2 binding to TAAT sites present within contaminating bacterial sequences, and the remaining three sequences are unidentifiable by BLAST query of the mouse genome.

Both clone 2 and clone 12 contain partial intronic sequences from the *Dyrk4* and *Tasp* genes, respectively. Many homeobox proteins regulate cell- and tissue-specific transcription through recognition of intronic regulatory elements (Dusing et al., 2001; Kim et al., 2002; Lampe et al., 2004). *In vitro* binding and transfection analysis indicated that Hoxa2 binds to the *Tasp* intronic element and mediates repression of transcription. Recognition of the target sequence may also involve several different cofactors. *Hoxa2*$^{-/-}$ mutation results in an upregulation of *Tasp* expression within the CNS of newborn mice, indicating that Hoxa2 can act as a transcription repressor *in vivo*. This activity may be mediated by competition for DNA binding sites within the target

sequence between Hoxa2 and another positive regulatory factor. Also, protein-protein interactions may result in the masking of the Hoxa2 activation domain and exposure of a repressor domain. Many Hox genes are known to function as both a transactivator and as a repressor depending on their cellular environment. This has been previously demonstrated for other homeodomain proteins such as HOXA9 (Lu et al., 2003), En (Alexandre and Vincent, 2003), HOXB1 (Saleh et al., 2000), and Pdx (Asahara et al., 1999).

Tasp is a serine protease family member that has been previously shown to interact with TGFβ signaling factors (Tocharus et al., 2004). This signaling pathway is involved in determining dorsoventral patterning within *Xenopus*, and left-right symmetry within the murine system (reviewed in: Heasman, 1997; Whitman and Mercola, 2001). TGF signaling pathways have a key factor in regulating CNS development (reviewed in Böttner et al., 2000). Several members of this superfamily have been implicated in determination of neuronal phenotypes, neuronal proliferation and differentiation, as well as regulation of oligodendroglial differentiation and cell adhesion (reviewed in Böttner et al., 2000). Hoxa2 also has been implicated in specification of neuronal phenotypes (Hao et al., 1999; Ohnemus et al., 2001), and may mediate this role by regulation of the TGF signaling pathway via repression of *Tasp* in specific cell types at later stages of development.

In contrast to the repression of *Tasp* by Hoxa2, *Dyrk4* appeared to be positively regulated by Hoxa2 in transfection assays. *In vitro* binding assays with recombinant protein indicate that this action may be mediated through direct recognition of the intronic sequence by Hoxa2. Expression of *Dyrk4* within the postnatal CNS appeared to

be quite low and further analyses are required to determine if Hoxa2 does indeed play a role as a transcription activator for *Dyrk4 in vivo*.

Although the precise function of Dyrk4 has not been characterized, it potentially may have a function analogous to its family members which are involved in phosphorylation of proteins such as transcription factors. Also, Dyrk4 in cell culture has been implicated in neuronal differentiation and several of its family members function in late neuronal differentiation, neurogenesis and proliferation of neuronal progenitors (reviewed in Hammerle et al., 2003). Hoxa2 regulation of *Dyrk4* may affect the activity of other transcription factors or signaling molecules involved in neuronal development pathways.

Within the hindbrain several target genes such as *Pax6* and *MDK1* are believed to be regulated by Hoxa2. Also, there are many examples of *Hox* auto and crossregulation as already observed for *Hoxa2* (Lampe et al., 2004). It may therefore be expected that isolation of *in vivo* downstream targets of Hoxa2 from the developing CNS would perhaps result in isolation of regulatory elements from these genes. However, these genes are found to coincide with Hoxa2 expression early in CNS development and generally play key roles in A-P patterning and morphogenesis early on in development. Although Hoxa2 may function as a regulator of anteroposterior patterning in early development, it may also play a role in dorsoventral patterning at later stages of development. Also, previous studies of Hoxa2 expression and the effects of its mutation have revealed a possible role in the specification of neuronal cell types (Hao et al., 1999; Ohnemus et al., 2001). Therefore, regulation of the *Dyrk4* and *Tasp* genes may reflect a

cell specific role for Hoxa2 in neuronal specification within the later stages of CNS development.

5.9 Future Directions

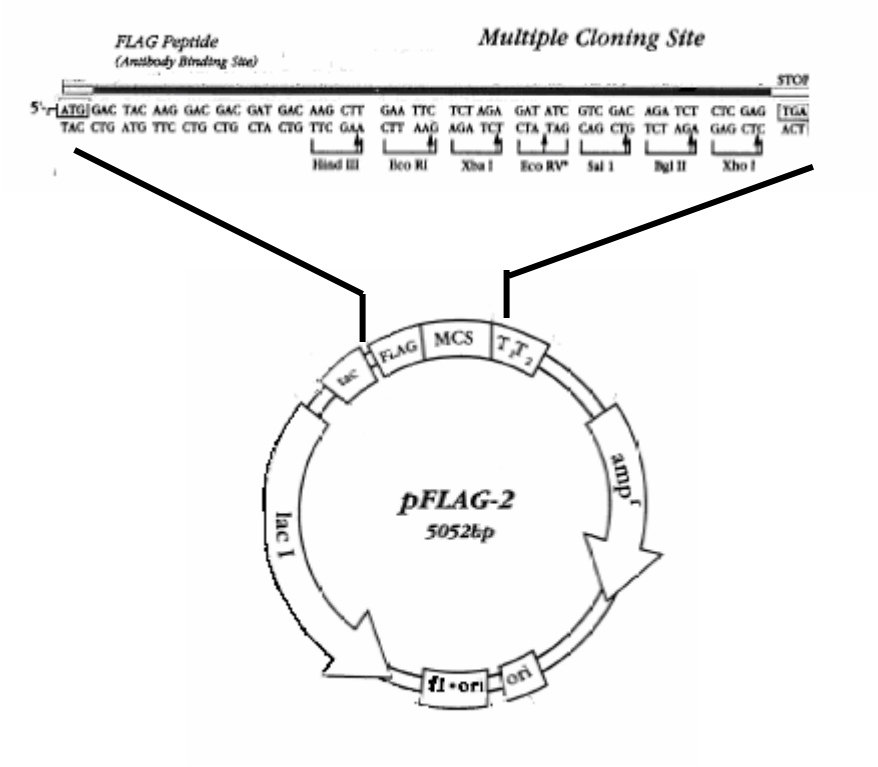
To verify that the intronic sequences isolated for *Tasp* and *Dyrk4* contain Hox binding elements that are bound by and regulated by Hoxa2 several criteria should be fulfilled. This could be assessed by assays using a DNA binding deficient Hoxa2 mutant protein in both transfection and *in vitro* DNA binding experiments (Lampe et al., 2004). *DNase* I footprinting reactions using recombinant protein would identify a specific *in vitro* Hoxa2 binding site within clone 12 and clone 2. ChIP coupled with PCR may also be used to characterize a specific *in vivo* binding site for Hoxa2 (Johnson and Bresnick, 2002; Wells and Farnham, 2002). Also, the CCATCA/T sites present within clone 12 merits further analysis to identify if Hoxa2 binds specifically to these sites and what cofactors are involved in recognition of this sequence. This may be performed by DNA binding assays such as EMSA where supershifts using an anti-Pbx antibody will identify if Hoxa2 binds to this sequence with Pbx as a partner. Mutation analysis of the consensus site will identify a Hoxa2 binding site which may in the future be helpful in identifying potential target genes using bioinformatics.

It is important to examine the *in vivo* role of the *Tasp* and *Dyrk4* intronic sequences to determine whether Hoxa2 is involved in tissue or cell specific regulation. As there are potentially multiple transcripts for both *Dyrk4* and *Tasp*, it is important to identify if both or only one transcript is regulated by Hoxa2 *in vivo*. This may be assessed by comparative quantitative RT-PCR in *Hoxa2*$^{-/-}$ and wildtype mice (Lowe et al., 2003). Also, an expression profile of these target genes within the developing

murine CNS may reflect a dorsoventral specific pattern similar to that of Hoxa2. *In situ* hybridization of mouse tissues will provide a more detailed analysis of the expression profiles for both targets within specific CNS structures. It is possible that regulation of these target genes may occur more specifically within the hindbrain or spinal cord, rather than within the entire CNS. Also, functional redundancy between Hox paralog members is well documented, and mutation analyses of *Hoxa2* and *Hoxb2* mutants indicate a functional overlap (Davenne et al., 1999). Therefore, in the absence of Hoxa2 its paralog Hoxb2 may act to regulate these target genes. Analysis of target gene expression in the absence of both *Hoxa2* and *Hoxb2* in the murine CNS may demonstrate further the function of Hoxa2 in regulation of these targets.

Although it is apparent that Hoxa2 is post-translationally modified in some manner, it is important to determine whether this modification alters its function as a transcription factor or if it is a mechanism for post-translational regulation of Hoxa2 protein. Therefore, assessment of the phosphorylation and glycosylation state of Hoxa2 would be valuable. Identification of glycosylated or phosphorylated protein may be performed by a variety of different experiments. Both glycosylation and phosphorylation of proteins may be detected by radiolabelling of translated protein and a variety of staining techniques (reviewed in: Patton, 2002; Larsen and Roepstorff, 2000). Ubiquitin conjugates may be identified by the His_6 tagged ubiquitin method described by Salghetti et al. (1999), where cells are co-transfected with His_6-ubiquitin and the protein of interest for subsequent affinity purification and western blot analysis. An analysis of the binding capacity of Hoxa2 when post-translationally modified with the target sequences would be informative.

Identification of specific temporal and spatial Hoxa2 target genes may be enhanced by utilizing ChIP on CHIP assays, where chromatin immunoprecipitation is followed by Microarray analysis of the target genes (Kirmizis et al., 2004). Also, subtractive hybridization Microarray analysis using tissues from *Hoxa2*$^{-/-}$ mice would identify several targets of Hoxa2 that are affected *in vivo*.

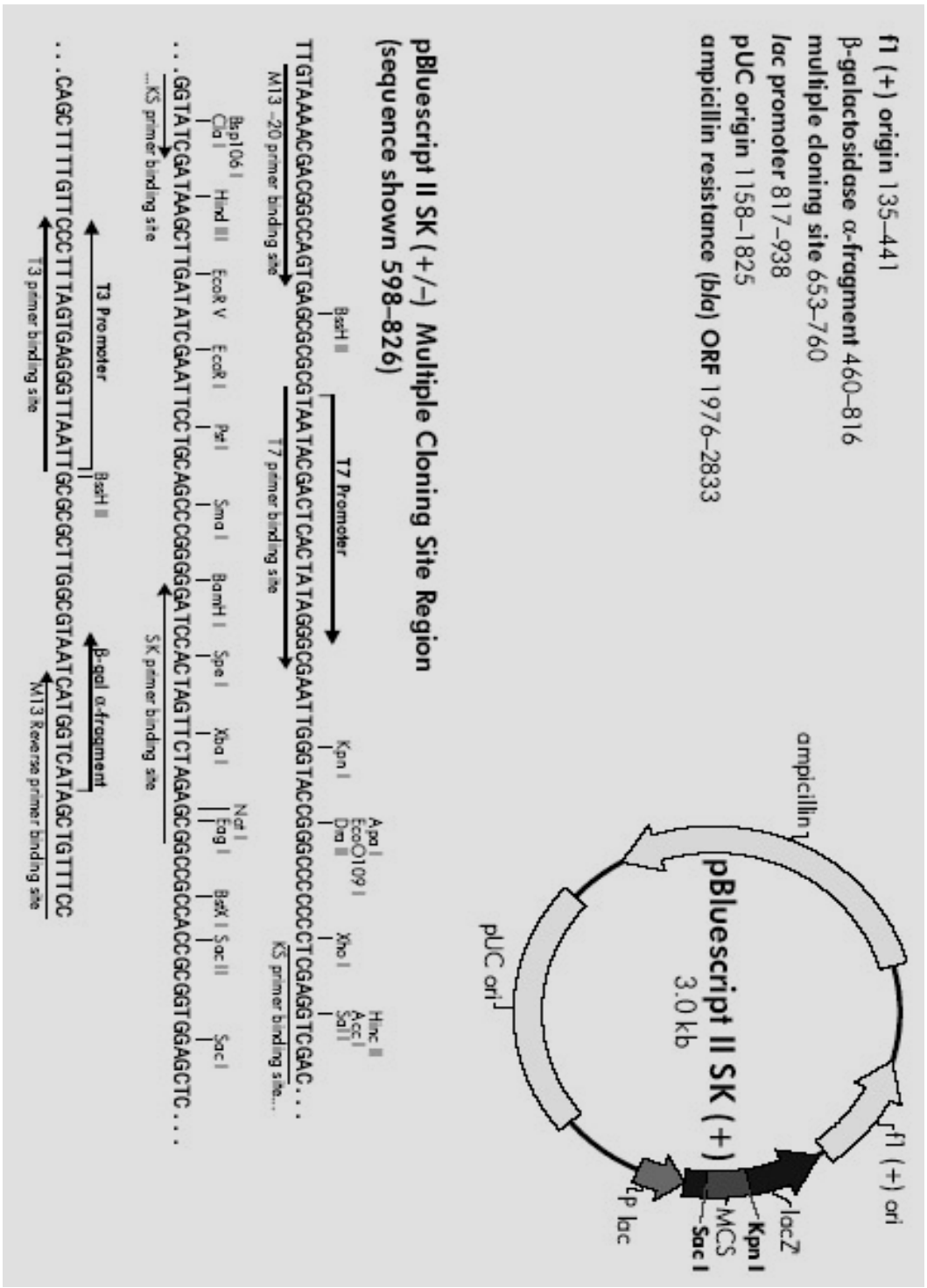


Appendix I Map of the pFLAG-2 expression vector (Eastman Kodak)

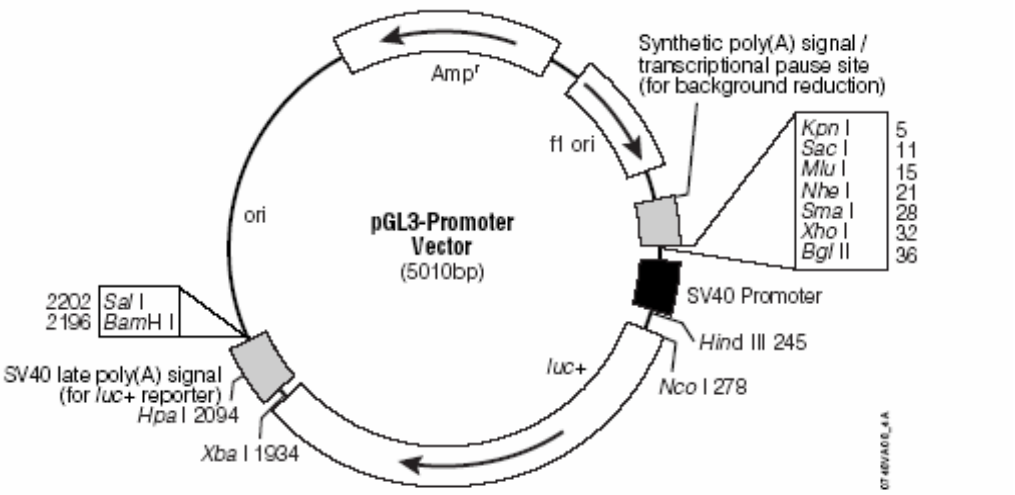
CD spectra for the protein was analyzed by Contin program version 1.0 of Provencher and Glockner. single precision

Sample		a-helix	b-sheet	b-turn	remainder	scale factor
FLAG-HOXA-2	(1)	0.12(.013)	0.56(.014)	0.30(.013)	0.02(.021)	0.994
	(2)	0.20(.012)	0.55(.016)	0.24(.014)	0.02(.034)	1.006
	(3)	0.22(.010)	0.26(0.11)	0.29(.004)	0.23(.006)	0.996
HOXA-2	(1)	0.11(.013)	0.56(.014)	0.30(.013)	0.02(.021)	0.995
	(2)	0.19(.012)	0.55(.018)	0.24(.014)	0.02(.034)	1.007
	(3)	0.21(.010)	0.26(0.11)	0.29(.004)	0.23(.006)	0.995
FLAG-HOXA-2 50% TFE	(1)	0.23(.015)	0.51(.016)	0.27(.015)	0.00(.025)	0.990
	(2)	0.29(.012)	0.50(.009)	0.21(.010)	0.00(.000)	1.005
	(3)	0.32(.011)	0.23(0.13)	0.26(.005)	0.19(.007)	1.002
HOXA-2 50% TFE	(1)	0.21(.015)	0.51(.016)	0.28(.015)	0.00(.000)	0.990
	(2)	0.29(.012)	0.50(.009)	0.22(.010)	0.00(.000)	1.005
	(3)	0.31(.011)	0.24(0.13)	0.26(.005)	0.20(.006)	1.001

Appendix II Tabular Data from the Circular Dichroism Scan of recombinant Hoxa2 protein



Appendix III pBluescript SKII+(Stratagene) vector used for subcloning target sequences



Appendix IV Map of the pGL3 Promoter vector

Clones 2 and 12 were subcloned into the *Sal* I and *Bam*H I restriction sites present downstream from the luciferase coding region and the SV40 poly(A) signal.

Parameter	Value	Data Set-B	Data Set-C
Table Analyzed			
Tasp 1.0			
Repeated Measures ANOVA			
P value	0.1048		
P value summary	ns		
Are means signif. different? (P < 0.05)	No		
Number of groups	3		
F	8.538		
R squared	0.8952		
Was the pairing significantly effective?			
R squared	0.01587		
F	0.3077		
P value	0.6349		
P value summary	ns		
Is there significant matching? (P < 0.05)	No		
ANOVA Table	SS	df	MS
Treatment (between columns)	0.3700	2	0.1850
Individual (between rows)	0.006667	1	0.006667
Residual (random)	0.04333	2	0.02167

	0μg pR-Hoxa2	0.5μg pR-Hoxa2	1.0μg pR-Hoxa2
Number of values	2	2	2
Minimum	1.600	1.200	1.200
25% Percentile			
Median	1.750	1.250	1.200
75% Percentile			
Maximum	1.900	1.300	1.200
Mean	1.750	1.250	1.200
Std. Deviation	0.2121	0.07071	0.0
Std. Error	0.1500	0.05000	0.0
Lower 95% CI of mean	-0.1559	0.6147	1.200
Upper 95% CI of mean	3.656	1.885	1.200
Sum	3.500	2.500	2.400

Appendix V Results of one way ANOVA for luciferase transfection data with 1 μg: 4 ng pGL3-c12 to pCMV-RL

Parameter	Value	Data Set-B	Data Set-C
Table Analyzed			
Tasp 2			
Repeated Measures ANOVA			
P value	0.0875		
P value summary	ns		
Are means signif. different? (P < 0.05)	No		
Number of groups	3		
F	10.43		
R squared	0.9125		
Was the pairing significantly effective?			
R squared	0.6154		
F	36.57		
P value	0.0263		
P value summary	*		
Is there significant matching? (P < 0.05)	Yes		
ANOVA Table	SS	df	MS
Treatment (between columns)	0.2433	2	0.1217
Individual (between rows)	0.4267	1	0.4267
Residual (random)	0.02333	2	0.01167
Total	0.6933	5	

	0μg pR-Hoxa2	0.5μg pR-Hoxa2	1.0μg pR-Hoxa2
Number of values	2	2	2
Minimum	1.600	1.300	1.300
25% Percentile			
Median	1.950	1.550	1.500
75% Percentile			
Maximum	2.300	1.800	1.700
Mean	1.950	1.550	1.500
Std. Deviation	0.4950	0.3536	0.2828
Std. Error	0.3500	0.2500	0.2000
Lower 95% CI of mean	-2.497	-1.627	-1.041
Upper 95% CI of mean	6.397	4.727	4.041
Sum	3.900	3.100	3.000

Appendix VI Results of one way ANOVA for luciferase assay of 2 μg: 40 ng pGL3-c12 to pCMV-RL

Parameter	Value	Data Set-B	Data Set-C
Table Analyzed			
Tasp 4 with 4			
Repeated Measures ANOVA			
P value	0.0591		
P value summary	ns		
Are means signif. different? (P < 0.05)	No		
Number of groups	3		
F	15.92		
R squared	0.9409		
Was the pairing significantly effective?			
R squared	0.1875		
F	7.811		
P value	0.1077		
P value summary	ns		
Is there significant matching? (P < 0.05)	No		
ANOVA Table	SS	df	MS
Treatment (between columns)	1.963	2	0.9817
Individual (between rows)	0.4817	1	0.4817
Residual (random)	0.1233	2	0.06167
Total	2.568	5	

	0μg pR-Hoxa2	0.5μg pR-Hoxa2	1.0μg pR-Hoxa2
Number of values	2	2	2
Minimum	2.900	2.100	1.700
25% Percentile			
Median	3.200	2.550	1.800
75% Percentile			
Maximum	3.500	3.000	1.900
Mean	3.200	2.550	1.800
Std. Deviation	0.4243	0.6364	0.1414
Std. Error	0.3000	0.4500	0.1000
Lower 95% CI of mean	-0.6119	-3.168	0.5294
Upper 95% CI of mean	7.012	8.268	3.071
Sum	6.400	5.100	3.600

Appendix VII Results for one way ANOVA of luciferase assay data from transfections with 4 μg:4 ng pGL3-c12 to pCMV-RL

Parameter	Value	Data Set-B	Data Set-C
Table Analyzed			
Dyrk 1.0			
Repeated Measures ANOVA			
P value	0.1842		
P value summary	ns		
Are means signif. different? (P < 0.05)	No		
Number of groups	3		
F	4.429		
R squared	0.8158		
Was the pairing significantly effective?			
R squared	0.5682		
F	14.29		
P value	0.0634		
P value summary	ns		
Is there significant matching? (P < 0.05)	No		
ANOVA Table	SS	df	MS
Treatment (between columns)	0.1033	2	0.05167
Individual (between rows)	0.1667	1	0.1667
Residual (random)	0.02333	2	0.01167
Total	0.2933	5	

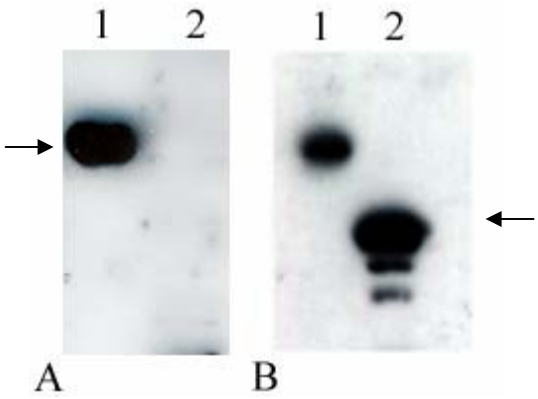
	0μg pR-Hoxa2	0.5μg pR-Hoxa2	1.0μg pR-Hoxa2
Number of values	2	2	2
Minimum	1.200	1.600	1.600
25% Percentile			
Median	1.450	1.700	1.750
75% Percentile			
Maximum	1.700	1.800	1.900
Mean	1.450	1.700	1.750
Std. Deviation	0.3536	0.1414	0.2121
Std. Error	0.2500	0.1000	0.1500
Lower 95% CI of mean	-1.727	0.4294	-0.1559
Upper 95% CI of mean	4.627	2.971	3.656
Sum	2.900	3.400	3.500

Appendix VIII Results for the one way ANOVA test of pGL3-c2 at 1.0 μg concentration to 4 ng of pCMV-RL

Parameter	Value	Data Set-B	Data Set-C
Table Analyzed			
Dyrk2with40			
Repeated Measures ANOVA			
P value	0.1250		
P value summary	ns		
Are means signif. different? (P < 0.05)	No		
Number of groups	3		
F	7.000		
R squared	0.8750		
Was the pairing significantly effective?			
R squared	0.3043		
F	7.000		
P value	0.1181		
P value summary	ns		
Is there significant matching? (P < 0.05)	No		
ANOVA Table	SS	df	MS
Treatment (between columns)	0.1633	2	0.08167
Individual (between rows)	0.08167	1	0.08167
Residual (random)	0.02333	2	0.01167
Total	0.2683	5	

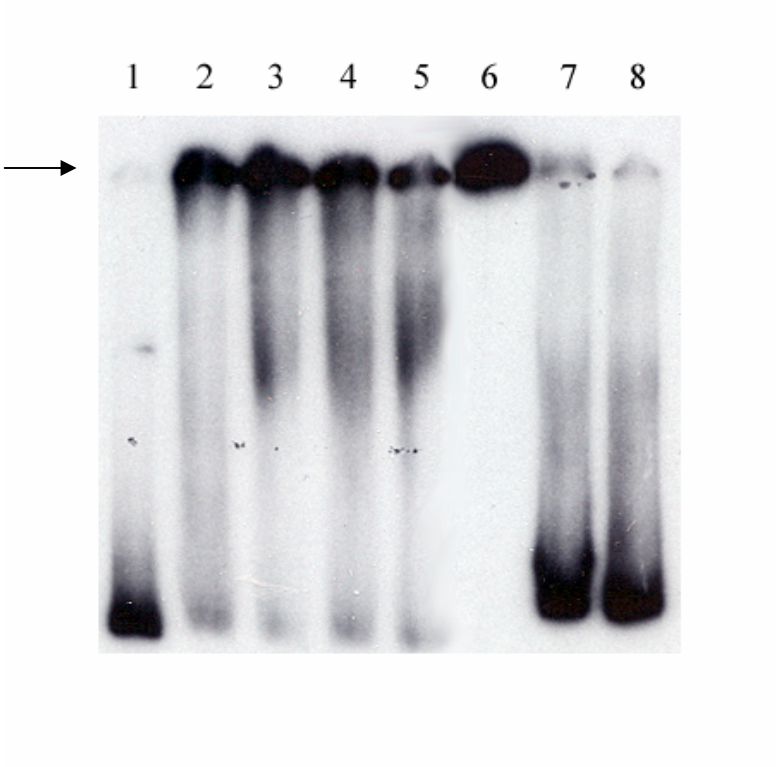
	0μg pR-Hoxa2	1.0μg pR-Hoxa2	2.0μg pR-Hoxa2
Number of values	2	2	2
Minimum	2.500	2.800	2.800
25% Percentile			
Median	2.600	2.850	3.000
75% Percentile			
Maximum	2.700	2.900	3.200
Mean	2.600	2.850	3.000
Std. Deviation	0.1414	0.07071	0.2828
Std. Error	0.1000	0.05000	0.2000
Lower 95% CI of mean	1.329	2.215	0.4588
Upper 95% CI of mean	3.871	3.485	5.541
Sum	5.200	5.700	6.000

Appendix IX One way ANOVA test for luciferase assay using 2 μg: 40 ng of pGL3-c2 to pCMV-RL



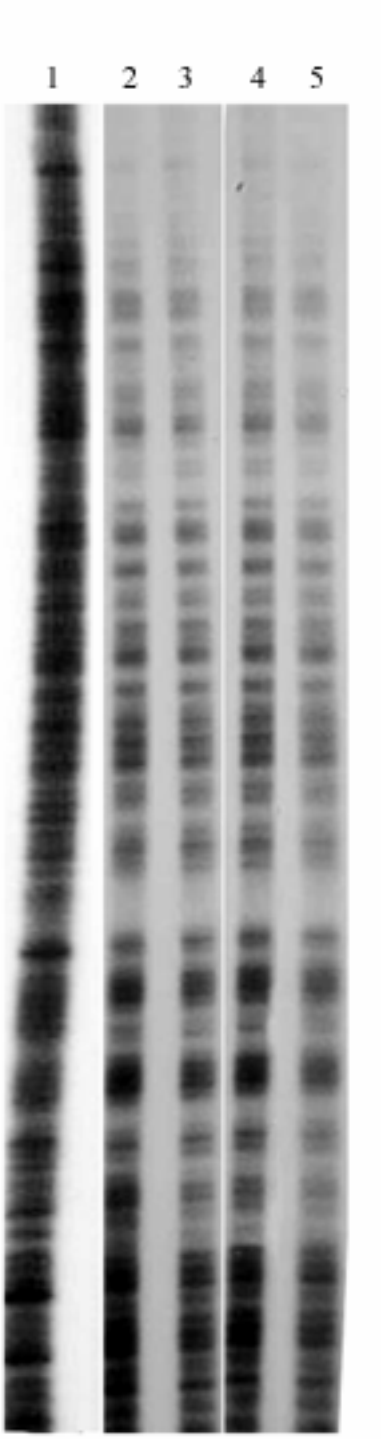
Appendix X Specificity of anti-Hoxa2 antibodies purified from J3 rabbit polyclonal serum by Western Blot analysis

Antibodies purified from the J3 rabbit polyclonal antiserum by affinity chromatography with recombinant FLAG-Hoxa2 protein was used for immunoblotting (Figure A) against whole bacterial protein extract from FLAG-Hoxa2 induced cultures (*lane 1*) and FLAG-Hoxd1 induced cultures (*lane 2*). Antibodies specifically recognize a 40 kDa size protein (*black arrow*) in FLAG-Hoxa2 induced cultures, but no bacterial protein is detected in cultures expressing FLAG-Hoxd1. Both FLAG-Hoxa2 (*lane 1*, Figure B) and FLAG-Hoxd1 (*lane 2*, Figure B) induced cultures were analyzed by immunoblotting using the M2 anti-FLAG specific monoclonal antibody as a control. Both proteins are detected at the expected migration of 40 kDa (*lane 1*) for Hoxa2 and 16 kDa for Hoxd1 (*lane 2*, *black arrow*).



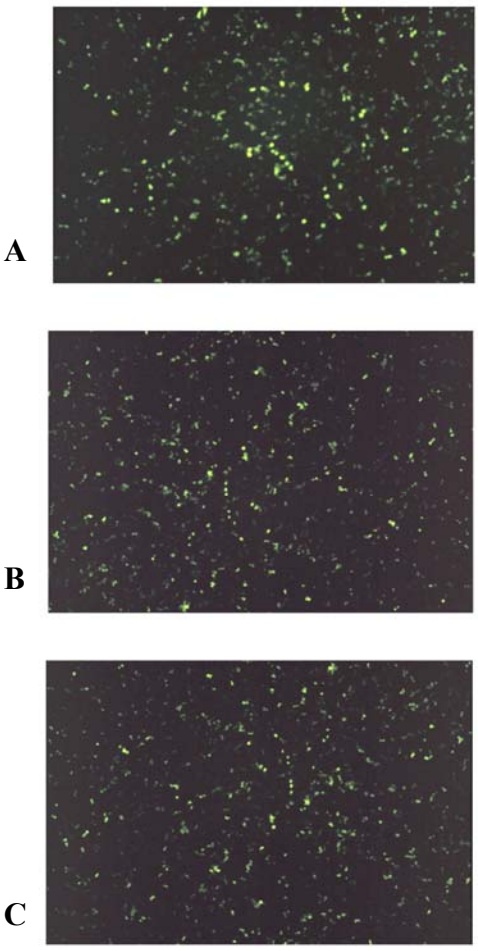
Appendix XI *In vitro* binding analysis of clone 3 using E18 NE

EMSA analysis was performed on clone 3 using NE from E18 mice resulting in no resolution of shift bands. In the absence of protein the probe migrates to the bottom of the gel (*lane 1*), but after incubation with of decreasing amounts of NE protein at 5, 3, 2 and 1 μg (*lanes 2-5*) the protein-DNA complex is retained at the top of the gel (black arrow) and could not be resolved within the gel. Addition of anti-Hoxa2 antiserum increased the amount of probe retained at the top of the gel (*lane 6*), Competition results in removal of the protein binding allowing for migration of the probe (*lane 7 and 8*).



Appendix XII DNA footprinting analysis of clone 12 using E18 NE

Digestion of clone 12 with *DNase* I in the presence of differing concentrations of NE (*lanes 2-5*) does not appear to result in protection of >4 DNA bases at any specific site when compared to the control reaction (*lane 1*).



Appendix XIII Visualization by flourescence microscopy of GFP expression in transfected HeLa cells

HeLa cells were cotransfected with pRSV-Hoxa2 at concentrations of 0, 1, and 2 μg pRSV-Hoxa2 (**A**, **B**, and **C** respectively). RNA samples were used for RT-PCR analysis of *Tasp* expression. Transfection efficiency was assessed by cotransfection of cells with pGFP, and the number of GFP expressing cells/total cell count was visualized by fluorescence microscopy.

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