CHARACTERIZATION OF THE Hoxa2 BINDING SITE IN DUAL SPECIFICITY TYROSINE KINASE 4 (*Dyrk4*) AND HIGH TEMPERATURE REQUIREMENT FACTOR A 3 (*HtrA3*) GENES

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By

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ABSTRACT

Hox proteins are evolutionarily conserved transcription factors that control important developmental pathways in morphogenesis of the embryo. The *Hoxa2* gene is expressed in the developing central nervous system in rhombomeres 2 to 7 and affects cellular differentiation. Few target genes of Hoxa2 protein have been identified so far and its mechanisms of regulating gene expression remain elusive. Previous work in our laboratory isolated Hoxa2 protein binding sequences from the E18 mouse spinal cord and hindbrain tissues using chromatin immunoprecipitation (ChIP). All isolated DNA fragments contain conserved GATG motifs. Sequence analysis revealed that one fragment belongs to the high temperature requirement factor A 3 *(HtrA3)* gene and another fragment belongs to the Dual specificity tyrosine kinase 4 (*Dyrk4*) gene. In this study, direct binding of Hoxa2 protein to the *HtrA3* and *Dyrk4* fragments was confirmed by electrophoretic mobility shift assays (EMSA). Site-directed mutagenesis and EMSA studies revealed that Hoxa2 protein binds to the multiple GATG motifs within these fragments. *HtrA3* fragment also repressed luciferase gene expression in transient transfection and luciferase assays. Mutation of the DNA fragment showed that the repressive activity was affected by the GATG motifs, suggesting Hoxa2 protein regulated gene expression by binding to the GATG motif in the *cis*-regulatory element. In contrast to the inhibitory activity of Hoxa2 protein, a Hoxa2-VP16 fusion protein (Hoxa2 fused with an activation domain of a virion protein from herpes simplex virus) transactivates the luciferase expression by binding to GATG sites. RT-PCR and immunohistochemistry analysis revealed an upregulation of *HtrA3* expression in *Hoxa2*$^{-/-}$ mice. This observation correlates with the inhibitory role of Hoxa2 protein acting upon the *HtrA3* fragment in luciferase assays. Our data suggest that *HtrA3* is a direct *in vivo*

downstream target of Hoxa2 protein and support the activity regulation model in which Hox proteins selectively regulate target genes through occupation of multiple monomer binding sites.

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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
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
cDNA:	complementary DNA
ChIP:	Chromatin Immunoprecipitation
CMV:	Cyto-Megalo Virus promoter
CNCCs	Cranial Neural Crest Cells
CNS:	Central Nervous System
CRE:	cAMP Response Element
CREB:	cAMP Response Element Binding Protein
Dfd	Hox protein Deformed
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA:	Deoxyribonucleic acid
dpc:	days post coitum
DS	Down Syndrome
DSCR	Down Syndrome Critical Region

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
Eph:	Ephrin
Exd:	Extradenticle homeodomain protein
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
GFP:	Green Fluorescent protein
GR:	Glucocorticoid Receptor
GST:	Glutathione-S-Transferase protein tag
HB9:	Human *homeobox gene 9*
HBS:	Homeodomain binding site
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
HRP:	Horse Radish Peroxidase
IgG:	Immunoglobulin G
IPTG:	Isopropyl-β-d-Thiogalactopyranoside
KCl:	Potassium chloride

kDa:	kiloDalton
MDK1:	Mouse developmental kinase 1
Meis:	*Myeloid ecotropic insertion site* genes
$MgCl_2$:	Magnesium chloride
min:	minutes
Mirk	Minibrain-related kinase
mnb	minibrain
Msx:	*Msh*-like homeobox
NaCl:	Sodium Chloride
NaH_2PO_4:	Sodium di-hydrogen phosphate
nM:	nanoMolar
Na_3PO_4:	Sodium phosphate
NaSCN:	Sodium thiocyanate
NCCs	Neural Crest Cells
NE:	nuclear extract
NFκB:	Nuclear Factor kappa B
NKL:	Homeobox gene cluster containing the *NK1*, *NK3*, *NK4*, *Lbx*, *Tlx*, *Emx*, *Vax*, *Hmx*, *NK6*, and *Msx* genes
NMR:	Nuclear Magnetic Resonance
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

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
PKA:	Protein kinase A
PMSF:	Phenylmethanesulfonyl Fluoride
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	Room Temperature
RT-PCR:	Reverse Transcriptase PCR
S:	Shift complex
SDS:	Sodium Dodecyl Sulfate
siRNA:	small interference RNA
SS:	Supershift complex
TALE:	Three amino acid loop extension homeodomain protein
Tasp:	Toll associated serine protease
TESS:	Transcription element search system
TGF:	Transforming Growth Factor

TTF:	Thyroid Transcription Factor homeodomain protein
UTR:	Untranslated region
UV:	Ultraviolet light
μM:	microMolar
μl:	microliter

1. INTRODUCTION

Hox genes were first identified in *Drosophila*, where they play a key developmental role in specifying different body segments (Akin and Nazarali, 2005). The critical roles of *Hox* genes are conserved in animals (Peifer and Wieschaus, 1990; McGinnis and Krumlauf, 1992; Rijli et al., 1998; Akin and Nazarali, 2005). The murine *Hoxa2* gene and its paralog *Hoxb2* are homologs of the *Drosophila proboscipedia* gene. *Hoxa2* is expressed in the neural tube and neural crest cells (during embryogenesis), which contribute to the formation of the second brachial arch, as well as tissues and organs derived from it (Prince and Lumsden, 1994b; Davenne et al., 1999a; Hao et al., 1999; Barrow et al., 2000; Grammatopoulos et al., 2000). The anterior expression boundary of *Hoxa2* is located at the rhombomere (r) 1/2 interface within the neural tube (Davenne et al., 1999a; Barrow et al., 2000) (Figure 2). *Hoxa2* expression is initiated within the ventral mantle region at embryonic day 10 (E10) and extends from the hindbrain caudally throughout the spinal cord.

Disruption of *Hoxa2* gene causes 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., 1998; Davenne et al., 1999a; Barrow et al., 2000). Additionally, defects of the branchial arch derivatives, such as cleft palate (Gendron-Maguire et al., 1993; Barrow and Capecchi, 1999; Nazarali et al., 2000), are observed in *Hoxa2* mutant mice. This evidence suggests that Hoxa2 protein is also involved in the cell differentiation process. Hoxa2 protein is further shown to promote the formation of cartilage and prevent ossification during craniofacial development

(Kanzler et al., 1998; Grammatopoulos et al., 2000; Plant et al., 2000; Trainor and Krumlauf, 2001; Creuzet et al., 2002). More recently, Grosschedl and his colleagues (Dobreva et al., 2006) reported that SATB2, a member of the nuclear matrix-attachment region (MAR) protein family, functioned as a molecular joint in a transcriptional network regulating craniofacial patterning and osteoblast differentiation, partially, if not exclusively, through directly inhibiting *Hoxa2* expression. This finding suggested a molecular mechanism that influences patterning and differentiation process during bone formation (Ellies and Krumlauf, 2006).

Many genes may work together with *Hoxa2* in specifying early development of the hindbrain. For example, *Hoxa2* and *MDK1/EphA7* showed coinciding spatial and temporal patterns of expression within the hindbrain (Taneja et al., 1996). In later stages of development, the expression of *Hoxa2* was directed by an enhancer sequence located at the 3′ end of *Hoxa2* containing a 10 bp Hox/Pbx binding element, which was activated by Hoxa2 in the presence of cofactors Pbx1a and Prep1 (Frasch et al., 1995; Ren et al., 2002; Lampe et al., 2004). Co-expression of *Hoxa2*, *Hoxd1* and *Pax6* was found in the diencephalon. Hoxa2, in coordination with Hoxd1 and Pax6, may play a role in specifying the cytoarchitechture of the developing diencephalon (Wolf et al., 2001).

Hoxa2 expression is controlled by the transcription factor Krox20 (Nonchev et al., 1996; Tumpel et al., 2002a). Krox20 governs the role of *Hoxa2* in early anteroposterior patterning through 5′ cranial neural crest enhancer region. However, *Hoxa2* expression in neural crest cell of the second branchial arch is regulated by multiple *cis*-regulatory elements in the regulatory region. For instance, mutation or deletion of one element in

the regulatory region abolishes expression in cranial neural crest cells but not in the hindbrain, which means that *Hoxa2* gene is differently regulated in different regions. It also reveals mechanisms on how neural crest cells can lead to development of various tissues and respond to the environment through which they migrate (Trainor, 2003). Another regulator of *Hoxa2* gene is SATB2 (Dobreva et al., 2006). SATB2 inhibits *Hoxa2* gene expression during skeletal development. Interestingly, SATB2 represses *Hoxa2* gene expression through a distinct *cis*-regulatory element at the 3′ of the gene. Hence, it is suggested that distinct *cis*-regulatory elements account for regulation of the *Hoxa2* gene expression in neural crest and osteoblast cells.

The regulation of *Hoxa2* gene expression has been well studied, however, very few target genes of Hoxa2 protein has been identified so far. In order to illustrate the pathways through which Hoxa2 protein function, a former Ph.D student in our lab employed chromatin immunoprecipitation (ChIP) to isolate new downstream target genes of Hoxa2 protein from E18 hindbrain and spinal cord tissue (Akin Z, Ph.D Thesis, University of Saskatchewan, 2004). Seven clones were obtained and sequenced. BLAST query of these sequences identified two potential target genes, the murine homolog of the human dual specificity tyrosine kinase 4 (*Dyrk4*) gene and high temperature requirement factor A 3 (*HtrA3*) gene.

1.1 Hypothesis

The hypothesis to be tested is that Hoxa2 protein will bind to specific target sequences in dual specificity tyrosine kinase 4 (*Dyrk4*) gene and high temperature requirement factor A 3 (*HtrA3*) gene and inhibit their transcription.

1.2 Objectives

(1) Expression and purification of Hoxa2 protein in bacteria using Glutathione S-transferase (GST) gene fusion system.

(2) Examination of DNA-Protein interactions using electrophoretic mobility shift assays (EMSA). In this experiment the mutant and wild-type oligonucleotide probes will be designed according to the putative target sequences.

(3) Based on the information obtained from step (2), the *Dyrk4* and *HtrA3* sequences will be mutated in specific sites and transient transfection experiments will be conducted to examine the *cis*-regulatory effect of Hoxa2 protein on *Dyrk4* and *HtrA3* wild-type and mutant sequences.

2. LITERATURE REVIEW

2.1 Homeobox Genes

Homeotic genes were first identified in *Drosophila* (Akin and Nazarali, 2005), where they influenced segmental identity (McGinnis and Krumlauf, 1992). A homeobox is a 180 base pair (bp) DNA sequence that encodes a conserved 60 amino acid motif called homeodomain (Gehring et al., 1994). *Hox* genes is a subgroup of homeobox genes and generally function as transcription regulators that govern various aspects of morphogenesis and cell differentiation (McGinnis and Krumlauf, 1992). In *Drosophila,* a single homeotic complex (HOM-C) consists of two clusters [the Bithorax (BX-C) and Antennapedia (ANT-C) cluster] located on chromosome 3 (Kaufman et al., 1990). In mice and humans, the *Hox* complex includes 39 genes located on different chromosomes as four separate clusters named *Hox* a, b, c and d (Krumlauf, 1993; Akin and Nazarali, 2005). These genes are classified into 13 paralog groups based on their homology to *Drosophila* HOM-C. During early embryonic development in the mouse all *Hox* genes are expressed in central nervous system and adjacent mesoderm. Expression of the *Hox* genes is limited within the mouse hindbrain at early stages of development. The hindbrain is divided into metameric units referred to as rhombomeres (r) and the anterior boundaries of *Hox* expression coincide with that of the rhombomeric boundaries (Figure 2.1) (Lumsden and Krumlauf, 1996; Rijli et al., 1998). General colinearity exists among the expression pattern of *Hox* genes, particularly in the developing spinal cord (Figure 2.2). Thus, the 3′ anterior *Hox* genes have rostral expression restriction in the

hindbrain (Figure 2.1) and the expression extends through the spinal cord to varying extents. The 5′ Hox groups (5-13) generally present anterior boundaries of expression restricted to domains within the spinal cord (Figure 2.2) (Akin and Nazarali, 2005). The expression of *Hox* genes in the developing spinal cord represents a role for *Hox* genes in spinal cord patterning. *Hox* genes play an important role in central nervous system development, especially in determining neuronal organization within the hindbrain (Lumsden and Krumlauf, 1996; Studer et al., 1996; Rijli et al., 1998; Pasqualetti and Rijli, 2001; Pattyn et al., 2003) and the spinal cord (Carpenter, 2002).

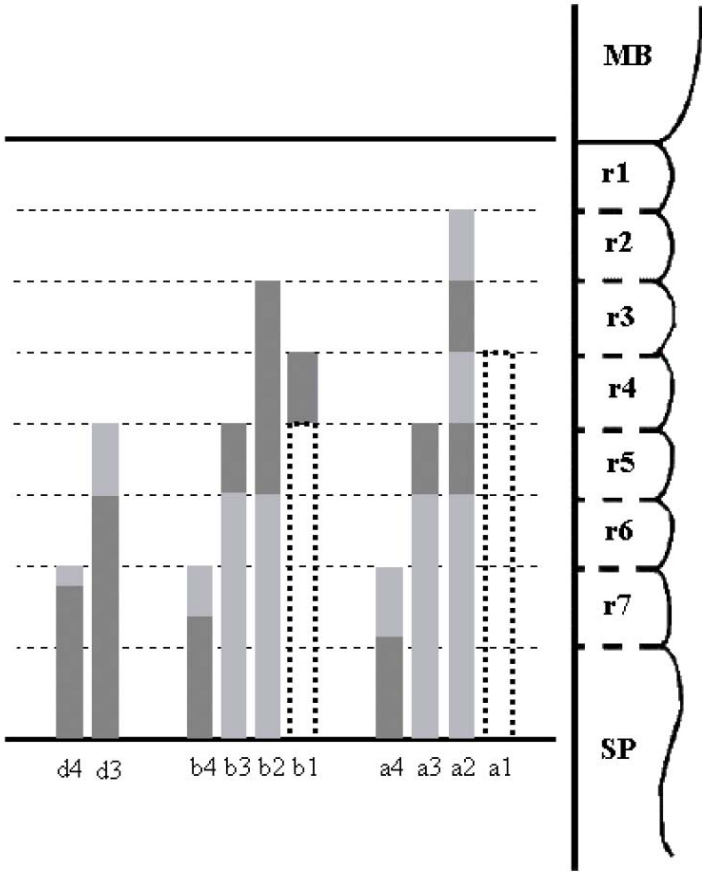


Figure.2.1 Schematic diagram of *Hox* gene expression patterns in the rhombomeres. Dash lines represent the interface between different rhombomeres. Dashed bars indicate transient expression of *Hoxa1* and *Hoxb1*. Light grey bars represent lower lever of expression and dark grey bars represent higher expression. The above figure is taken from Akin and Nazarali (2005) with kind permission from Springer Science and Business Media: Cellular and Molecular Neurobiology，*Hox* Genes and Their Candidate Downstream Targets in the Developing Central Nervous System, 25, 697-741, figure 2.

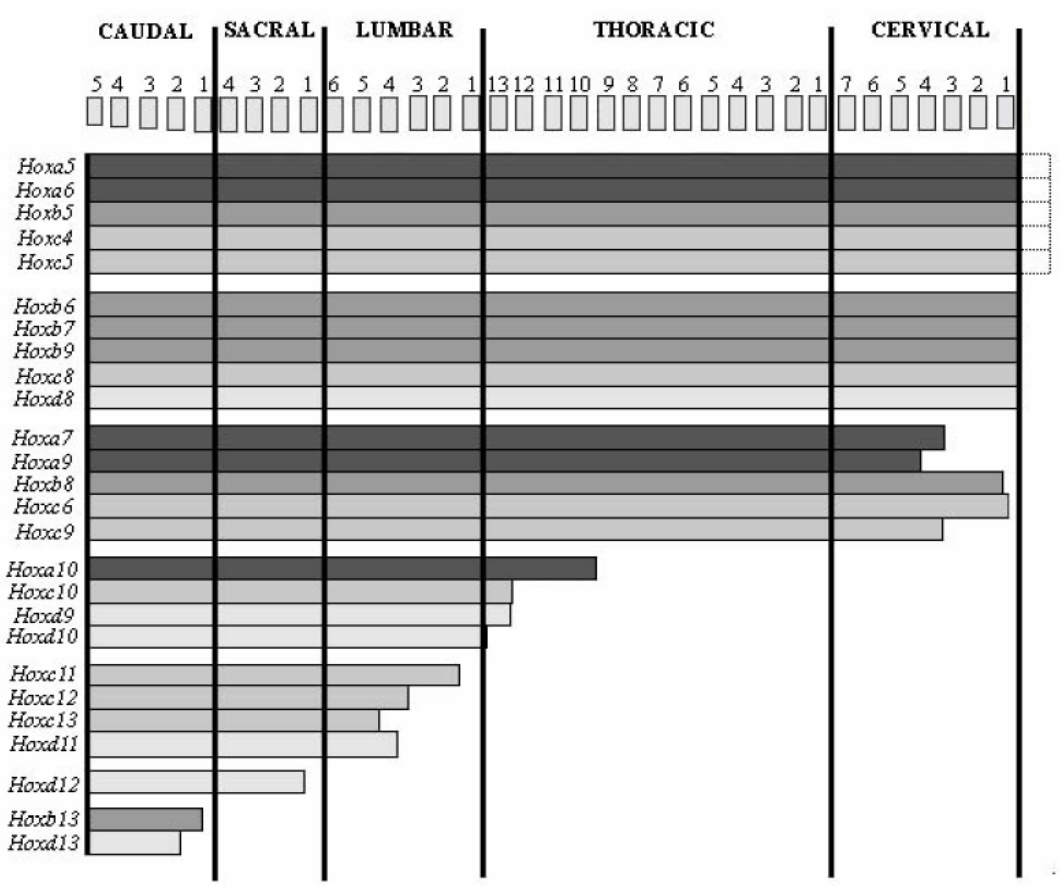


Figure 2.2 *Hox* gene expression in the spinal cord
Schematic drawing of *Hox* gene expression pattern in the developing murine spinal cord at E 12.5. Genes are grouped based on their anterior boundary limit. Spinal cord was represented by separated region according to the vertebrae (cervical, thoracic, lumbar, sacral, caudal). The different shades of grey represent was used to represent paralog members. The above figure is taken from Akin and Nazarali (2005) with kind permission from Springer Science and Business Media: Cellular and Molecular Neurobiology，*Hox* Genes and Their Candidate Downstream Targets in the Developing Central Nervous System, 25，697 – 741, figure 3.

2.2 Structure of Antennapedia Homeodomain

The most common class of homeodomain is the Antp class of homeodomain, classified based on its similarity to the homeodomain in *Drosophila Antennapedia*. This class of homeodomain proteins is characterized by a conserved helix-turn-helix motif (Gehring et al., 1994). The structure of the Antp homeodomain was determined by nuclear magnetic resonance (NMR) spectroscopy (Qian et al., 1989; Billeter et al., 1990). The structure consists of three α helical regions and a more disordered and flexible fourth helix extended following the third helix. The three helical regions are folded in a globular structure. A conserved hexapeptide motif precedes the first helix in Antp homeodomain and is reported to be involved in protein-protein interactions (Sprules et al., 2000; Sprules et al., 2003). The first two helices are present anti-parallelly. The second and third helix form the helix-turn-helix motif which is conserved among many *Hox* transcription factor family. The fourth helix is perpendicularly aligned to the first two helices (Gehring et al., 1990). *In vitro* DNA-binding studies found that proteins encoded by the class I *homeobox* (Antp) genes recognized a 5′-TAAT-3′ (β strand) core motif with varying flanking sequences (Kalionis and O'Farrell, 1993; Gehring et al., 1994; Kumar and Nazarali, 2001). NMR spectroscopy analysis of the Antp homeodomain protein-DNA complex showed that the third helix (recognition helix) binds to the major groove of the DNA having the TAAT motif. The N-terminal flexible arm forms additional connections to the bases in the minor groove. The loop connecting the first and second helices is associated with the DNA backbone. The recognition helix is responsible for the specific DNA-binding with the TAAT motif. For instance, in the

recognition helix, the conserved Gln-50, Ile-47 and Met-54 are critical to establishing contacts with bases within the target sequence (Gehring et al., 1994; Kumar and Nazarali, 2001). These residues are also reported to function in the binding of DNA by Hoxb1 within the Hoxb1-Pbx1-DNA complex, as determined by X-ray crystallography (Piper et al., 1999).

2.3 DNA-binding of Hox Proteins

Although it is well recognized that Hox transcriptional factors govern body patterning along the anteroposterior body axis during animal development, it is not fully known how Hox proteins exert such function *in vivo*. Inconsistency exists between the high developmental specificity and low DNA-binding specificity of Hox proteins. Two models, selective binding model and activity regulation model (Nasiadka et al., 2000), have been proposed so far to elucidate how Hox proteins select the appropriate target genes.

The selective binding model suggests that cofactors work together with Hox proteins and direct Hox proteins to different binding sequences by raising their DNA-binding specificity. Consistent with this model are the extradenticle (Exd/Pbx) family of the homoeodomain proteins have been identified as cofactors and have crucial impact on the diversity of Hox function (Peifer and Wieschaus, 1990; Rauskolb et al., 1993; Van Dijk et al., 1993; van Dijk and Murre, 1994). For instance, the selective binding of Hoxb1 to r4 enhancer is determined by the interaction of Pbx and Hoxb1 (Berthelsen et al., 1998). In luciferase assays, cotransfection of Pbx with Hoxb1 greatly increases its regulatory activity through r4 enhancer elements (Di Rocco et al., 1997). Furthermore, additional cofactors other

than Pbx, Prep and Meis exist (Berthelsen et al., 1999; Jacobs et al., 1999). The presence of Prep1 in ternary Prep1–Pbx–HOXB1 complex greatly increases the transcriptional activity of Pbx/HOXB1 complex, although Prep1 does not need to bind to the DNA (Figure 2.3). Meis1 was shown to function in a trimeric complex involving Hoxb1. But different from Prep1, it enhances the regulatory activity of Hoxb1 in a DNA-binding manner (Figure 2.4). Evidence shows that Exd/Pbx proteins can interact with Hox proteins and increase their DNA-binding affinity on a specific DNA site (Chan et al., 1994; Chang et al., 1995; Popperl et al., 1995; Neuteboom and Murre, 1997). A bipartite 10 pb Hox-PBC consensus binding site, 5′ TGATNNAT [G/T] [G/A] 3′, has been defined in which the first half site is for PBC binding and the second half site is for Hox binding (Popperl et al., 1995; Chan et al., 1997). A Pbx/Hoxa2 composite site [(T/A) GAT (T/G) GA (T/A) G] was identified in *Hoxa2* gene and the Pbx was found to significantly contribute to the autoregulation of *Hoxa2* gene through this site in COS-7 cells (Lampe et al., 2004).

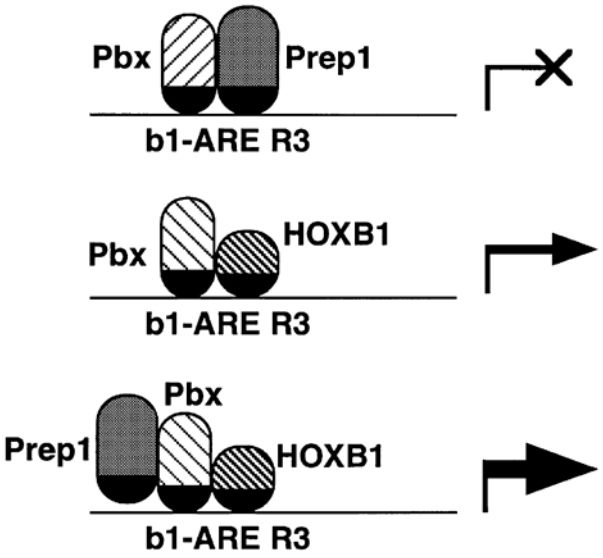


Figure 2.3 Hox, Pbx and Prep interaction on the b1-ARE
Interaction between HOXB1 and Pbx on the b1-ARE enhancer results in the activation of *Hoxb1* transcription. This transcription is dependent on the DNA-binding of both Hox and Pbx proteins, not Meis/Prep. Reprinted by permission from Macmillan Publishers Ltd: [EMBO Journal], (Berthelsen, J., Zappavigna, V., Ferretti, E., Mavilio, F., and Blasi, F. The novel homeoprotein Prep1 modulates Pbx-Hox protein cooperativity. 17, 1434-1445), copyright (1998).
http://www.nature.com/emboj/index.html

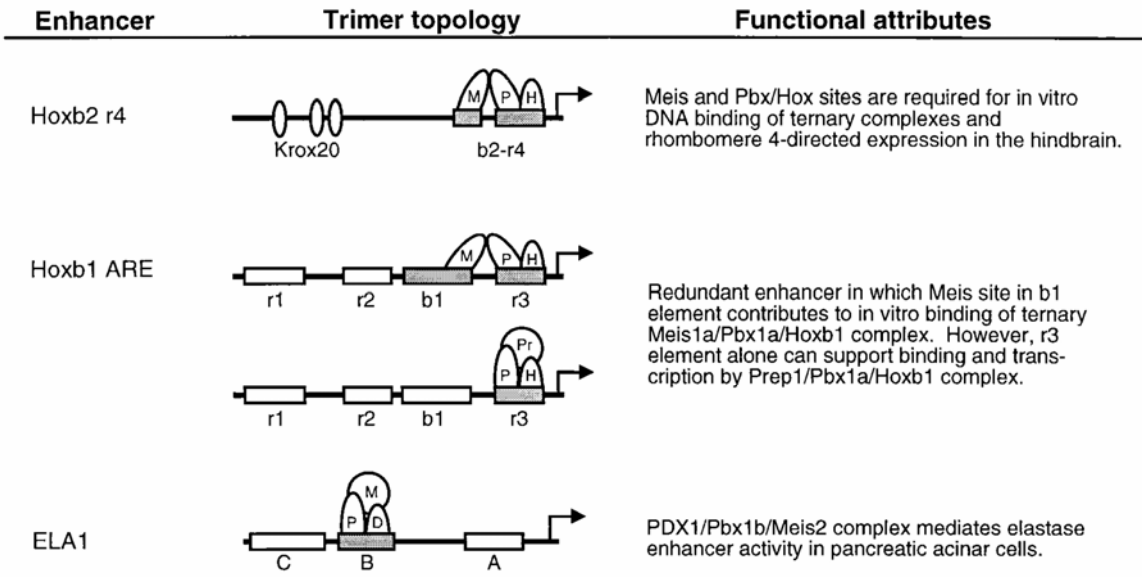


Figure 2.4 Trimeric interactions of homeobox proteins on enhancers
Complexes of Hox/Pbx and Meis proteins results in the activation of various enhancer. This activity is denpendent on the DNA-binding of Meis. This figure is taken from Jacobs Y, Schnabel CA, Cleary ML (1999) with kind permission from Molecular and Celluar Biology: Trimeric Association of Hox and TALE Homeodomain Proteins Mediates Hoxb2 Hindbrain Enhancer Activity, figure 8.

The activity regulation model proposes that cofactors (e.g. Pbx) affect the transcriptional activity of Hox protein through the sequence which they are already bound, instead of influencing DNA-binding affinity (Biggin and McGinnis, 1997). The low DNA-binding specificity means that Hox protein can bind to many sites across the genome; however, they will remain in a neutral state and not exert an independent influence until a cofactor appears. In favor of this model, the homeodomain in the Hox protein Deformed (Dfd) has an inhibitory affect on the Dfd activation function (Li et al., 1999). Thus, Dfd binds to the regulatory region of a variety of genes *via* its homeodomain, however; it does not activate transcription because of the inhibitory activity of its homeodomain (Figure 2.5). The suppression mechanism of homeodomain remains unclear and it has been proposed to be mediated by a masking factor. The interaction between Exd/Pbx and Dfd homeodomain releases the Dfd activation capacity (Figure 2.5). Additional evidence supporting activity regulation model is that the Dfd-VP16 fusion protein can regulate same downstream target genes of Dfd in the absence of Exd/Pbx in embryos (Li et al., 1999). VP16 is a virion protein from herpes simplex virus. It has a strong 77-aa activation domain and is joined with the Hox protein to isolate Hox protein downstream target genes. The rationale of this approach is that Hox-VP16 (activation domain) fusion protein can function and affect the expression of Hox protein target genes (or more genes in some cases) without indispensable or unknown cofactors (Li and McGinnis, 1999; Li et al., 1999).

Although some of the supporting evidence of these two models is contradictory, they are not mutually exclusive. The disparities could be due to the differences in

experimental methods. These two models might work under different circumstances and further research is needed to clarify the differences.

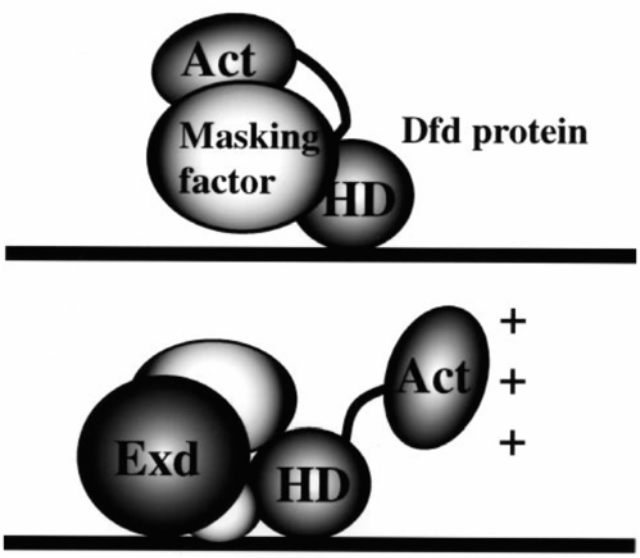


Figure 2.5 The activity regulation model for Dfd protein
A hypothesized masking factor inhibits the transcriptional activity of Dfd protein. The binding between Dfd and Exd releases the activation domain function. Reprinted by permission from Macmillan Publishers Ltd: [EMBO Journal], (Li, X., Murre, C., and McGinnis, W. Activity regulation of a Hox protein and a role for the homeodomain in inhibiting transcriptional activation. 18, 198-211), copyright (1999). http://www.nature.com/emboj/index.html

2.4 *Hoxa2* gene

The *Hoxa2* gene and its paralog *Hoxb2* are homologs of the *Drosophila proboscipedia* gene. They belong to the most 3′ paralogous group of the *Hox* gene family (Figure 2.6). *Hoxa2* gene is expressed during embryogenesis in the neural tube and neural crest cells, which contribute to formation of the second brachial arch, as well as other tissues derived from it (Prince and Lumsden, 1994b; Davenne et al., 1999a; Hao et al., 1999; Barrow et al., 2000; Grammatopoulos et al., 2000). The anterior expression boundary of *Hoxa2* gene is located at the interface of rhombomere (r) 1 and 2 within the neural tube (Davenne et al., 1999a; Barrow et al., 2000). *Hoxa2* gene expression is initiated within the ventral mantle region at embryonic day 10 (E10) and extends from the hindbrain caudally throughout the spinal cord. Hoxa2 protein is found within the dorsal horn (Hao et al., 1999) and may potentially contribute to both anterior-posterior (A-P) positioning as well as dorsal-ventral (D-V) patterning (Hao et al., 1999). Hoxa2 protein is also involved in the patterning of cranial neural crest cells (Trainor and Krumlauf, 2001; Tumpel et al., 2002b; Creuzet et al., 2005). *Hoxa2* gene is expressed in subsets of premigratory and migratory crest cells populating the second and more caudal branchial arches (Prince and Lumsden, 1994a; Nonchev et al., 1996; Mallo and Brandlin, 1997). Interestingly, *Hoxa2* transcripts are detected in r2, however, *Hoxa2* gene is not expressed in the neural crest until these cells start to migrate from r2 to the first branchial arch (Prince and Lumsden, 1994b).

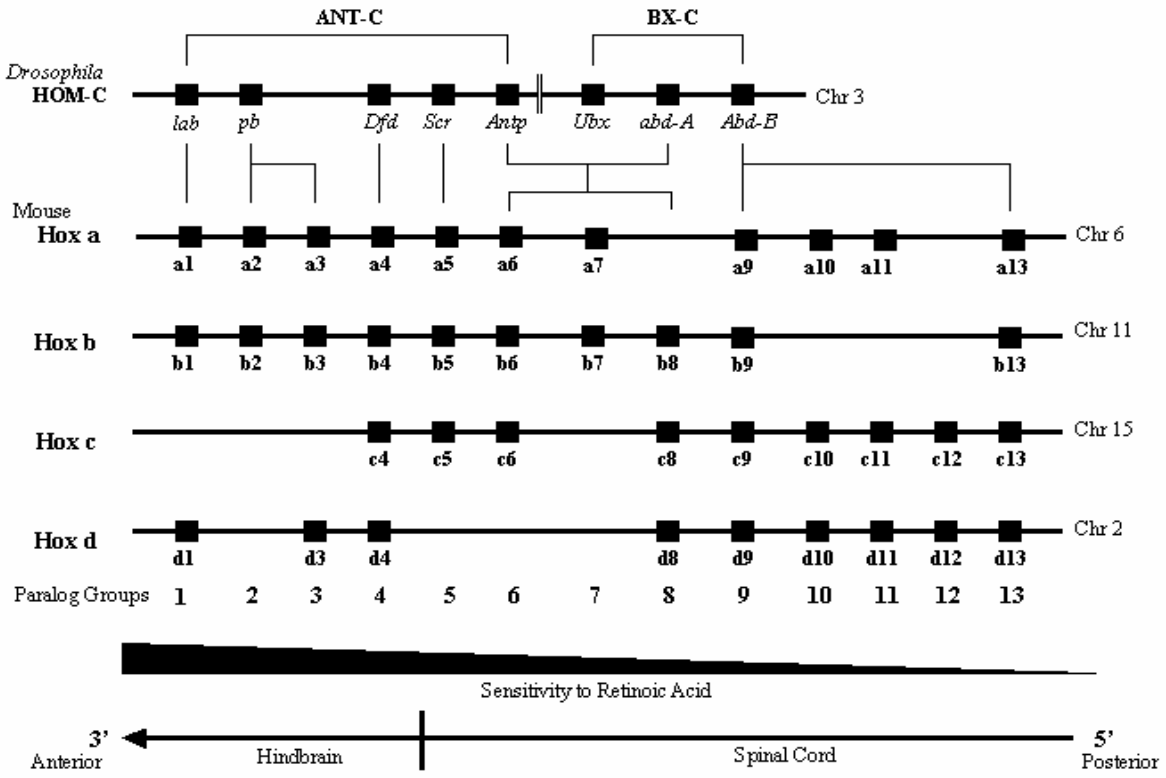


Figure 2.6 Schematic representation of Hox gene clusters
The 39 murine *Hox* genes are present on four separate chromosomal clusters (Hox a, Hox b, Hox c, Hox d). The above figure is taken from Akin and Nazarali (2005) with kind permission from Springer Science and Business Media: Cellular and Molecular Neurobiology，*Hox* Genes and Their Candidate Downstream Targets in the Developing Central Nervous System, 25，697 – 741, figure 1.

Studies using haploinsufficient *Hoxa2* mutant mice revealed a dose-dependent mechanism of development within the hindbrain and branchial arches (Ohnemus et al., 2001). For instance, the branchial arches, in particular the second arch, are highly sensitive to a reduction in Hoxa2 protein activity. In contrast, the anterior hindbrain is not affected at all even with an extreme decline in Hoxa2 protein levels. Therefore, general anterior-posterior and dorsal-ventral patterning of the CNS is

maintained even at low levels of Hoxa2 protein activity, which is possibly due to functional redundancy between anterior *Hox* genes or the presence of parallel pathways in segmental regulation (Ohnemus et al., 2001). However, differential sensitivity to *Hoxa2* gene inactivation between specific neuronal subtypes has been observed at the molecular level, which when considered with previous studies of neuronal expression suggests *Hoxa2* gene involvement in the specification of neuronal phenotypes (Davenne et al., 1999a; Hao et al., 1999; Ohnemus et al., 2001).

2.5 Dyrk Kinase family

One of the largest protein superfamilies is the family of protein kinases that are mostly identified from eukaryotic sources. Protein kinases amplify extracellular and intracellular signals and thus play important regulatory roles in diverse cellular processes, such as metabolism, transcription, cell cycle progression, apoptosis, and neuronal development (Karin and Hunter, 1995; Johnson and Lapadat, 2002). Protein kinases function by transferring phosphate from ATP to a serine, threonine or tyrosine hydroxyl group on a protein substrate, thereby influencing the the protein conformation and, as a result, downstream signaling pathways (Johnson and Lewis, 2001; Huse and Kuriyan, 2002; Lu et al., 2002). The protein kinases are related according to their homologous kinase domains (also known as catalytic domains), which consist of 250-300 amino acid residues (Hanks et al., 1988; Hanks and Quinn, 1991). Based on the amino acid sequence similarity of their kinase domains, this superfamily of protein kinases can be further divided into the phylogenetically related subfamilies (Hanks and Hunter, 1995).

Dyrk1 has been identified independently by three research groups. First, its yeast homolog (*Yak1*) was identified as a functional antagonist of the Ras/PKA pathway (Garrett and Broach, 1989; Garrett et al., 1991). Second, its *Drosophila* homolog (*mnb*) has been identified by positional cloning of the *minibrain* (*mnb*) mutations, which exhibit specific behavioural defects and a reduced number of neurons in distinct areas of the brain (Tejedor et al., 1995). Third, rat *Dyrk1* was cloned from a rat brain cDNA library and identified as a dual-specificity protein kinase (Kentrup et al., 1996). Subsequently, six additional mammalian Dyrk-related kinases were identified, namely Dyrk1B, Dyrk1C, Dyrk2, Dyrk3, Dyrk4 and Dyrk4B (Becker et al., 1998).

The acronym "Dyrk" (Dual-specificity Yak-related kinase) refers to the unusual ability of these kinases to phosphorylate serine/threonine and tyrosine residue and to the sequence similarity with the protein kinase Yak1. Like Dyrk1 and Mnb, recombinant Yak1 is autophosphorylated on tyrosine residues, and is thus recognized as the yeast homolog of the Dyrk family (Kassis et al., 2000). Based on the function of the conserved tyrosine residues in the activation loop, "Dyrk" can alternatively be interpreted as "dual–specificity tyrosine-phosphorylation regulated kinase". One thing to note is that tyrosine phosphorylation has been only found to occur from autophosphorylation catalyzed by Dyrk itself (Kentrup et al., 1996; Kassis et al., 2000). DNA sequences of many Dyrk family kinases are listed (Table 2.1). Benefiting from the genome sequencing project, many studies have been carried out in model organisms such as fruit fly, eelworm, yeast, rat and mouse.

These studies have greatly elucidated the function of Dyrk family. In mammals, study has been mainly focused on *Dyrk1A* and *Dyrk1B*.

Table 2.1 Dyrk family and Dyrk-related kinases

Gene product	*Species*	*Accession No.*	*Reference*
DYRK1A	*Homo sapiens*	NM_001396	(Guimera et al., 1996; Shindoh et al., 1996; Song et al., 1996)
DYRK1B	*Homo sapiens*	NM_004714	[a]
DYRK2	*Homo sapiens*	NM_006482	[a]
DYRK3	*Homo sapiens*	NM_001004023	[a]
DYRK4	*Homo sapiens*	NM_003845	[a]
DYRK5	*Homo sapiens*	AF327561	[a]
Dyrk1a	*Mus musculus*	NM_007890	[a]
Dyrk1b	*Mus musculus*	Y18280	[a]
Dyrk1c	*Mus musculus*	U49952	[a]
Dyrk2	*Mus musculus*	AY399074	[a]
Dyrk3	*Mus musculus*	NM_145508	[a]
Dyrk4	*Mus musculus*	NM_207210	[a]
Mnb	*Drosophila melanogaster*	X70798	(Tejedor et al., 1995)
dDYRK2	*Drosophila melanogaster*	NP_995711	(Lochhead et al., 2003)
Yak1	*Saccharomyces cerevisiae*	X16056	(Garrett and Broach, 1989)
YakA	*Dictyostelium discoideum*	AF045453	[a]
Pom1	*Schizosaccharomyces pombe*	Z50142	[a]
Mbk-2	*Caenorhabditis elegans*	AY090019	[a]
Mbk-1	*Caenorhabditis elegans*	AY064464	[a]
Hipk1	*Homo sapiens*	NM_198268	[a]
Hipk2	*Homo sapiens*	AF207702	[a]
Hipk3	*Homo sapiens*	NM_005734	[a]

[a] Sequences have been identified from genome sequencing projects.
The above table is adapted from Becker and Joost, 1999

2.5.1 Dyrk1A/mnb in mammals

Dyrk1A is considered as one of the candidate genes in Down syndrome (DS). Down syndrome is a major genetic cause of mental retardation. The brain of Down syndrome patients presents diverse macroscopic and microscopic alterations such as reduction in the size of brain, decreased neuronal number/density, abnormal neuronal differentiation processes and precocious Alzheimer-like neurodegeneration (Becker et al., 1991; Korenberg et al., 1994). Although the molecular bases of all these alterations remain unknown, Down syndrome is generally caused by the trisomy of chromosome 21(HC21). A Down syndrome Critical Region (DSCR) has been identified and suggested to be responsible for the etiology of Down syndrome (Rahmani et al., 1989). *Dyrk1A* gene is mapped to the Down syndrome Critical Region (Guimera et al., 1996; Song et al., 1996). In mice, *Dyrk1A/mnb* is mapped to chromosome 16 (Song et al., 1997).

The structural and functional similarity between *Drosophila* mnb and vertebrate Dyrk1A makes the fly an attractive model for genetic and functional analysis. *Mnb* is distinctly expressed in central nervous system (CNS) of *Drosophila* throughout development. In embryo Mnb protein is present in the CNS but not in the peripheral nervous system (PNS). During post-embryonic development, *mnb* is preferentially expressed in the proliferative centers of the CNS, the tissue where adult neurons are generated. In the adult brain, *mnb* is expressed in several neuropile areas. In contrast to the preferential expression in the *Drosophila* brain, Northern and RT-PCR analysis indicated that *Dyrk1A* mRNA is ubiquitously expressed in different tissues and in most brain regions of rodents and humans (Guimera et al., 1996; Song et al., 1996; Guimera et al., 1999; Okui et al., 1999). *In situ* hybridization of rodent brain

sections shows preferential expression in brain, particularlly in the olfactory bulb, cerebellum, cortex, hippocampus and the hypothalamus (Song et al., 1996; Guimera et al., 1999). Dyrk1A immunostaining partially contrasts with the *in situ* hybridization results since protein expression is high not only in the olfactory bulb and cerebellum, but also in the spinal cord and most motor nuclei of midbrain and brain stem (Marti et al., 2003). For the developmental pattern of expression, Western and Northern blot analyses also show different results. The levels of Dyrk1A protein are high during development, but gradually decrease to very low levels during postnatal stages, whereas mRNA levels do not vary as much (Okui et al., 1999).

The pattern of *Dyrk1A* expression during vertebrate brain development is more complex than *Drosophila*. Two waves of expression have been observed. In early embryos, it is expressed before the onset of neurogenesis in the three general locations where neuronal precursors originate: neuroepithelia of the neural tube, neural crest, and cranial placodes (Hammerle et al., 2002). A second wave of *Dyrk1A* expression has been found in chick embryonic brain (Hammerle et al., 2003a). This takes place in the CNS of intermediate and late vertebrate embryos. The *Dyrk1A* expression appears to be limited to neurons since no consistent expression was detected in astroglial or oligodendroglial cells (Galceran et al., 2003). The beginning of *Dyrk1A* expression in these neurons seems to precede the onset of dendritic tree differentiation (Hammerle et al., 2003a).

A great deal of knowledge about the function of *Dryk1A* comes from the study carried out in *Drosophila mnb* mutants. These mutants exhibit a reduced brain size, especially in optic lobes and central brain hemispheres. This appears to be due to a

decrease in the number of cells generated during the proliferative processes of post-embryonic development compared to wild-type flies. *Mnb* is expressed in proliferative centers of the larval brain and its loss of function causes alterations in the arrangement of neuroblasts in these centers (Tejedor et al., 1995). Altogether, these data suggest the involvement of *mnb* in neurogenesis. During pupal stages *mnb* mutants exhibit an increased number of degenerating neurons in the optic lobe. Although this could account for a decrease in the number of neurons, it cannot be ruled out that loss of neurons may be a consequence of alterations in neuronal differentiation. In addition, *mnb* mutants exhibit behavioral defects, such as the poor visual pattern fixation and poor odor-discrimination learning (Tejedor et al., 1995).

Dyrk1A$^{-/-}$ mice show a large reduction in the embryo size, which appears to be due to a developmental delay (Fotaki et al., 2002). The brain of *Dyrk1A*$^{-/-}$ mice exhibit a reduced number of postmitotic neurons, but the early embryonic lethality precludes the analysis of phenotypic alterations in cell proliferations and neurogenesis (Fotaki et al., 2002). Nevertheless, haploinsufficient *Dyrk1A*$^{+/-}$ mice are viable and exhibit a significant reduction in brain size. This size reduction seems to be region-specific since it is more prominent in midbrain and hindbrain than in the forebrain, and in ventral (hypothalamus, pons, medulla oblongata) than in dorsal (neocortex and cerebellum) directions (Fotaki et al., 2002). Surprisingly, these changes do not seem to correlate with the developmental pattern of *Dyrk1A* expression found by other researchers (Song et al., 1996; Hammerle et al., 2002). Despite the brain size reduction, most brain regions of *Dyrk1A*$^{+/-}$ mice lack clear changes in neuronal components and cytoarchitecture. Thus, the reduced brain size

could be explained by a decrease in the neuropile areas rather than by a decline of neuronal number.

The identification of physiological substrates of Dyrk1A is necessary for understanding its biological function. In addition to its autophosphorylation ability on tyrosine residue, Dyrk1A phosphorylates serine and threonine residues in substrate peptides or proteins (Himpel et al., 2000). Several putative substrates of Dyrk1A have been identified (Table 2.2). Four of them are transcription factors (FKHR, CREB, STAT3, GLi1), which exhibit increased activity after phosphorylation. All of these factors play key roles in cell development. Different mechanisms have been described by which Dyrk1A modulates the activity of these transcription factors.

FKHR is an important regulator of cell survival and proliferation. Dyrk1A phosphorylates FKHR specifically on Ser-329, an *in vivo* phosphorylation site that is conserved in related homologues (FKHR-L1 and AFX) (Woods et al., 2001) However, stimulation of FKHR-dependent promoter activity was found to be independent of Dyrk1A kinase activity (von Groote-Bidlingmaier et al., 2003). This observation suggests that Dyrk1A may act as a scaffolding protein and ascribes a potential function to its non-catalytic domain. This situation is also observed when Dyrk1A interacts with other non-substrate partners. CREB is a transcription factor that plays a role in neuronal development and differentiation, e.g. synaptic plasticity. Yang et al. (2001) reported that Dyrk1A/mnb directly phosphorylates CREB and thereby promotes neuronal differentiation of the hippocampal progenitor cell line, H19-7. STAT3 is a downstream effector of cytokines such as interleukin-6 (IL-6),

leukemia inhibitory factor (LIF), or oncostatin M, and in many systems controls genes that determine the regulation from cell growth to differentiation. STAT3 is phosphorylated by Dyrk1A on Ser-727 in the transactivation domain (Matsuo et al., 2001; Wiechmann et al., 2003). Phosphorylation of Ser-727 is known to modulate the transcriptional activity of STAT3. GLi proteins are key downstream signaling components of the *hedgehog* signaling pathway, which controls cell proliferation and pattern formation. GLi1-dependent promoter activity is enhanced by Dyrk1A in part through retaining GLi1 in the nucleus, but also by stimulating GLi1 transcriptional activity directly (Mao et al., 2002). In all these cases, further work is required to define the detailed role of Dyrk1A in transcriptional regulation.

Table 2.2 Substrate and binding partners of Dyrk1A

Substrate or interacting protein	Substrate		Interaction	Effect	References
	Evidence	P-site[a]	Evidence[b]		
Forkhead in rhabdomyosarcoma (FKHR)	In vitro	Ser-329	Co-IP	Stimulation of nuclear export	Woods et al. 2001a; Von Groote-Bidlingmaier et al. 2003
Signal transducer and activator of transcription 3 (STAT3)	In vivo[d]	Ser-727	N/A	N/A	Matsue et al. 2001; Wiechmann et al. 2003
cAMP responsive element binding protein (CREB)	In vitro In vivo[d]	Ser-133	Y2H, Co-IP	Stimulation of transcription[c]	Yang et al. 2001
Glioma-associated oncogene (GLi1)	In vitro	n.d.	N/A	Stimulation of transcription[c]	Mao et al. 2002
Adenovirus E1A oncoprotein	-	n.d.	Pull down assay	E1A stimulates activity of Dyrk1A	Zhang et al.2001
Elf2Bε (eukaryotic initiation factor 2B epsilon)	In vitro	Ser-539	N/A	Priming phosphorylation for GSK3	Woods et al. 2001b
tau	In vitro	Thr-212	N/A	Priming phosphorylation for GSK3	Woods et al. 2001b
Cyclin L2	In vitro In vivo[d]	n.d.	Pull down assay	Translocate to site of transcription/pre-mRNA processing.	de Graaf et al., 2004
Glycogen synthase	In vivo[d]	Ser-640	N/A	Inactivate glycogen synthase	Skurat and Dietrich 2004
phytanoyl-CoA alpha-hydroxylase-associated protein 1 (PAHX-AP1)	N/A	n.d.	Y2H, Co-IP	Translocate Dyrk1A from nucleus to the cytoplasm	Bescond and Rahmani, 2005
Androgen receptor-interacting protein 4 (Arip4)	N/A	n.d.	Y2H, Co-IP	Synergistically activate androgen receptor- and glucocorticoid receptor-dependent transcription	Sitz et al., 2004
Ras, B-Raf, and MEK1	N/A	n.d.	Co-IP	Prolong the kinetics of ERK activation	Kelly and Rahmani 2005
Dynamin	In vitro	n.d.	Pulldown assay	Regulation of protein/protein interaction	Cheng-Hwang et al. 2002; Huang et al. 2004

[a] phosphorylation site (n.d., not determined); [b] Co-IP, co-immunoprecipitation; RNAi, RNA interference;Y2H, yeast two -hybrid system; [c] co-transfection of Dyrk1A causes enhanced activity in reporter gene assays;[d] co-transfection of Dyrk1A causes enhanced phosphorylation. The above table is adapted from Galceran et al., 2003

Cyclin L2 is a nuclear factor and contains a N-terminal cyclin domain and a C-terminal arginine/serine-rich domain, which is a hallmark of many proteins involved in pre-mRNA processing (Dickinson et al., 2002). The phosphorylation of Cyclin L2 by Dyrk1A indicates that Dyrk1A may regulate mRNA splicing (de Graaf et al., 2004).

Glycogen synthase is a key enzyme in the regulation of glycogen synthesis by insulin and controlled by multisite phosphorylation. Several Dyrks (Dyrk1A, 1B, Dyrk2) were found to phosphorylate glycogen synthase at Ser-640 and inactivate the enzyme activity in muscle (Skurat and Dietrich, 2004). This may represent a new pathway for regulation of glycogen synthase. Interestingly, high level expression of *Dyrk1B* was observed in skeleton muscle (Deng et al., 2004). Most information so far indicates *Dyrk1B* assists cell arrest in G_0/G_1, and subsequently mediate transition from growth to differentiation and survival of myoblasts. This function of phosphorylating glycogen synthase may be involved in its regulatory activity in the cell cycle.

Three cytoplasmic proteins have been shown to be *in vitro*-substrates of Dyrk1A: the protein-synthesis initiation factor eIF2Bε, the microtubule-associated protein tau and dynamin 1. Tau and eIF2Bε phosphorylation by Dyrk1A creates recognition sites for subsequent phosphorylation by glycogen synthase kinase 3. The phosphorylation of dynamin 1 by Dyrk1A modulates its capacity to interact with components of the endocytotic apparatus (Chen-Hwang et al., 2002). Interestingly, Dyrk1A and dynamin1 colocalize in the growing dendritic tree of differentiating neurons. This finding suggests that phosphorylation of dynamin1 by Dyrk1A may be related to the molecular processes underlying neuronal differentiation (Hammerle et al., 2003a). This fits with recent evidence indicating that a fraction of the Dyrk1A molecule is located outside the

nucleus (Hammerle et al., 2003a; Marti et al., 2003). It is likely that new putative substrates of Dyrk1A will be identified and the task will be to determine which of them is the key mediator on cell differentiation and proliferation.

Several other interaction partners of Dyrk1A are identified. Skurat and Dietrich (2004) reported that the C-terminus of Dyrk1A interacts with a brain specific protein, phytanoyl-CoA α-hydroxylase-associated protein 1 (PAHX-AP1, also named PHYHIP) which is known to interact with phytanoyl-CoA α-hydroxylase (PAHX, also named PHYH), a Refsum disease gene product (Bescond and Rahmani, 2005). Dyrk1A and PAHX-AP1 are both expressed in similar regions of the brain that are known to be affected in Down syndrome patients. Interestingly, PAHX-AP1 is located to cytoplasm and in the presence of PAHX-AP1, Dyrk1A is re-localized to cytoplasm and can no longer phosphorylate the nuclear factor CREB. These observations suggest PAHX-AP1 and Dyrk1A may together contribute to the neurological abnormalities in Down syndrome.

Arip4 (androgen receptor-interacting protein 4) is a SNF2-like steroid hormone receptor cofactor. It contains chromatin remodeling activity, interacts with the androgen receptor (AR), and modulates androgen-mediated transactivation (Rouleau et al., 2002). In general, SNF2-like proteins are believed to modify the structure of chromatin in a noncovalent manner through rearrangement of nucleosomes and are able to render condensed chromatin accessible to sequence-specific transcription factors (Becker and Horz, 2002; Lusser and Kadonaga, 2003). Arip4 is an interacting partner of Dyrk1A (Sitz et al., 2004). Transactivation assays show that either Dyrk1A or Arip4 alone has an activating effect on androgen receptor- and glucocorticoid receptor-mediated

transactivation, and Dyrk1A and Arip4 together act synergistically (Sitz et al., 2004). However, these effects are independent of the kinase activity of Dyrk1A.

Kelly and Rahmani report another effect of Dyrk1A which is independent of its kinase activity (Kelly and Rahmani, 2005). *Dyrk1A* overexpression potentiates NGF-mediated PC12 neuronal differentiation by upregulating the Ras/MAP kinase signaling pathway. Further investigation shows that Dyrk1A prolongs the kinetics of ERK activation by interacting with Ras, B-Raf, and MEK1 to facilitate the formation of a Ras/B-Raf/MEK1 multiprotein complex. These data indicate that *Dyrk1A* may play a critical role in steroid hormone signaling or Ras-dependent transducing signals and suggest that overexpression of *Dyrk1A* may contribute to the neurological abnormalities observed in Down syndrome patients.

In summary the available information demonstrates that *Dyrk1A* plays important roles in neurogenesis and neuronal differentiation during brain development. Among the genes of the Down syndrome Critical Region, the consideration of *Dyrk1A* as a candidate gene for mental retardation is relatively well supported by the phenotype of transgenic mice that overexpress it and by the analysis of its neuro-developmental roles (Hammerle et al., 2003b).

2.5.2 Dyrk1B/Mirk in mammals

The full-length cDNA sequence of *Dyrk1B* was first cloned with the use of RACE (rapid amplification of cDNA ends) technique with the help of a homology-based PCR cloning (Leder et al., 1999). Three splicing variants of *Dyrk1B* were identified in mouse (Leder et al., 2003). The amino acid sequences of Dyrk1A and Dyrk1B are 84% identical in the N-terminal domain and the catalytic domain, but show no extended

sequence similarity in the C-terminal region. Both Dyrk1A and Dyrk1B contain PEST regions that are believed to determine a rapid turnover of proteins (Rogers et al., 1986). Dyrk1B contains all motifs characteristic for the Dyrk family of protein kinases. Like Dyrk1A, the sequence of Dyrk1B comprises a bipartite nuclear localization motif. Dyrk1B is also designated by the acronym Mirk (minibrain-related kinase). Dyrk1B is expressed at low levels in normal tissue, being found at high levels only in skeletal muscle and testes (Leder et al., 1999; Lee et al., 2000). Ten-fold elevated levels of Dyrk1B protein are found within a subset of colon cancers compared to paired normal tissue. Increased expression of Dyrk1B is also observed in lung carcinomas, ovarian carcinomas and melanomas, suggesting that Dyrk1B is often upregulated in tumor tissue. So far most of the research has focused on determining its role in carcinoma cell and muscle development. The main function of Dyrk1B/Mirk is to assist cell arrest in G_0/G_1 and regulate the transition from growth to differentiation in skeletal muscle tissue (Deng et al., 2005).

Until now, two upstream pathways were found to regulate Dyrk1B/Mirk. Dyrk1B/Mirk is down-regulated by the activation of the Ras-MEK-Erk pathway (Lee et al., 2000). Removal of serum mitogens and blocking any residual Erk activation from autocrine growth factors elevates Dyrk1B levels 20-fold, whereas activation of erks with IGF-I reduces Dyrk1B levels to those observed in cells cultured in serum containing medium, leading to cell proliferation. *Dyrk1B* is induced by members of the Rho-family in myoblasts, and Dyrk1B is active in skeletal muscle differentiation (Deng et al., 2003). The Rho family of small GTPases regulate numerous signaling pathways that control

the organization of the cytoskeleton, transcription factor activity, and differentiation of skeletal myoblasts (Charrasse et al., 2003).

Several proteins have been found to be substrates of or to interact with Dyrk1B (Table 2.3). Five potential targets are identified so far. These include hepatocyte nuclear factor 1α (HNF1α), cyclin-dependent kinase (CDK) inhibitor $p27^{kip1}$, Cyclin D1, Class II histone deacetylases (HDACs) and CDK inhibitor $p21^{cip1}$. DCoHm (dimerization cofactor of hepatocyte nuclear factor 1α (HNF1α) from muscle), a novel member of the DCoH family with 78% amino acid identity to DCoH, was identified as a Dyrk1B-binding protein. Dyrk1B, DCoHm, and HNF1α form a complex which enhances HNF1α transcriptional activity. In many colon carcinomas Dyrk1B and DCoH are co-expressed and may function as an activating complex for HNF1α to induce ectopic gene expression (Lim et al., 2002a). The expression of some of these genes may contribute to the ability of cell lines with stably overexpressed Dyrk1B protein to maintain serum-free proliferation (Lim et al., 2002a).

Table 2.3 Substrate and binding partners of Dyrk1B/Mirk

Substrate or interacting protein	Substrate		Interaction	Effect	References
	Evidence	P-site[a]	Evidence[b]		
Dimerization cofactor of hepatocyte nuclear factor 1 α (DcoHm)	N/A	n.d.	Co-IP Y2H GST Pull-down assay	Mirk, DcoHm form activating complex for HNF1 α	(Lim et al., 2002a)
hepatocyte nuclear factor 1 α (HNF1 α)	In vitro	Ser-247	GST Pull-down	Activate function of HNF1 α	(Lim et al., 2002a)
P38 MAP kinase	N/A	n.d.	Co-IP	Inhibition of transcription[c]	(Lim et al., 2002b)
Ran-binding protein M (RanBPM)	N/A	n.d.	Co-IP Y2H GST Pull-down assay Invivo cross linking	Inhibit Dyrk1B/Mirk	(Zou et al., 2003)
$p27^{kip1}$	In vitro in vivo [d]	Ser-10	immunohistochemistry RNAi	Stabilize $p27^{kip}$ in G_0	(Deng et al., 2004)
Cyclin D1	In vitro in vivo [d]	Thr-288	Co-IP RNAi	Stimulation of nuclear export	(Zou et al., 2004)
Class II histone deacetylases (HDACs)	In vitro	Ser-279	N/A	Stimulation of nuclear export	(Deng et al., 2005)
$p21^{cip1}$	In vivo	Ser-153	N/A	Stimulation of nuclear export	(Mercer et al., 2005)

[a] phosphorylation site (n.d., not determined); [b] Co-IP, co-immunoprecipitation; RNAi, RNA interference;Y2H, yeast two hybrid system; [c] co-transfection of Dyrk1B causes reduced activity in reporter gene assays;[d] co-transfection of Dyrk1B causes enhanced phosphorylation

$p27^{kip1}$ is an inhibitor of CDK and acts as a brake on the proliferation program (Deng et al., 2004). Elevated levels of $p27^{kip1}$ block the cell in G_0/G_1 until mitogenic signals activate G_1 cyclins and initiate proliferation. Phosphorylation of $p27^{kip1}$ at Ser-10 during G_0 by Dyrk1B stabilizes $p27^{kip1}$ and maintains $p27^{kip1}$ within the nucleus where it can bind to CDK2 (Deng et al., 2004). Dyrk1B phosphorylation of $p27^{kip1}$ may assist differentiating myoblasts to arrest in G_0 by stabilizing $p27^{kip1}$. In contrast to the role of $p27^{kip1}$ to block CDK pathway, D-type cyclins , D1, D2 and D3 facilitate the import of CDK4 into the nucleus (Diehl and Sherr, 1997) and assemble combinatorially with CDK4 or CDK6 into complexes that phosphorylate the retinoblastoma protein, releasing factors needed for the progression into S phase. Cyclin D1 is translocated into the cytoplasm during S phase where it is destroyed by the proteasome following phosphorylation. Therefore, phosphorylation by Dyrk1B can increase the turnover of cyclin D1 and assist cell arrest in G_0/G_1. Thus, Dyrk1B has the function of both stabilizing a CDK inhibitor and destabilizing a G_1 cyclin to assist cells to remain arrested in G_0 (Zou et al., 2004).

Dyrk1B is essential for the transcription of myogenin and induces myogenin transcription through indirect activation of the MEF2 transcription factor. Myogenin is a member of a family of myogenic regulatory genes, which includes *myoD*, *myf5* and *mrf4*. These genes encode a set of transcription factors, which are essential for muscle development. Expression of myogenin is restricted to cells of skeletal muscle origin. Deacetylation of histones by histone deacetylases results in chromatin condensation and transcriptional repression. Dyrk1B relieves the inhibition of MEF2 by phosphorylating the class II histone deacetylases on a conserved serine within the highly conserved

nuclear localization sequence which reduces the nuclear localization of histone deacetylases (Deng et al., 2005).

Dyrk1B phosphorylates $p21^{cip1}$ within its nuclear localization domain at Ser-153 causing a portion of the typically nuclear $p21^{cip1}$ to localize in the cytoplasm (Mercer et al., 2005). Translocation to the cytoplasm enables $p21^{cip1}$ to block apoptosis through inhibitory interaction with pro-apoptotic molecules (Mercer et al., 2005). Dyrk1B/Mirk mediates survival during the differentiation of myoblasts.

All of the evidence suggests that Dyrk1B/Mirk assist cell arrest in G_0/G_1, to subsequently mediate transition from growth to differentiation and survival of myoblasts. This seems to be a common role of some members in Dyrk family. As mentioned before, both YakA and Yak1 regulate the transition from growth to differentiation. In myogenesis, Mirk is dramatically up-regulated when myoblasts are induced to differentiate, while depletion of endogenous Dyrk1B/Mirk by RNAi blocks myotube formation (Deng et al., 2005).

Some studies have been performed on *Dyrk*2, 3 and 4 in this family, although *Dyrk1A* and *1B* are the major focus of researchers. *Dyrk2* was found to be a candidate oncogene with high level expression in lung and gut cancer tumors (Miller et al., 2003; Koon et al., 2004; Gorringe et al., 2005). High level expression of *Dyrk3* was observed in erythroid cells and testes, suggesting that *Dyrk3* play a role in erythroid development (Zhang et al., 2005). The human *DYRK4* gene has been found to be 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). Retinoic acid is known to play an important role in neurogenesis in embryonic CNS and

the developing spinal cord. Interestingly, *Hox* genes are known to be transactivated by retinoic acid *in vivo* (Akin and Nazarali, 2005).

2.5.3 Other Dyrk family members

Some studies have been performed on *Dyrk* genes in other model organisms such as *Caenorhabditis elegans* (Pellettieri et al., 2003; Raich et al., 2003), *Schizosaccharomyces pombe* (Bahler and Pringle, 1998; Bahler and Nurse, 2001), *Saccharomyces cerevisiae* (Garrett et al., 1991; Smith et al., 1998; Kassis et al., 2000; Griffioen et al., 2001; Moriya et al., 2001; Martin et al., 2004), and *Dictyostelium discoideum* (Clarke and Gomer, 1995; Reymond et al., 1995; Parent and Devreotes, 1996; Souza et al., 1998; Souza et al., 1999; van Es et al., 2001; Taminato et al., 2002). Dyrk family members exhibit similar functions in these model organisms, assisting or inducing life cycle arrest in cells and further regulating the transition from growth to differentiation.

2.6 *HtrA3* gene

HtrA is a highly conserved family of serine proteases found in species ranging from bacteria to human (Clausen et al., 2002). The HtrA family is characteristic of the combination of a catalytic domain with at least one C-terminal PDZ (present in PSD-95, Dlg, and ZO-1/2) domain (Clausen et al., 2002; Schlieker et al., 2004). HtrA was initially identified in *E. coli* by two phenotypes of null mutants. These mutants failed to degrade misfolded proteins in the periplasm (Strauch and Beckwith, 1988), resulting in the family's first name DegP. The discovery that the mutants did not grow at temperatures above 42°C led to the family name of HtrA (High Temperature

Requirement Factor A) (Lipinska et al., 1988). It was later shown that bacterial HtrA had a molecular chaperon activity at low temperatures and a serine protease activity that digested misfolded proteins at high temperatures (Spiess et al., 1999).

Human or mouse genome contains four *HtrA* genes, named *HtrA1*, *2*, *3*, and *4*. HtrA2 protein has a mitochondrial localization signal and a transmembrane domain in the N-terminal region and is localized in mitochondria as a membrane protein with the protease domain believed to be protruding into the intermembrane space (Suzuki et al., 2001; Li et al., 2002). It was recently reported that a protease-deficient mutation of HtrA2 causes hereditary neuromuscular degeneration disease (Mnd2) in mouse (Jones et al., 2003), suggesting possible roles of HtrA2 in mitochondrial protein quality control akin to that of bacterial HtrAs. HtrA2 has also been reported to induce apoptosis in caspase-dependent and -independent manners (Suzuki et al., 2001; Martins et al., 2002).

There are two alternatively spliced variants of *HtrA3* mRNA (long and short forms) (Nie et al., 2003a). The protein sequence of the short form is identical to that of the sequence of the long form except that it does not have the PDZ domain (Nie et al., 2003a). HtrA3 was discovered initially as a pregnancy-related serine protease that is up-regulated dramatically during mouse placental development (Nie et al., 2003b); HtrA3 is selectively expressed at the maternal-fetal interface during placentation in the mouse (Nie et al., 2006b). More recently, human HTRA3 is found to exert a similar function in human placentation (Nie et al., 2006a).

Research findings show that *HtrA1* and *HtrA3* are expressed mostly in the same embryonic organ but exhibit complementary expression patterns in various tissues (Tocharus et al., 2004). As HtrA1 and HtrA3 share a high degree of domain homologies,

they also share a functional similarity (Nie et al., 2003a). Both HtrA1 and HtrA3 are inhibitors of transforming growth factor-β (TGF-β) signaling and bind to various superfamily members (including TGF-β1, BMP2, BMP4) (Oka et al., 2004; Tocharus et al., 2004). HtrA1 and HtrA3 exhibit similar substrate specificity toward β-casein and certain extracellular matrix (ECM) proteoglycans (Tocharus et al., 2004). Both HtrA1 and HtrA3 act as tumor suppressors. *HTRA1* and *HTRA3* mRNA and protein levels decrease with increasing grades of human endometrial cancer (Bowden et al., 2006). They are both upregulated in the arthritic cartilage (Tocharus et al., 2004). Their protease activity and the ability to degrade extracellular matrix proteins (including cartilage) account for their role in both tumor suppression and arthritis (Baldi et al., 2002; Chien et al., 2004; Tsuchiya et al., 2005; Bowden et al., 2006; Grau et al., 2006). Interestingly, the Hoxa2 regulatory binding element found in this study is partially conserved in both genes. However, whether the regulatory role of Hoxa2 protein on *HtrA3* is conserved in *HtrA1* remains to be demonstrated.

2.7 BMP signaling in craniofacial development

TGF-β (Transforming Growth Factor beta) superfamily members are major regulators of a broad range of developmental events occurring from pre-gastrula embryo to adult (Whitman and Raftery, 2005). The family includes BMPs, TGF-βs, activins, inhibins, and myostatin (Massague, 1998; Piek and Roberts, 2001). Given the fact that HtrA1 and HtrA3 (potential targets of Hoxa2 protein) act as inhibitors of transforming growth factor-β (TGF-β) signaling and bind to at least TGF-β1, BMP2 and BMP4 (Oka et al., 2004; Tocharus et al., 2004), it would be of great importance to identify the possible development process(es) which might involve all these factors. One such event

is craniofacial skeletogenesis. A great deal of literature demonstrates that Hoxa2, TGF-β1, and BMP2, and BMP4 are all involved in this process (Gendron-Maguire et al., 1993; Wan and Cao, 2005; Kanaan and Kanaan, 2006). The knowledge of these factors and their roles in craniofacial development would help us illustrate the potential signaling pathway(s) *Hoxa2* gene may be involved in.

BMPs (Bone Morphogenetic Proteins) induce the formation of bone and cartilage (Nie et al., 2006c). The presence of BMPs was originally implicated from the work of Urist in which he revealed bone autoinduction by bone matrix (Urist, 1965). Later, a number of BMPs were isolated and cloned (Wozney et al., 1988). BMPs are currently recognized as multifunctional growth factors that are essential for gastrulation, organogenesis and embryonic and postnatal growth. BMPs are also involved in craniofacial development. The BMP signaling pathway is involved in a number of developmental processes and is critical for the formation of various craniofacial elements such as cranial neural crest, facial primordia, tooth, lip and palate. Members of the BMP family are referred to as either BMPs, osteogenic proteins, cartilage-derived morphogenetic protein, or growth and differentiation factor (GDF) (Wan and Cao, 2005; Nie et al., 2006c). These members are classified into subfamilies based on their sequence similarity. So far, more than 20 members have been identified in the BMP family (Kishigami and Mishina, 2005).

BMPs mainly function through BMP receptor type I and type II (BMPRI and BMPRII). Three type I (ALK2, ALK3 or BMPRIA and ALK6 or BMPRIB) and three type II receptors (BRII, ActRIIA and ActRIIB) have been identified (Nohe et al., 2004; Kanaan and Kanaan, 2006). In general, the type I receptors are the high-affinity binding

receptors, whereas the type II receptors bind BMPs with lower affinity. Binding of BMP ligand and receptor results in phosphorylation of downstream Smad proteins and triggers the intracellular signal cascade (Kanaan and Kanaan, 2006). The BMP signal activates Smad1, Smad5 and Smad8, which individually can form a heterodimeric complex with Smad4. This complex is further translocated to the nucleus where it activates transcription of target genes (Nohe et al., 2004). The Smad pathway is a well-characterized BMP signaling pathway. However, BMPs also initiate non-Smad interacellular signaling pathways. BMPs activate the MAPK family signaling molecules, i.e., ERK1/2, p38, and stress-activated protein kinase/Jun N-terminal kinase (Nakamura et al., 1999; Hassel et al., 2003; Nohe et al., 2004). Activated MAPK molecules lead to activation of alkaline phosphatase and stimulation of osteocalcin expression in osteoblastic cells (Guicheux et al., 2003).

BMPs are associated with the formation, migration, and differentiation of neural crest cells (NCCs) (Tzahor et al., 2003; Glavic et al., 2004). The neural crest is a pluripotent population of cells that is produced in the dorsal neural tube as a result of inductive interactions between the neural plate and the surface ectoderm (Olsen et al., 2000). After neurulation, NCCs delaminate and migrate along defined pathways to differentiate into a variety of cells and tissues. NCCs originating from the anterior neural tube (forebrain, midbrain, and anterior hindbrain), referred to as the cranial neural crest cells (CNCCs), populate the facial region and the first and second branchial arches. These cells eventually differentiate into bone, cartilage, cranial ganglia, and connective tissue of the head and neck.

The BMP signaling has been recognized as a patterning signal for the neural crest. *BMP4* and *BMP7* are detected in the ectoderm and can induce non-neural ectoderm to form neural crest cells (Liem et al., 1995). BMP4 is responsible for the maintenance of a number of dorsal neural tube genes such as *Msx1, Msx2 and Slug* (Trainor et al., 2003; Tribulo et al., 2003) and a gradient of BMP seems crucial for proper patterning of the neural plate and neural crest (Tribulo et al., 2003). Furthermore, BMP signaling is also essential for migration of CNCCs to the facial primordia (Kanzler et al., 2000; Knecht and Bronner-Fraser, 2002; Tribulo et al., 2003). Blockage of BMP2/BMP4 in mouse cranial neural crest results in depletion of CNCCs from the targeted areas; as an outcome, the branchial arches populated by NCCs are hypomorphic and their skeletal and neural derivatives fail to develop (Kanzler et al., 2000). During early craniofacial development, *BMP4*, *BMP2* and *BMP7* are prominently expressed (Francis-West et al., 1994; Bennett et al., 1995; Francis-West et al., 1998). Later, *BMP4* is also expressed in the mesenchyme of facial primordia. Ectopic application of recombinant BMP2 or BMP4 protein can activate the expression of *Msx* genes at sites where *Msx* expression does not occur (Barlow and Francis-West, 1997). This signaling cascade is associated with altered expression of *Fgf4* and *Shh* and can cause abnormal development of the facial primordia (Barlow and Francis-West, 1997).

BMP signaling is involved in determination, migration, condensation, proliferation, differentiation and apoptosis of skeletal cells (Nie et al., 2006c). BMP signaling is conserved in craniofacial skeletons regulating both the endochondral and intramembranous bone formation. It is crucial for the formation of skeletogenic precursor cells in the neural crest and their migration to the programmed destinations

(Kanzler et al., 2000). Overexpression of *BMP* or application of BMP proteins changes the skeletal patterning, resulting in altered size and morphology of the skeleton in both the face and limb (Duprez et al., 1996; Barlow and Francis-West, 1997). Moreover, interruption of BMP signaling in mouse cranial neural crest shows multiple defects in craniofacial skeletons (Dudas et al., 2004). These data demonstrate a patterning role of *BMPs* in craniofacial skeletogenesis.

After early development, *BMPs* maintain their expression in the skeletons and skeletal growth centres and play an important role. The cranial sutures are critical growth sites for the calvarias. Premature fusion of these sutures leads to a pathologic condition, known as craniosynostosis. The BMP signal is an important player in regulating the sutural morphogenesis and function (Kim et al., 1998; Holleville et al., 2003). Both BMP2 and BMP4 are present in the osteogenic fronts of cranial sutures (Kim et al., 1998). Application of BMP4 protein increases the tissue volume in the suture and induces the expression of *Msx* genes (Kim et al., 1998).

The crucial roles of BMP signaling in skeletogenesis have improved our knowledge in skeletal tissue regeneration and engineering. Application of BMP proteins, BMP-induced or BMP-expressing stem cells, or *BMP* gene transfer techniques have significantly progressed the regeneration process of bone and cartilage (Lieberman et al., 1998; Lieberman et al., 1999; Suzuki et al., 2002; Chang et al., 2004).

2.8 TGF-β1 and skeletogenesis

Transforming Growth Factor β1 (TGF-β1) exerts its functions in both embryogenesis and adult organism. TGF-β1 regulates cell proliferation, differentiation, motility and apoptosis. Although TGF-β2 and -3 are detectable, TGF-β1 is the most

abundant growth factor in human bone (Hering et al., 2001). Similar to BMPs, TGF-β1 can initiate both Smad and non-Smad interacellular signaling pathways (Kanaan and Kanaan, 2006).

TGF-β1 is a prototypic multifunctional cytokine, which regulates a wide range of biological processes including cell proliferation, migration, differentiation, apoptosis, and extracellular matrix deposition. Interleukin (IL)-13 which is a major inducer of fibrosis in many chronic infectious and autoimmune diseases induces TGF-β1 (Kanaan and Kanaan, 2006). TGF-β1 plays a role in craniofacial skeletal development. TGF-β promotes neural crest cell proliferation and extracellular matrix production (Ito et al., 2002). During the epithelial-mesenchymal interaction, TGF-β1 together with BMP-2/4, Msx-1 and tenascin mainly control these interactions (Hall and Miyake, 2000).

2.9 Strategy Used To Identify Downstream Targets of Hox Factors

To elucidate pathways through which *Hox* genes regulate development specification, we must first identify the downstream targets of homeobox proteins. Various strategies have been applied to identify homeobox protein targets (Pradel and White, 1998; Martinez and Amemiya, 2002). Each method has its advantages as well as shortcomings. 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 or phenotype in *Hox* mutants (Graba et al., 1997). This method may potentially identify regulators of *Hox* genes or parallel factors within the same developmental pathway. So it 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). 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. However, it suffers the same drawbacks as it is difficult to determine whether the control by Hox protein is direct or indirect. Therefore, direct regulation of the potential targets and identification of the binding sites by a particular Hox protein still needs to be demonstrated by DNA-binding analysis and Hox regulation of the target promoter *in vivo*. Chromatin immunoprecipitation has emerged as a very popular method to isolate target of transcription factors, since it facilitates the identification of a target in an *in vivo* context. It can also identify the likely DNA-binding site from multiple potential sites (Salsi and Zappavigna, 2006).

Targets of *Hox* 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). Moreover, it can be used for investigating the transcription activation of Hox proteins on previously identified targets (Li et al., 1999; Nasiadka 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 reported that the use of Hoxa2-

VP16 fusion protein in cell culture resulted in VP16-mediated squelching (Matis et al., 2001).

After the initial isolation of the core TAAT binding motif of Hox proteins, 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 luciferase assays or by expression pattern analysis. For instance, the *EphA7* gene was recently identified as a direct target of Hoxd13 and Hoxa13 (Salsi and Zappavigna, 2006). The promoter sequence of the *EphA7* gene was first analyzed and multiple TAAT motifs were found. The actual direct binding site between these motifs and Hox proteins was identified and confirmed by ChIP, EMSAs and luciferase assays. As mentioned earlier, all of the above methods have advantages and limitations when applied individually. The findings are more indicative when multiple methods are used to characterize the target gene.

The subtractive hybridization and the mutant screening methods have been applied to isolate downstream targets of Hoxa2 protein (Bobola et al., 2003; Kutejova et al., 2005; Santagati et al., 2005). Several downstream targets have been identified, including *Six*, *Ptx1* and *Msx1* all of which function in cranial development. A Hoxa2/Pbx consensus binding site was also reported [(T/A) GAT (T/G) GA (T/A) G]. However, this site was deduced from the Pbx/Hox composite site and has only been tested for function in luciferase assays (Lampe et al., 2004). More experiments need to be conducted to determine its physiological importance.

Although subtractive hybridization and the mutant screening methods were applied previously to isolate downstream targets of Hoxa2 protein (Bobola et al., 2003;

Kutejova et al., 2005; Santagati et al., 2005), there are no reports where ChIP has been used to identify Hoxa2 targets. In our laboratory, ChIP has been used to isolate Hoxa2 targets from chromatin preparations in the mice spinal cord and hindbrain. Two potential targets: the *high temperature requirement factor A3 (HtrA3)* and the *Dual specificity tyrosine kinase 4* (*Dyrk4*) genes were identified. In this study I have used various techniques to identify the binding site and address the physiological relevance of this regulation. Our findings provide additional evidence for the activity regulation model and contribute to the understanding of pathways through which *Hoxa2* gene functions in regulating regional specification.

3. IDENTIFICATION AND CHARACTERIZATION OF *Dyrk4* AS A PUTATIVE DOWNSTREAM TARGET OF Hoxa2 PROTEIN

3.1 Abstract

1. Currently very few Hoxa2 protein downstream target genes have been identified and little is known of the genetic pathways through which Hoxa2 protein regulates CNS development.

2. *Dual specificity tyrosine kinase 4* (*Dyrk4*) was isolated as a potential target gene of Hoxa2 protein in the developing CNS by chromatin immunoprecipitation.

3. We found that Hoxa2 protein binds to a "CATCATG" site in the *Dyrk4* sequence *in vitro*.

4. A Hoxa2-VP16 fusion protein activates luciferase reporter expression in the presence of this specific binding site.

5. Our results showed that Hoxa2 protein may regulate *Dyrk4* expression in the developing CNS.

KEY WORDS: Hoxa2, chromatin immunoprecipitation, Dyrk4, CNS, EMSA, transcription factor.

3.2 Introduction

Hox genes are conserved transcription factors that function to control embryonic morphogenesis (McGinnis and Krumlauf, 1992; Favier and Dolle, 1997; Prince, 2002). Vertebrate *Hox* genes are considered to specify the regional identity by regulating

common cellular processes such as cell death, adhesion, proliferation, and migration (Akin and Nazarali, 2005). It appears that many *Hox* genes are the 'selector' genes, 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).

The *Hoxa2* gene and its paralog *Hoxb2* are homologs of the *Drosophila proboscipedia* gene (Akin and Nazarali, 2005). Dominant expression of *Hoxa2* gene during embryogenesis occurs in the neural tube and neural crest cells that contribute to the second brachial arch, as well as other tissues (Prince and Lumsden, 1994b; Gavalas et al., 1997; Hao et al., 1999; Barrow et al., 2000; Grammatopoulos et al., 2000). Within the neural tube, the anterior boundary of *Hoxa2* gene expression is situated at the rhombomere (r) 1/2 interface (Davenne et al., 1999b; Barrow et al., 2000). *Hoxa2* gene expression extends from the hindbrain caudally throughout the spinal cord, with expression beginning at first within the ventral mantle region at embryonic day 10 (E10). However, expression of *Hoxa2* gene at E18.5 is predominantly found within the dorsal horn (Hao et al., 1999). Hence, *Hoxa2* gene may potentially contribute to both anteroposterior (A-P) positioning as well as dorsoventral (D-V) patterning (Hao et al., 1999). Although the anterior hindbrain is unaffected even by an extreme decline in Hoxa2 protein levels, at the molecular level differential sensitivity to *Hoxa2* gene inactivation between specific neuronal subtypes has been observed (Ohnemus et al., 2001). This evidence suggests that *Hoxa2* gene might be involved in the specification of neuronal phenotypes within the CNS (Hao et al., 1999; Ohnemus et al., 2001).

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. Several direct downstream target genes of the anteriorly expressed *Hox* genes have been isolated with regard to CNS development (Akin and Nazarali, 2005). Targets identified for the *proboscipedia* class of homeobox genes include: regulation of the homeobox *Otx1* gene by Hoxb2 (Guazzi et al., 1998), regulation of *Six2* by Hoxa2 protein (Kutejova et al., 2005), and *Hoxa2* gene expression autoregulation within the rhombomeres (Guazzi et al., 1998; Lampe et al., 2004; Kutejova et al., 2005). Various strategies have been utilized to identify targets of homeobox proteins in both vertebrates and *Drosophila* (Akin and Nazarali, 2005). In *Drosophila,* Gould et al. (1990) isolated *in viv*o targets of the Ubx homeotic protein by immunoprecipitation of chromosomal-protein DNA complexes. This method has been successfully used in the murine system for the isolation of Hoxc8 (Tomotsune et al., 1993) and Hoxb5 (Safaei, 1997) candidate target genes. This technique is advantageous over other approaches in that it allows isolation of a target gene *in vivo*. Isolation of targets by immunoprecipitation also 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. We have employed the chromatin immunoprecipitation method to identify a downstream target gene regulated by Hoxa2 protein during the later stages of hindbrain and spinal cord development. *Dyrk4*, a member of the Dyrk (dual-specificity tyrosine-phosphorylated regulated kinase) protein kinase family, was isolated as a target gene of Hoxa2 protein.

3.3 Materials And Methods

Plasmid Construction

The *Hoxa2* gene expression vector pRSV-Hoxa2 was available (Nazarali et al., 1992) and the plasmid pRG50 with the entire VP16 coding region was kindly provided by Dr. Vikram Misra of the Department of Veterinary Microbiology at the University of Saskatchewan (Akhova et al., 2005). The plasmid pRG50-Hoxa2/VP16 encodes Hoxa2 fusion protein with a 77-aa VP16 activation domain. The *Hoxa2* cDNA without the stop codon was amplified using forward primer 5′ CCCAAGCTTATGAATTACG AATTTGAG 3′ and backward primer 5′ GAAGATCTGTAATTCAGATGCTGTAG 3′. Then, the DNA encoding the N-terminal region (amino acid 1-412) of VP16 was replaced by the *Hoxa2* PCR product to create the *Hoxa2-VP16* fusion gene. To construct the recombinant protein expression vector, the *Hoxa2* cDNA sequence (Nazarali et al., 1992; Tan et al., 1992) was cloned into the pFLAG-2 vector and pGEX-KG vector at the *Eco*R I and *Xho* I sites downstream of the FLAG epitope and GST coding region respectively. The *Dyrk4* sequence was amplified by PCR using primer: 5′ ACGCGTCGACCCAGTCTGCTTAGACTC 3′ and 5′ CGCGGATCCCCATGATGC CTGGTT TT 3′. The *Dyrk4* PCR product was cloned into the pGL3-promoter plasmid at the *Sal* I and *Bam*H I sites to generate pGL3-Dyrk. Mutagenesis of the putative Hoxa2 protein response element localized at the end of *Dyrk4* sequence was performed by PCR with the following mutant oligonucleotides: 5′ CGCGGATCCCCCCGCCGCCTGG TTTT 3′. The resulting PCR product was cloned into the pGL3-promoter plasmid at the *Sal* I and *Bam*H I sites to generate pGL3-Dyrkdoublem. Mutations were confirmed by sequencing. Plasmids pRL-null and pRL-CMV were obtained from Promega (Promega, USA). The expression vector pCS2PBX was a gift from Dr. Mark Featherstone from McGill University (Shanmugam et al., 1999)

Protein Purification

All chemicals were obtained from Sigma (Oakville, ON) unless otherwise specified. All enzymes were purchased from Invitrogen (Burlington, ON). Expression of recombinant FLAG-Hoxa2 protein was induced in XL1-Blue supercompetent *E.coli* (Stratagene, CA, USA) with isopropyl-ß-thiogalactoside (IPTG) and extracted as inclusion bodies from bacteria (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 ^oC. After centrifugation at 87,000 g for 30 min (4^oC), the supernatant was dialyzed against HEMGN with 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 used for subsequent purification by affinity chromatography with columns containing anti-FLAG M2 antibody affinity gel (Sigma, Oakville, ON). Purified recombinant Hoxa2 protein was eluted with 0.1 M glycine (pH 3.5) and then neutralized with 1 M Tris-HCl (pH 8.0).

GST-tagged Hoxa2 protein was expressed in *E .coli* strain BL21-Codon Plus-RIPL. Bacteria were cultured overnight in Luria-Bertani (LB) medium containing 100 µg/ml ampicillin. The cultures were then diluted 1:100 with fresh pre-warmed LB and incubated at 37 °C with vigorous shaking (300 rpm). When the OD_{600} had reached a value between 0.6-1.0 (about 2 h), expression of the fusion protein was induced by adding IPTG to a final concentration of 0.1 mM. Uninduced cultures were also included

to compare protein expression. The culture was then incubated at 37 °C for an additional 2 h and the cells were harvested by centrifugation (7000 rpm, 10 min, 4 °C). Cell pellets were resuspended in 50 µl of phosphate-buffered saline (PBS) per ml of bacterial culture, incubated in the presence of 0.1 mg/ml lysozyme, 1 mM PMSF and 0.1% Triton X - 100 (10 min, RT), and lysed by ultrasonication at 4 °C. Bacterial debris was then removed by centrifugation (14,000 rpm, 10 min, 4 °C) and the supernatant was collected for further purification. The bacterial proteins in the supernatant were analyzed by SDS–PAGE. For purification of the GST–Hoxa2 fusion protein, clarified bacterial lysates were applied to a 50% slurry of glutathione–Sepharose 4B (Amersham Pharmacia) in PBS for 30 min with gentle rotation at room temperature. The beads were washed three times with cold PBS before the recombinant protein was eluted (10 min, 4 °C) three times using 1 × volume of glutathione elution buffer (50 mM Tris–HCl, pH 8.0, 10 mM reduced glutathione). The yield of the Hoxa2 fusion proteins was estimated by measuring their absorbance at 280 nm. Purity of purified protein was analyzed on 10-12% SDS-PAGE gels and identified by Western Blot analysis.

Antibody Purification

A 17-amino acid oligopeptide (J3 peptide) derived from Hoxa2 protein was previously used to generate Hoxa2 peptide specific polyclonal antiserum (B579) in rabbits (Hao et al., 1999). Antibody specific for Hoxa2 protein was purified from polyclonal antiserum (B579) using affinity chromatography. Recombinant FLAG-Hoxa2 protein was conjugated to AffiGel-10 affinity support (BioRad Laboratories, CA, USA) in the coupling buffer [PBS containing 80 mM $CaCl_2$] for 4 h at 4 ^{o}C. The

remaining active ester sites were blocked with 0.1 M Tris (pH 8.0). The J3 antiserum was inactivated at 56 ^{o}C for 30 min and then diluted 1: 2 in the washing buffer [50 mM Tris-HCl (pH 7.5)]. The column was washed alternately by washing buffer alone or washing buffer containing 0.5 M NaCl. Hoxa2- specific antibody was eluted with 0.1 M glycine and neutralized with 0.1 M triethylamine (pH 11.5). The antibody was dialyzed against 0.1 M HEPES (pH 8.0). Eluants were tested by electrophoresis on 12% SDS-PAGE gels and western blot analysis.

Chromatin Preparation and Immunoprecipitation

Target DNA sequences of Hoxa2 protein were isolated from the E18 hindbrain and spinal cord chromatin preparations using a modified immunoprecipitation method (Tomotsune et al., 1993; Safaei, 1997). Day eighteen (E18) gestational embryos were removed from CD-1 dams and staged as per Kaufman (1992) and Theiler (1989). The spinal cord and surrounding tissue were removed from the embryos and fixed by immersing in 4% paraformaldehyde. Chromatin was prepared from the tissues by homogenization in the binding buffer (Safaei et al., 1997). The chromatin-containing supernatant was digested with 10,000 units of *Hae*III for 2 h at 37 ^{o}C, followed by DNase I (10 U) digestion for 5 min at 4 ^{o}C. The reactions were stopped with 2.5 mM EGTA. Hoxa2-DNA complexes were immunoprecipitated from the chromatin preparations by affinity chromatography with purified anti-Hoxa2 antibody conjugated to AffiGel-10 matrix. 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 Na H_2PO_4

(pH 7.0). Protein was removed by incubation with 0.1 mg/ml proteinase K in 10 mM Tris-HCL, 5 mM EDTA and 0.5% SDS for 16 h at 37^{o}C. Samples were extracted with phenol-chloroform followed by passage through NAP-5 columns (Amersham Pharmacia Biotech, Canada). The resulting DNA was subcloned by blunt-end ligation into pBluescript SKII+ (Stratagene, CA, USA). *E.coli* DH5α cells were used for transformation with plasmids and subsequent plasmid DNA isolation. Target sequences were sequenced by the dideoxy-chain termination method (Sanger et al., 1977) using Sequenase-Version 2.0 (USB Corp., OH, USA) (DNA Technologies Unit, NRC-Plant Biotechnology Institute, Saskatoon, SK)

Electrophoretic Mobility Shift Assay (EMSA)

EMSA has been extensively used to in protein DNA-binding study. The following schematic drawing (Figure 3.1) represents the experimental procedures of EMSA and super shift gel electrophoresis assay.

In this study, EMSA was performed using the isolated 103 base pairs (bp) fragment of *Dyrk4* with nuclear extract and FLAG-Hoxa2 protein. Oligonucleotides were incubated with recombinant GST-Hoxa2 protein in buffer. Oligonucleotide probes used in this study were synthesized based on the sequence listed in Table 3.1. The probes were made double-stranded by annealing with equimolar amount of complementary DNA in 1 × annealing buffer (10 mM Tris-HCl, pH 7.5-8.0, 50 mM NaCl, 1 mM EDTA). Double-stranded probes (5 pmol) were then end-labeled by incubating with 10 U T4 polynucleotide kinase, 2.5 µl [γ-^{32}P] ATP (10 Ci/µl, 3000 Ci/mmol) in reaction buffer (25 µl total volume) for 30 min at 37 °C. The probes were

purified using a Qiaquick nucleotide removal kit. The DNA-binding reactions were performed for 30 min on ice with a volume of 20 µl containing 2µl GST or GST-Hoxa2 protein where indicated, 10^5 cpm ^{32}P-labeled probe, 1 µg poly [dI:dC], and 10 µl 2 × binding buffer (1 × buffer: 10 mM Hepes-KOH, pH 7.9, 10% glycerol, 50 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT).

The anti-Hoxa2 polyclonal rabbit antiserum (B579) was used for supershift reactions, and non-immune rabbit antiserum was used in control reactions. Competition reactions were performed using 100 X concentration of unlabelled target sequence probes with Hoxa2 protein. A 100-fold excess of unlabeled competitor DNA, antibody or nonspecific serum was added 1h prior to adding the probes. The entire contents of each reaction were loaded on a 5% polyacrylamide (38:2) gel, pre-cooled to 4 °C in 1 × TGE buffer. Samples were subjected to electrophoresis for 40 min at 8 volts per centimeter. Gels were dried and exposed to x-ray film overnight at -70 °C.

Nuclear Extract Preparation

Nuclear extracts were prepared from spinal cord and hindbrain tissue of E18 mice by the method described in Thompson et al. (1998). Briefly, embryonic hindbrain and spinal cord were homogenized in PBS (pH7.4), 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, ON), and 0.2% Nonidet P-40]. Cells were lysed for 10 min at 4 ^{o}C and the nucleus pelleted by centrifugation in a 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 M KCl, 0.2 mM EDTA, 1 mM DTT, 0.5

mM PMSF, 25% glycerol] and incubated for 1 hr at 4 ^{o}C on an orbitron shaker. The 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. The DNA probe for the 103 bp isolated *Dyrk4* fragment was produced by digesting with *Xho* I and *Xba* I and labeling with [α-^{32}P]dATP using Klenow fragment of DNA polymerase I. Binding reactions with nuclear protein 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 poly(dI-dC), and 50,000 cpm of probe at room temperature for 20 min.

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′ GTAAAA CGACGGCCAGT 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 m at 95 ^{o}C followed by 20 cycles of 95 ^{o}C for 30 s, 58 ^{o}C for 15 s, and 72 ^{o}C for 40 s. Varying amounts of nuclear extract 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 poly(dI-dC) at 4 ^{o}C overnight or at room temperature for 2 h. Samples were treated with 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 precipitated with ethanol for several hours at –20 ^{o}C. The DNA pellets were then 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 a 6% denaturing sequencing gel and exposed to Kodak X-OMAT film at –70 ^{o}C.

Cell culture, transfection and luciferase activity assays

COS-7 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (Gibco), 100 UI/ml of penicillin and 100 µg/ml streptomycin. Transfections were performed using polyfect transfection reagent (Qiagen) in 6-well plates as per the manufacturer's instructions. For internal control, 10 ng of pRL-CMV or 100 ng of pRL-null were used as indicated. In a typical transfection experiment 1.5 µg DNA was used, containing 100 ng of reporter plasmid (pGL3, pGL3-Dyrk or pGL3-Dyrkdoublem), 200 ng of pRG-Hoxa2-VP16 or pRSV-Hoxa2, internal control, and pcDNA to complement. Cells were harvested 24-48 hr after transfection, lysed, and assayed for renilla and firefly luciferase expression as described in the manual included with Dual-Luciferase Reporter Assay System (Promega).

3.4 Results

Isolation of *Dyrk4* as a putative downstream target gene of Hoxa2 protein using ChIP

Hoxa2-DNA complexes were isolated by immunoprecipitation from chromatin preparations from the hindbrain and spinal cord tissue of E18 embryos, and a 103 bp fragment was subcloned into the *Sma* I site of pBluescript SKII+. The 103 bp fragment (Figure 3.2) is present within the intronic segment of the *Dyrk4* gene.

Hoxa2 protein binds to a specific site in *Dyrk4* sequence

EMSAs were performed to confirm the direct binding of Hoxa2 protein to the isolated sequence. Incubation of the probe with the crude FLAG-Hoxa2-expressing bacterial extract results in the formation of a band-shift (lane 2, Figure 3.3). This shift complex represents the interaction between Hoxa2 protein and the sequence, as it can be supershifted by anti-Hoxa2 antibody (lane 4, Figure 3.3). This complex was also competed in the presence of 100-fold unlabeled probe (lane 6, Figure 3.3). A consistent result in the EMSA was observed when using nuclear extract from E18 embryos. However, instead of producing the supershift band, the addition of anti-Hoxa2 antibody abolished the interaction between Hoxa2 protein and the probe (lane 5, Figure 3.4). To identify the specific binding site in the isolated *Dyrk4* sequence we performed DNase I footprinting. Nuclear extract isolated from E12 whole embryos was used for footprinting analysis due to high level of *Hoxa2* gene expression at this stage of development. Protection from DNase I digestions was observed within the sequence 5′ TACCGTAGTACC 3′ (Figure 3.5). Wild-type and mutant probes (Table 3.1) were incubated with GST-Hoxa2 protein in EMSA to further characterize the binding site. The “TAAT” site is known to bind to all Hox proteins *in vitro* (Kalionis and O'Farrell, 1993; Gehring et al., 1994; Kumar and Nazarali, 2001). A probe with multiple “TAAT”

site, named Pctrl, was included as a positive control. The binding between GST-Hoxa2 protein and the Pctrl (Figure 3.6, A) probe demonstrates the binding activity of GST-Hoxa2 protein *in vitro*. Consistent with previous EMSA results, incubation of the probe Pdyrk482-521 results in the formation of a band-shift. This band is supershifted by the addition of anti-Hoxa2 antibody, which indicates the specific binding between GST-Hoxa2 protein and the probe (Figure 3.6B, lane 2). This binding is competed by adding 100-fold unlabeled probe (Figure 3.6B, lane 5). Mutant probes with the sequences "CGGCATG" and "CATCGGG" can still bind to the protein (Figure 3.6C, lane 3, 4), but the alteration of the two "TA" nucleotides simultaneously (CGGCGGG) results in the loss of the binding ability (Figure 3.6C, lane 5).

Hoxa2-VP16 fusion protein activates transcription of luciferase expression through the specific binding site in the *Dyrk4* fragment

Transient transfection experiments were used to examine the effect of Hoxa2 protein and Hoxa2-VP16 fusion protein on the Hoxa2 protein responsive element in isolated *Dyrk4* sequence. Hox-VP16 fusion protein was previously used to examine the regulatory activity of Hox proteins on their responsive elements (Friedman-Einat et al., 1996; Li et al., 1999; Nasiadka et al., 2000). The isolated *Dyrk4* sequence is present within an intronic rather than a promoter region of *Dyrk4*. Hence, this sequence was subcloned into the pGL3-promoter vector, in which firefly luciferase expression is regulated by a SV40 minimal promoter. To avoid experimental variations caused by different transfection efficiencies, an internal control reporter containing renilla luciferase gene (pRL-CMV) was included in the cotransfection experiments.

Cotransfection of COS-7 cells with a *Hoxa2* gene expression plasmid (pRSV-Hoxa2), pRL-CMV and *Dyrk4* reporter plasmid (pGL3-Dyrk) was first performed. However, no responsive activity was observed with the overexpression of Hoxa2 protein (data not shown). Since Pbx is a common cofactor of Hox proteins and contribute to the binding specificity of Hox protein to target genes (Chan et al., 1994), a Pbx expression plasmid pCS2PBX was further added in the cotransfection experiments to test if the cofactor would significantly contribute to the influence of Hoxa2 protein on the *Dryk4* fragment. No significant effects were observed (data not shown). The Hoxa2-VP16 fusion protein was then used in transient transfection experiments. Matis et al. (2001) have shown that the fusion protein between Hoxa2 protein and the VP16 activation domain inhibits transcription from the strong promoter/enhancer of cytomegalovirus (CMV) and Rous sarcoma virus (RSV). We did observe varying amounts of inhibition of the CMV promoter when using pRL-CMV plasmid as an internal control (data not shown). To avoid this problem, we replaced pRL-CMV with a promoterless vector, pRL-null, to stabilize the renilla luciferase level. An activation effect of Hoxa2-VP16 on the *Dyrk4* intronic sequence was observed (Figure 3.7). Mutations in the binding site significantly inhibit this activation (Figure 3.7).

3.5 Discussion

Several putative target sequences of Hoxa2 protein were isolated by chromatin immunoprecipitation (Akin Z, Ph.D Thesis, University of Saskatchewan, 2004). Of these target sequences, one clone was identified by BLAST query (Altschul et al., 1990) of the Ensemble Mouse Genome Database (EBI/Sanger Institute) as the murine

homolog of the human *Dual Specificity Tyrosine Kinase 4 (DYRK4)* gene (Figure 3.2A. and B.). The Hoxa2 protein target sequence (Figure 3.2C.) is present within the intronic segment of the *Dyrk4* gene. It is a member of the phosphorylation regulated kinase family (Becker et al., 1998). The analysis involves the use of a GeneWise or GenScan prediction followed by confirmation of the exons by comparison to protein, cDNA and EST databases (Ensemble, EBI/Sanger Institute).

No specific *in vivo* binding site has previously been reported for Hoxa2 protein, although a consensus DNA-binding sequence has been reported for the Hox/Pbx complex. Here, for the first time, a specific binding site for Hoxa2 protein was identified. EMSA analysis shows recognition of the target sequence by recombinant Hoxa2 protein (Figure 3.3) and nuclear extract (Figure 3.4). Hoxa2 protein is involved in the formation of the DNA-protein complex as determined by supershift assays using anti-Hoxa2 antibody. Both wild-type and mutant probes were used to test the specific Hoxa2 protein response element. DNase I Footprinting and EMSA results demonstrate that the core specific binding site is "CATCATG". Mutations of both "AT" sites in the middle prevent the binding in EMSA, which is consistent with the previous suggestion of the importance of "AT" site in the Hox/Pbx consensus binding sequence (Chan and Mann, 1996; Lampe et al., 2004); this site is different from the consensus Hox/Pbx or Hoxa2/Pbx site: "(T/A) GAT (T/G) GA (T/A) G" in which Pbx and Hox each binds to an overlapping half site respectively (Chan and Mann, 1996; Lampe et al., 2004). In our case Hoxa2 protein did bind to "GATG" site which is consistent with the half Hox binding in the Hox/Pbx consensus binding sequence (Figure 3.6). Interestingly, only

simultaneous mutations of the two "AT" residues abolished binding, suggesting GST-Hoxa2 protein may bind to both "GATG" and "CATG" site.

Transient transfection experiments were applied to address the activity of this Hoxa2 protein responsive element in COS-7 cells. Hoxa2 protein did not affect the reporter gene through the target sequence even with the presence of the Pbx cofactor (data not shown), which could be due to the lack of the other half of the Pbx binding site. Although many experiments (Chan et al., 1994; van Dijk and Murre, 1994; Chan and Mann, 1996; Mann and Chan, 1996; Neuteboom and Murre, 1997; Lampe et al., 2004) suggest the involvement of Pbx in regulatory functions of Hox family proteins on their targets, these do not exclude the involvement of other unknown cofactors (Li et al., 1999). To bypass the needs of unknown cofactors, a Hoxa2-VP16 fusion protein was used in transient transfection assays. After using the fusion protein of Hoxa2 protein and the VP16 activation domain, an activation on the reporter gene expression was observed and the mutant oligonucleotide significantly decreased this activation. This indicates there may be unknown cofactors involved other than Pbx, because Hoxa2 protein alone cannot affect the reporter and neither can Pbx and Hoxa2 protein together. This could be due to the fact that Hoxa2 protein binds to the site, but is unable to initiate an effect. Fusion between Hoxa2 protein and a strong VP16 activation domain somehow bypasses the needs for cofactors and activates the reporter gene. The Hoxa2-VP16 fusion was reported to inhibit the CMV promoter (Matis et al., 2001). We observed various levels of inhibition in our experiments which led us to replace the CMV promoter renilla internal control with a promoterless renilla plasmid.

Dyrk family members exhibit similar functions in several model organisms.They assist or induce life cycle arrest in cells and regulate the transition from growth to differentiation. In mammalian cells, *Dyrk1A* plays important roles in the neurogenesis and neuronal differentiation during brain development and in learning/memory in adulthood (Galceran et al., 2003; Hammerle et al., 2003b). Among the genes of the Down syndrome Critical Region, the consideration of *Dyrk1A* as a candidate gene for mental retardation is relatively well supported by the phenotype of transgenic mice that overexpress it and by the analysis of its neurodevelopmental roles (Hammerle et al., 2003b). Dyrk1B/Mirk assists cell arrest in G_0/G_1 and subsequently mediates transition from growth to differentiation and survival of myoblasts (Diehl and Sherr, 1997; Deng et al., 2004; Zou et al., 2004; Deng et al., 2005). *Dyrk2* is a candidate oncogene with a high level of expression in lung and gut cancer tumors (Miller et al., 2003; Koon et al., 2004; Gorringe et al., 2005). A high level of expression of *Dyrk3* was observed in erythroid cells and testes, suggesting that *Dyrk3* might play a role in erythroid development (Zhang et al., 2005). 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 investigated (Leypoldt et al., 2001). Retinoic acid is known to play an important role in neurogenesis in embryonic CNS and the developing spinal cord. Interestingly, *Hox* genes are known to be transactivated by retinoic acid *in vivo* (Boncinelli et al., 1991; Simeone et al., 1991; Conlon and Rossant, 1992). *Vyak*, a Dyrk family member in chicken, was identified as a potential target of chicken *Hoxa4* by ChIP in an unpublished observation (Shang et al., 2000), however, further experiments are necessary to test if there is a relationship between these two

families. Although the precise function of Dyrk4 has not been characterized, it may play a role in the phosphorylation of proteins such as its other family members (Gwack et al., 2006). Our results show that Hoxa2 protein may be involved in the regulation of *Dyrk4* transcription in the developing CNS, where it may affect the activity of other transcription factors or signaling molecules involved in neuronal development pathways.

3.6 Figures

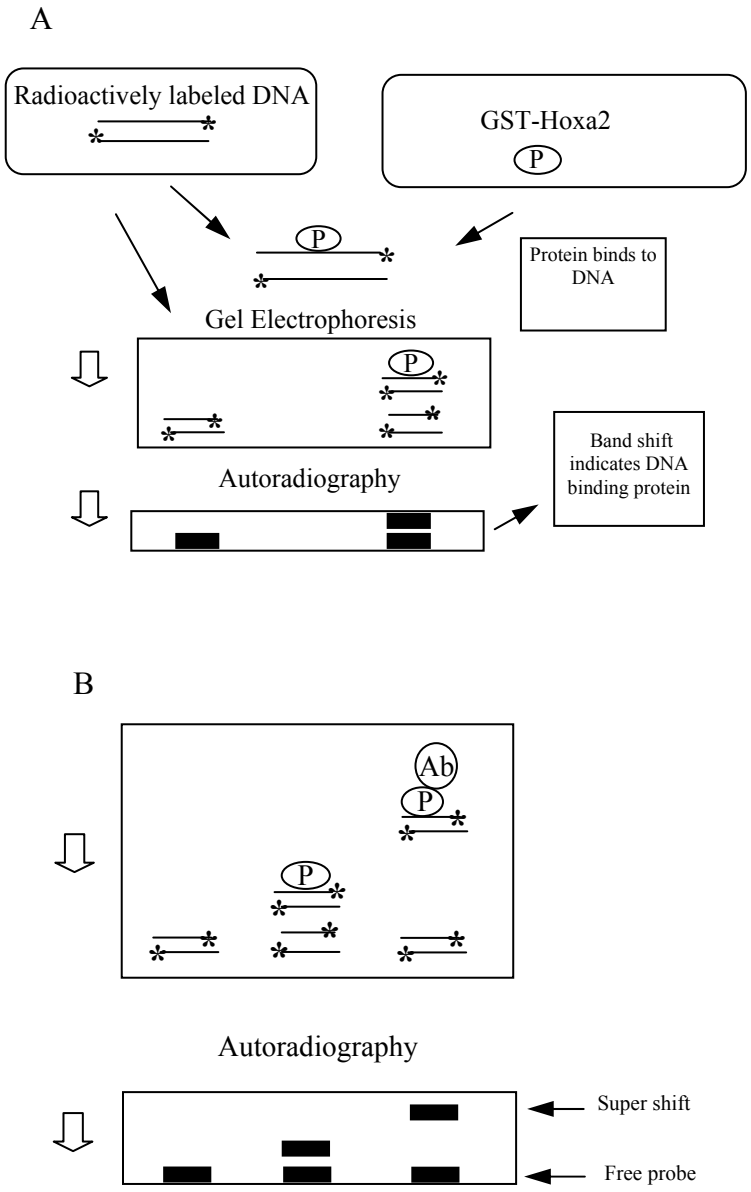
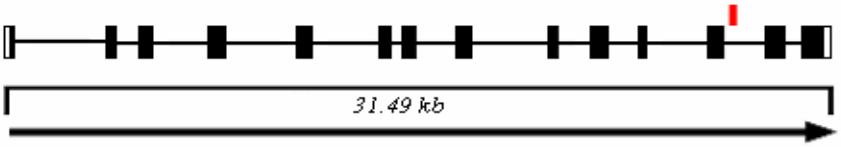
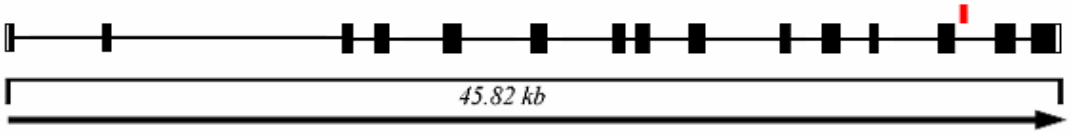


Figure 3.1 Schematic drawing of EMSA A, procedures for EMSA B, additional procedures for super shift gel electrophoresis

A.



B.



C.

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gtgagtttgttggggtgtctttgtttgtgggtctcttaacttttaagtaccatgctttcc 60
tgtgtgtttgctggtaagagtcaaggctgggaggtgggtgcaagagcccatcaggacact 120
gtcttcactgtcaggaagagctttcaagtattcaagtcatttcacgtacgtgtgcatgtg 180
tgtgtgtaaagattgttaccttcttaatcccaagctttccaacagattgtaggtcctaat 240
gtctaatacttcatgaactaactatatagccagctcttcagtgggcttaacgtactgtgt 300
gcaaagctatgcagtacacatgggcaccaggacacttttgtttagtccctcaatttagtg 360
ttcagacctccacatactgggcagttaggggcaaggggtcatagactattgtgcacagc 420
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gcgaggtgttactaaggtgccttcagtcccactggtcctacaactgtttagtgttttcag 720
aagagtgtgcctttataatctctcactctcctggaagggggggggcacagaatgatgtca 780
gcaaagcactgtggaattcacacattctacaactcgagctgcagcccagagggtgatcag 840
ttaacagaagagtctgcttggctgacttcaggtggagtcacgtccacctgaagtcacccc 900
agactcttgctgagactacagaatgaatttctctcaaaaatgacagaaaccaacactgag 960
actgggtttgggggtaggggttacctggaaccccattcattcagaacacagttcaggagc 1020
aactcaggttttcattcattcatggatgcattgcaaagggtcaactgccttcaactcata 1080
```

Figure 3.2 Schematic representation of the murine *Dyrk4* gene.
The direction of transcription is indicated by the black arrow, exonic sequences are shown by closed black boxes, intronic sequences are represented by the black line running between the closed boxes, and UTRs by open boxes. A 103 bp fragment was isolated by immunoprecipitation of Hoxa2-DNA complexes from chromatin (indicated by red boxes). *Dyrk4*, which is present on chromosome 6 in mouse, shows alignment with the Hoxa2 protein target sequence and encodes two overlapping transcripts. Both transcripts have been predicted by the Ensemble analysis pipeline and show similarity with the human *DYRK4* gene. **A**. This transcript is designated as *Dyrk4*, ENSMUSG00000030345 and consists of 14 exons spanning 31.49 kb. **B.** This gene transcript is designated as ENSMUST00000078521 and consists of 15 exons spanning 45.82 kb. **C.** A 103 bp fragment was isolated by immunoprecipitation of Hoxa2 protein from chromatin preparations. The sequence of immunoprecipitated fragment which is present in the intron of the mouse *Dyrk4* homolog is indicated in grey. (Akin Z, Ph.D Thesis, University of Saskatchewan, 2004).

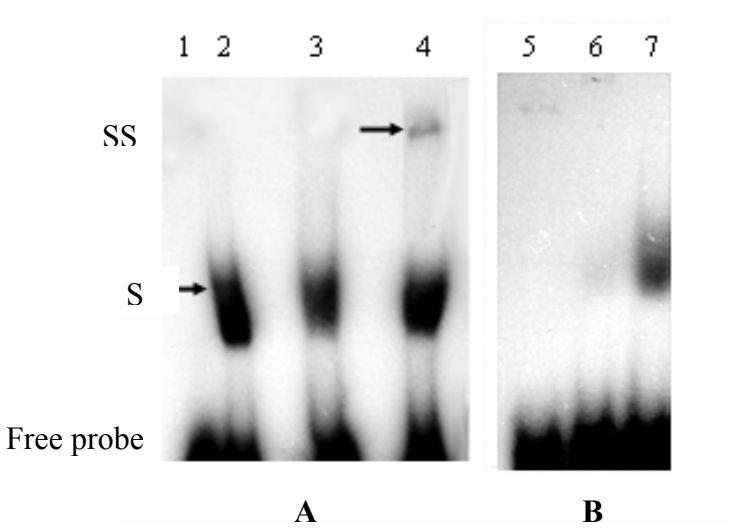


Figure 3.3 Binding of recombinant Hoxa2 protein to the 103 bp murine *Dyrk4* intronic sequence .

A. The target sequence was incubated with 10 μg of crude bacterial extract from FLAG-Hoxa2 protein expressing *E.coli* (lane 2-4). The addition of anti-Hoxd1 antibody did not affect band-shift formation (S) (lane 3), while the addition of anti-Hoxa2 antibodies resulted in a supershift (SS) (lane 4). B. The band-shift formed with crude recombinant FLAG-Hoxa2 protein extract (lane 7), is inhibited by the addition of 100 X unlabeled target sequence to the binding reaction (lane 6). Migration of the labeled unbound probe is shown in lanes 1 and 5. (Akin Z, Ph.D Thesis, University of Saskatchewan, 2004)

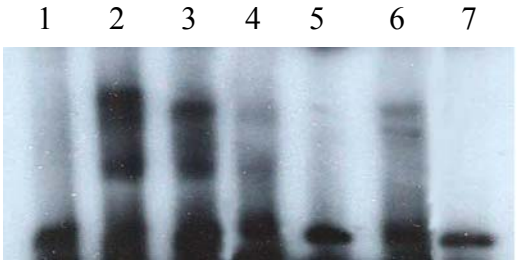


Figure 3.4 *In vitro* DNA-binding of nuclear factors to the *Dyrk4* intronic sequence. Lane 1: free probe (lane 1). Lanes 2, 3, and 4: probel with 15, 10 and 5 μg of nuclear extract respectively. Lane 5 and lane 6: probe with 4 μg and 2 μg of Hoxa2 protein anti-serum respectively. Lane 7: 100-fold unlabeled probe was added to binding reactions with 15 μg nuclear extract. (Akin Z, Ph.D Thesis, University of Saskatchewan, 2004)

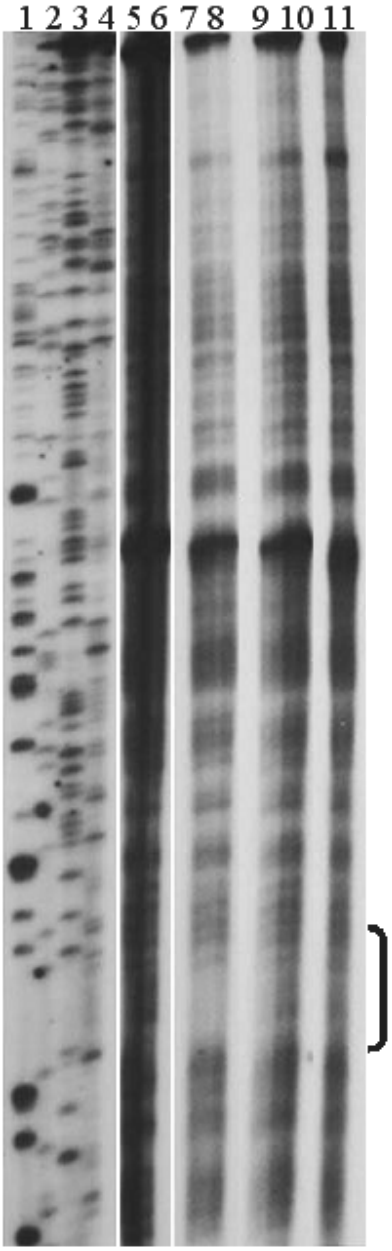


Figure 3.5 DNase I footprinting analysis of isolated Dyrk4 sequence. Thermocycle sequencing was used as a DNA ladder for footprinting reactions (lanes 1-4, GATC respectively). The probe was digested with increasing concentration of DNase I in the absence of protein (lanes 5 and 6). Binding reactions using 20 μg of E12 nuclear extract were digested with increasing concentrations of DNase I (0.002-0.08 U) in lanes 7 –11, respectively. DNase I footprinting sites sequence is 5′ TACCGTAGTACC 3′, marked with bracket. (Akin Z, Ph.D Thesis, University of Saskatchewan, 2004)

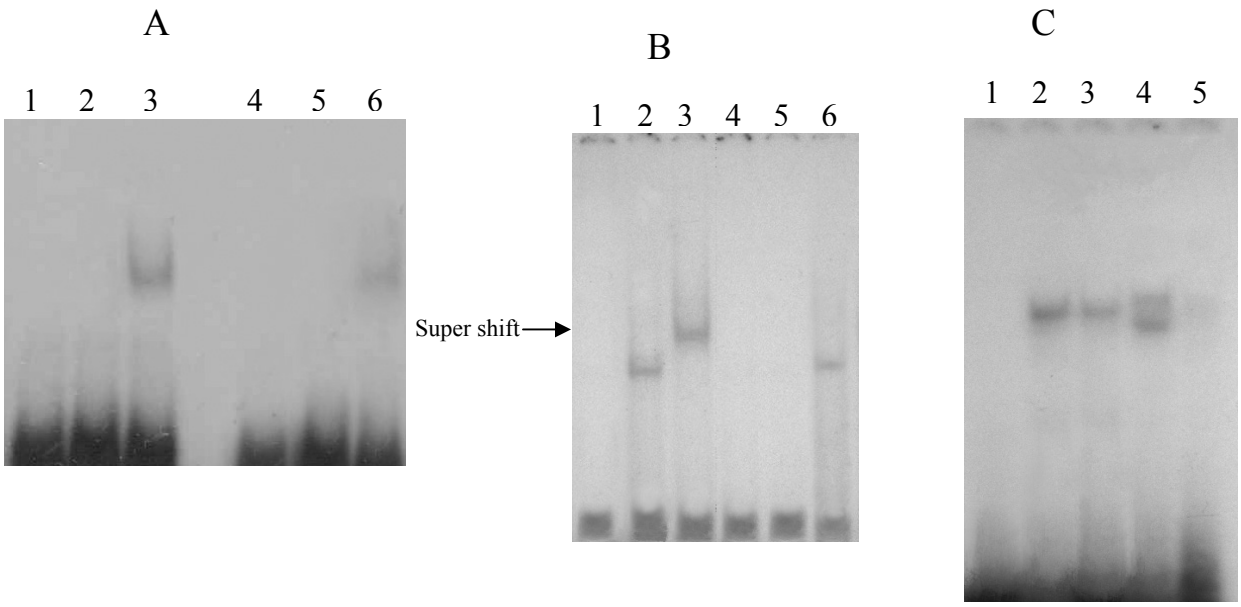
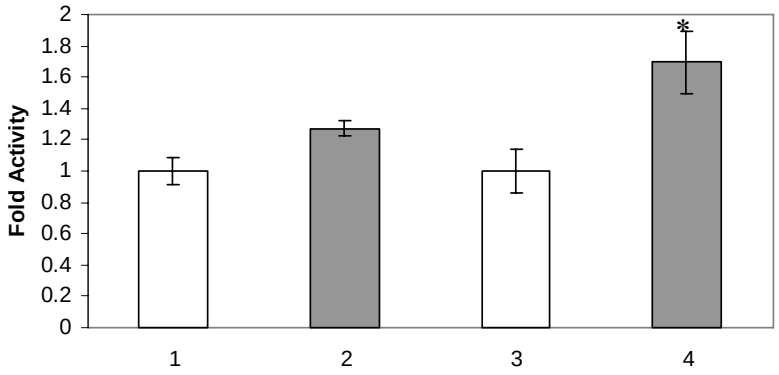


Figure 3.6 *In vitro* DNA-binding of GST-Hoxa2 protein to specific site in the *Dyrk4* intronic sequence. A: EMSA was used to examine the *in vitro* binding activity of *Dyrk4* with the recombinant Hoxa2 protein. Pctrl probe (see Table 3.1) was used as a positive control which is known to bind the homeobox domain *in vitro*. Lane 1, Free Pctrl probe; Lane 2, Pctrl probe + GST protein; Lane 3, Pctrl probe + recombinant Hoxa2 protein; Lane 4, Free Pdyrk482-521; Lane 5, Pdyrk482-521 + GST protein; Lane 6, Pdyrk482-521 + recombinant Hoxa2 protein. Binding of recombinant Hoxa2 protein was observed when using Pdyrk482-521. No binding was observed when using Pdyrk544-583 (data not shown).

B: Super shift (Lane 3, 6) and competition (Lane 5) experiments were carried out to verify the specificity of the binding between Pdyrk482-521 and recombinant Hoxa2 protein. Lane 1, Free Pdyrk482-521; Lane 2, Pdyrk482-521 + recombinant Hoxa2 protein; Lane 3, Pdyrk482-521 + recombinant Hoxa2 protein + Hoxa2 antibody; Lane 4, Pdyrk482-521 + GST; Lane 5, Pdyrk482-521 + 100-fold unlabeled probe + recombinant Hoxa2 protein; Lane 6, Pdyrk482-521+ recombinant Hoxa2 protein + nonimmune serum. The binding can be supershifted by the Hoxa2 antibody and the signal was inhibited by adding high amount of unlabeled probe. GST protein alone cannot bind to the probe.

C: Probes with mutations in the "GATG" site in *Dyrk4* sequence were included in EMSA analysis. Lane 1, Free probe Pdyrk482-521; Lane 2, Pdyrk482-521 + recombinant Hoxa2 protein; Lane 3, Pdyrkma482-521 + recombinant Hoxa2 protein; Lane 4, Pdyrkmb482-521 + recombinant Hoxa2 protein; Lane 5, Pdyrkdoublem + recombinant Hoxa2 protein.



pRL-null	+	+	+	+
pRG-Hoxa2-VP16	-	+	-	+
pGL3-Dyrk	-	-	+	+
pGL3-Dyrkdoublem	+	+	-	-

Figure 3.7 The Hoxa2-VP16 protein induces activation on the responsive element in *Dyrk4* in transient transfection experiments. pGL3-Dyrk and pGL3-Dyrkdoublem were transfected in COS-7 cell with pRG-Hoxa2-VP16 respectively. The relative luciferse activity obtained in the absence of pRG-Hoxa2-VP16 is set at 1. Values are expressed as fold activation over transfection of the reporter plasmid alone. Bars indicate the standard deviation of 4 independent experiments. "*" indicates that the difference between bar 2 and 4 was statistically significant ($P<0.05$) according to a two-tailed t-test.

Table 3.1 EMSA probes for *Dyrk4*

Probe Name	Sequence	Note
Pctrl	CAAATTTTTAATTTTATTTAATTGTAATTAATTTTAAGTGG	Positive control
Pdyrk544-583	CTGTGGCTGCTGTTTCCAGGGACTTTGCCAGCCTGTGTCT	Negative control
Pdyrk482-521	CCTCGTGAACATGGAGTGGCCATGATGCCTGGTTTTGTTC	Dyrk4
Pdyrkma482-521	CCTCGTGAACATGGAGTGGCCATGCCGCCTGGTTTTGTTC	Dyrk4 mutant
Pdyrkmb482-521	CCTCGTGAACATGGAGTGGCCCCGATGCCTGGTTTTGTTC	Dyrk4 mutant
Pdyrkdoublem	CCTCGTGAACATGGAGTGGCCCCGCCGCCTGGTTTTGTTC	Dyrk4 double mutant

Pctrl is designed based on published article (see Booth et al., 2007). Dyrk probes are designed based on isolated *Dyrk* sequence (Figure 3.2).

4. HOXA2 PROTEIN REPRESSES THE EXPRESSION OF *HTRA3*, A NEW DOWNSTREAM TARGET GENE, VIA NOVEL BINDING SITES IN THE DEVELOPING MOUSE CENTRAL NERVOUS SYSTEM

4.1 Abstract

In this study, we have used chromatin immunoprecipitation (ChIP) to isolate Hoxa2 protein binding sequences from the E18 mouse spinal cord and hindbrain tissues. All isolated DNA fragments contain a conserved GATG motif and one fragment belongs to the *high temperature requirement factor A 3 (HtrA3)* gene. Direct binding of Hoxa2 protein to the *HtrA3* fragment was confirmed by electrophoretic mobility shift assays (EMSA). Sequence analysis and EMSA revealed that Hoxa2 protein binds to the multiple GATG motifs within the *HtrA3* fragment. This fragment also repressed luciferase gene expression in transient transfection and luciferase assays. Mutation of the DNA fragment shows that this repressive activity was dependent on the GATG motifs, suggesting Hoxa2 protein regulates gene expression by binding to the GATG motif in the *cis*-regulatory element. In contrast to the inhibitory activity of Hoxa2 protein, a Hoxa2-VP16 fusion protein transactivates the luciferase expression by binding to GATG sites. RT-PCR and immunohistochemistry analysis revealed an upregulation of *HtrA3* expression in *Hoxa2*$^{-/-}$ mice. This observation correlates with the inhibitory role of Hoxa2 protein on the *HtrA3* fragment in luciferase assays. Our data suggest that *HtrA3* is a direct *in vivo* downstream target of Hoxa2 protein and support the activity regulation model as the mechanism by which Hox proteins selectively regulate target genes.

Homeobox genes were first identified in *Drosophila*, where they play a key developmental role in specifying different segmentations (Akin and Nazarali, 2005). These genes possess a conserved 60-aa homeodomain motif. Homeodomain proteins generally function as transcription factors that govern various aspects of morphogenesis and cell differentiation (McGinnis and Krumlauf, 1992). 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. In mice and human, the Hom-C complex is comprised of 39 *Hox* genes that are arranged into four separate chromosomal clusters. *Hox* genes are arranged into 13 paralog groups based on their homology to *Drosophila* HOM-C (Akin and Nazarali, 2005). During early embryonic development in the mouse all *Hox* genes are expressed in central nervous system 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. *Hox* genes play an important role in central nervous system development, especially in determining neuronal organization within the hindbrain (Lumsden and Krumlauf, 1996; Studer et al., 1996; Rijli et al., 1998; Pasqualetti and Rijli, 2001; Pattyn et al., 2003) and the spinal cord (Carpenter, 2002). In order to decipher pathways through which *Hox* genes function in regulating regional specification, especially in the spinal cord and hindbrain, we need to identify the downstream targets of Hox transcription factors.

Various strategies have been utilized to identify targets of Hox proteins in both vertebrates and *Drosophila* (Akin and Nazarali, 2005). The availability of naturally occurring mutants and transgenics in *Drosophila* has allowed for significant delineation of the morphogenic pathways controlled by *Hox* genes. The earliest and most common method for the 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). Another method is subtractive hybridization, based on differential expression patterns to identify potential targets. This method enables isolation of target genes that are up or downregulated in specific mRNAs of a cell system or tissues where a particular *Hox* gene is activated at a specific developmental stage. One limitation shared by the above two methods is that the isolated genes may not be direct targets and instead are parallel factors within the same developmental pathway. In *Drosophila,* Gould et al. (1990) isolated *in viv*o targets of the Ubx homeotic protein by chromatin immunoprecipitation (ChIP). This method has been successfully used in the murine system for the isolation of target genes of Hoxc8 (Tomotsune et al., 1993) and Hoxb5 (Safaei, 1997). It has been increasingly recognized that the dynamic structure of chromatin plays a crucial role in the regulation of development (Hsieh and Fischer, 2005). The advantage of ChIP technique over other approaches is that it allows isolating direct targets or targets controlled by Hox proteins in conjunction with cofactors from accessible regions of native chromatin (Stevens et al., 2004; Akin and Nazarali, 2005).

The *Hoxa2* gene and its paralog *Hoxb2* are homologs of the *Drosophila proboscipedia* gene. A dominant expression of *Hoxa2* gene during embryogenesis occurs in the neural tube and neural crest cells that contribute to the second brachial arch and other tissues (Prince and Lumsden, 1994b; Gavalas et al., 1997; Hao et al., 1999; Barrow et al., 2000; Grammatopoulos et al., 2000). Within the neural tube, the anterior domain of *Hoxa2* gene expression is situated at the r 1/2 boundary (Davenne et al., 1999b; Barrow et al., 2000) and evidence suggests that *Hoxa2* gene may potentially contribute to both anteroposterior (A-P) positioning as well as dorsoventral (D-V) patterning (Davenne et al., 1999b; Hao et al., 1999; Barrow et al., 2000; Ohnemus et al., 2001). Hoxa2 protein is also known to play a role in the patterning of cranial neural crest cells (Trainor and Krumlauf, 2001; Tumpel et al., 2002b; Creuzet et al., 2005).

Both the subtractive hybridization and the mutant screening methods have been used to isolate downstream targets of Hoxa2 protein (Bobola et al., 2003; Kutejova et al., 2005; Santagati et al., 2005), but no targets identified for Hoxa2 protein by ChIP have been reported. In this study, we have used ChIP to isolate Hoxa2 protein targets from chromatin preparations of mice spinal cord and hindbrain and indicated a fragment belonging to the *high temperature requirement factor A 3 (HtrA3)* gene was isolated. Hoxa2 protein binds to the conserved GATG binding sites in this *HtrA3* fragment and acts to repress the expression of luciferase gene in transient transfection and luciferase assays. Mutations of GATG motifs in this *cis*-regulatory element were able to abolish its transcriptional activity. Upregulation of *HtrA3* expression in the spinal cord and hindbrain in *Hoxa2*$^{-/-}$ mice was shown to coincide

with the negative regulatory effect of Hoxa2 protein on the *HtrA3 cis*-regulatory element. Our results establish that *HtrA3* is a direct downstream target gene of the Hoxa2 protein during embryo development and provide additional evidence for the activity regulation model in which Hox proteins exert a transcriptional effect through binding to multiple monomer binding sites.

4.2 Experimental Procedures

Plasmid Construction – The *HtrA3* sequence was amplified by PCR using the forward primer: 5′ ACGCGTCGACTGCAGCCCCCATCGGCAGCCC 3′ and the reverse primer 5′ CGCGGATCCCC CCCTTCCTAGATGGTGGTGTG 3′. This PCR product was then subcloned into the pGL3-promoter plasmid at the *Sal* I and *Bam*H I sites to generate pGL3-HtrA3. Mutagenesis of the putative Hoxa2 protein response elements localized at the two ends of *HtrA3* sequence was performed by PCR amplification with the following primers: 5′ ACGCGTCGACTGCAGCC CCCAGCGGCAGCCC 3′ and 5′ CGCGGATCCC CCCCTTCCTCGCTGGTGGT GTG 3′. Mutated sites are underlined. Mutagenesis of the Hoxa2 protein response elements in the middle of *HtrA3* fragment was conducted as described in the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutations were confirmed by DNA sequence analysis. The *HtrA3* luciferase vector with the mutant Hoxa2 protein response elements was named pGL3-HtrA3mu. The plasmids pRL-null and pRL-CMV were obtained from Promega (Fisher Scientific, Nepean, ON). The expression vector pCS2PBX was a gift from Dr. Mark Featherstone, McGill University (Shanmugam et al., 1999). See section 3 for other plasmid construction.

Protein Purification – See section 3

Antibody Purification –See section 3

Chromatin Preparation and Immunoprecipitation – See section 3.

Preparation of Nuclear Extracts – See Section 3

Electrophoretic Mobility Shift Assay (EMSA) – See Section 3

Cell Culture, Transfection and Luciferase Assays – See Section 3.

RNA Isolation and RT-PCR Analyses – Embryonic and adult mouse tissues were removed and homogenized in TRIZOL Reagent (Sigma, Oakville, ON) for total RNA extraction according to the manufacturer's instructions. RNA was stored in ethanol at –70 ^{o}C, and dissolved in water immediately before use. DNA digestion with 1 U DNase in 1 μg of total RNA sample was incubated at room temperature for 15 min. The digestion was terminated by the addition of stop buffer (2.5 mM EDTA) and heating at 70 ^{o}C for 10 min. Reverse transcription (RT) was performed in a volume of 20 μl using 1 μg of DNase I treated RNA and 200 U of Superscript II *RNase* H^{-} Reverse Transcriptase (Invitrogen, Oakville, ON) and 2.5 μM of random nonamers (Sigma, Oakville, ON). Subsequent cDNA amplification was performed using 4 μl of RT product and 2 U Taq DNA polymerase (Invitrogen, ON). Amplification of a 612 bp fragment of the mouse *β-actin* transcript was performed by RT-PCR with 0.2 μM of primers 5′ GGCATCGGATGGACTCCG 3′ and 5′ GCTGGAAGGTGGACAGCGA 3′ (Remacle et al., 2002). A 429 bp fragment of *HtrA3* was amplified using 0.5 μM of primers 5′ CCAACCCAGACTTTCCAGCG 3′ and 5′ AGCTGAAATTAAGGGT CA 3′, corresponding to positions 1361-1770 within the mouse *HtrA3* the 9530081K03RIKEN sequence (NCBI Accession#

AK035194). *HtrA3* fragment amplification from RT reactions was performed using touchdown PCR as follows: 10 cycles of 95 ^{o}C for 45 sec, 68 ^{o}C for 1 min, and 76 ^{o}C for 1 min, followed by 20 cycles of 94 ^{o}C for 45 sec, 62 ^{o}C for 1 min, and 76 ^{o}C for 1 min, and a final extension step at 78 ^{o}C for 10 min. PCR amplification of the *HtrA3* fragment from the RT reactions was quantitated through imaging with a BioRad gel doc system followed by analysis using the pixel density function of the Quantity 1 program. This fragment was cloned and verified by sequencing.

4.3 Results

Hoxa2 Protein Binds to the HtrA3 Fragment — Hoxa2-DNA complexes were isolated by chromatin immunoprecipitation from the hindbrain and spinal cord of E18 embryos. Seven putative targets were isolated (Akin Z, Ph.D Thesis, University of Saskatchewan, 2004) and sequence analyses indicated that one of these sequences belongs to *HtrA3* (Figure 4.1).

EMSA was performed to evaluate the direct binding of Hoxa2 protein to the isolated *HtrA3* sequence. Incubation of the radiolabeled probe with the E12 nuclear extracts produced a band-shift (Figure 4.2A, lane2). Co-incubation of probe with nuclear extracts and anti-Hoxa2 antibody resulted in a specific supershift band (Figure 4.2A, lane 3). To further investigate the specificity of the Hoxa2 protein binding, competition experiments were performed. The specific band can be competed using 100-fold unlabeled probe (Figure 4.2A, lane5). Incubation of probe with E18 nuclear extracts showed comparable results (Figure 4.2B). An interesting observation was the absence of the shift band after the addition of anti-Hoxa2 antibody, which might be due to antibody blocking the interaction between Hoxa2

protein and the probe (Figure 4.2B, lane 5, 6). These results suggested that Hoxa2 protein can bind to the isolated *HtrA3* sequence.

Hoxa2 Protein Binds to Specific Sites in the HtrA3 Fragment — GATG motif was highly conserved in the *HtrA3* fragment (Figure 4.3A, C) and all other isolated sequences (Akin Z, Ph.D Thesis, University of Saskatchewan, 2004). Since this is not the typical Hox or Hox/Pbx binding motif, it was interesting to test its Hoxa2 protein binding specificity. In order to include all GATG motifs on the *HtrA3* fragment (Figure 4.3A), 4 pairs of wild-type and mutant probes with altered GATG motif (Table 4.1) were synthesized. The DNA-binding activity of GST-Hoxa2 protein was tested by incubation with TAAT concatemer probe, which was known to bind Hox proteins *in vitro* (Kumar and Nazarali, 2001; Booth et al, 2007). Incubations of wild-type probes with GST-Hoxa2 protein resulted in a band-shift (Figure 4.4A and B, lane 2 and 7), which can be further shifted by Hoxa2 antibody (Figure 4.4A and B, lane 3 and 8) and competed by adding 100-fold unlabeled probe (Figure 4.4A and B, lane 4 and 9). Mutations in GATG motif of these probes eliminated the binding completely (Figure 4.4A and B, lane 5 and 10). These data indicated the specific binding of Hoxa2 protein to *HtrA3* through GATG motifs.

Hoxa2 Protein Affects Expression through GATG Motifs in the Cis-regulatory Element in Luciferase Assays — To further evaluate the regulatory capability of Hoxa2 protein through the *HtrA3* fragment, *Hoxa2* gene and *Hoxa2-VP16 fusion* were expressed in COS-7 cells and employed in Dual-Luciferase Reporter Assay System (Promega, ON). The *HtrA3* fragment was cloned into the pGL3 promoter vector, in which firefly luciferase expression is controlled by a SV40 minimal

promoter. To avoid different transfection efficiencies caused by experimental variations, an internal control reporter containing renilla luciferase gene (pRL-CMV) was included in the co-transfection experiments. As shown in Figure 4.5A, Hoxa2 protein inhibited the luciferase expression in presence of the *HtrA3* fragment.

To test if the inhibition was directly mediated by Hoxa2 protein binding to GATG motifs, we silenced all GATG motifs in the *HtrA3* fragment and generated mutant vector pGL3-HtrA3mu. Transfection of *Hoxa2* gene expression vector with pGL3-HtrA3mu significantly alleviated the inhibitory ability of Hoxa2 protein compared to the wild-type vector pGL3-HtrA3 (Figure 4.5A). On the other hand, in contrast to the inhibitory activity of Hoxa2 protein, the Hoxa2-VP16 fusion protein enhanced luciferase expression (Figure 4.5B). This enhanced luciferase expression was significantly decreased when the Hoxa2-VP16 expression vector was co-transfected with pGL3-HtrA3mu (Figure 4.5B). These results suggested that the presence of GATG motifs were critical for transcriptional activates of Hoxa2 protein.

A plasmid pCS2PBX, expressing Pbx cofactor, was also added in co-transfection experiments to investigate if this common cofactor would contribute to the regulatory activity of Hoxa2 protein. No significant effects were observed (data not shown). The reason may be a lack of the consensus Pbx/Hoxa2 binding site "(T/A) GAT (T/G) GA (T/A) G" in the *HtrA3* fragment (Lampe et al., 2004). It implies that the regulatory function of Hoxa2 protein might be independent of the cofactor in some case.

Matis et al (2001) showed that the Hoxa2-VP16 fusion protein inhibited transcription initiated by the strong promoter/enhancer of cytomegalovirus (CMV)

and Rous sarcoma virus (RSV). We also observed this phenomenon when the internal control plasmid pRL-CMV was used (data not shown). It leaded to luciferase activity fluctuation of the internal control. To solve this problem, we replaced pRL-CMV with a promoterless vector, pRL-null when the Hoxa2-VP16 fusion protein was applied in luciferase assays (Figure 4.5B). As expected, the fluctuations of the renilla luciferase expression were no longer present due to the removal of the CMV promoter.

Hoxa2 Protein Represses the Expression of HtrA3 in Developing Mice — HtrA3 expression in wild-type and *Hoxa2* $^{-/-}$ mice were assessed using RT-PCR. A 429 bp fragment spanning the 3′ end of exon 8, exon 9, and including a portion of the 3′ UTR was amplified (Figure 4.6A). Semiquantitation using density measurements of RT-PCR bands normalized to *β-actin* mRNA indicated that levels of *HtrA3* transcripts were significantly higher in mutant mice compared to wild-type mice (Figure 4.6B). This increase in the expression of *HtrA3* in the absence of Hoxa2 protein further suggested the negative regulatory effects of Hoxa2 protein on the *HtrA3*.

4.4 Discussion

Chromatin as a complex of DNA and protein plays a critical role during the course of transcription in eukaryotic cells. The dynamic structure of chromatin contributes to various aspects of transcription such as DNA-protein interaction, protein-protein interaction and molecular translocation. Using ChIP as a selection method, we have isolated Hoxa2-bound DNA from accessible regions of native chromatin from mice hindbrain and spinal cord. This allowed the identification of a

Hoxa2 protein target gene and further elucidated a likely mechanism by which Hoxa2 protein regulates gene expression.

A major objective of this study was to identify a direct *in vivo* downstream target gene of Hoxa2 protein. Our investigations reveal a strong preference for binding of Hoxa2 protein to elements that contain GATG motif. This motif is highly repeated all isolated clones (Akin Z, Ph.D Thesis, University of Saskatchewann, 2005), one of which belongs to *HtrA3*. We used a combination of *in vivo* and *in vitro* methods to effectively demonstrate the functional relevance of the new Hoxa2 protein binding sites. EMSA using both nuclear extracts and recombinant protein demonstrate the direct binding of Hoxa2 protein to this *cis*-regulatory element through GATG motifs. Transient transfections and luciferase assays correlated the Hoxa2 protein regulatory activity to the integrity of the GATG sites. RT-PCR shows the up-regulation of *HtrA3* expression in *Hoxa2*$^{-/-}$ mice, further supporting the regulatory role of Hoxa2 protein.

Although it is well recognized that Hox transcriptional factors govern body patterning along the anteroposterior body axis during animal development, the mechanism by which Hox proteins exert such function *in vivo* is not clear. One reason is the inconsistency between the specific developmental role and the low DNA-binding specificity of Hox proteins. Two models so far have been proposed to explain how Hox proteins select the appropriate target genes: selective binding model and activity regulation model (Nasiadka et al., 2000).

The selective binding model suggests that cofactors work together with Hox proteins and direct them to different binding sequences by raising their DNA-

binding specificity. Consistent with this model is the extradenticle (Exd/Pbx) family of the homoeodomain proteins that have been identified as important cofactors that have a crucial impact on the diversity of Hox function (Peifer and Wieschaus, 1990; Rauskolb et al., 1993; Van Dijk et al., 1993; van Dijk and Murre, 1994). The evidence in support of this model includes Exd/Pbx proteins that interact with Hox proteins and increase their DNA-binding affinity on a specific DNA site (Chan et al., 1994; Chang et al., 1995; Popperl et al., 1995; Neuteboom and Murre, 1997). A bipartite 10 bp Hox-PBC consensus binding site 5′ TGATNNAT [G/T] [G/A] 3′, has been defined, in which the first half site is for PBC binding and the second half site is for Hox binding (Popperl et al., 1995; Chan et al., 1997). A Pbx/Hoxa2 composite site [(T/A) GAT (T/G) GA (T/A) G] was identified in the *Hoxa2* gene and the Pbx was found to significantly contribute to the autoregulation of *Hoxa2* gene through this site in COS-7 cells (Lampe et al., 2004).

The activity regulation model argues that, instead of influencing DNA-binding affinity, cofactors (e.g. Pbx) affect the transcriptional activity of Hox protein through the sequence which they are already bound (Biggin and McGinnis, 1997). The low DNA-binding specificity indicates that Hox protein can bind to many sites across the genome; however, they will remain in a neutral state and not exert an independent influence until a cofactor appears. In favor of this model, the homeodomain in Hox protein Deformed (Dfd) was found to have an inhibitory affect on the Dfd activation function (Li et al., 1999). The interaction between Exd/Pbx and Dfd homeodomain releases the Dfd activation capacity. In addition, Dfd-VP16 fusion protein can

regulate many same downstream target genes of Dfd in *Drosophila* in the absence of Exd/Pbx (Li et al., 1999).

In this study we provide further evidence for the activity regulation model. Our results show that Hoxa2 protein binds to a 4 bp core sequence GATG in the *HtrA3* and is able to repress reporter expression through these motifs in COS-7 cells. But a Hox/Pbx binding site was not found in the isolated *HtrA3* fragment. Due to the small size of this core binding motif, the probability of its occurrence in the genome is high. However, only a small proportion of these motifs are likely to be bound by Hoxa2 protein *in vivo*, with even fewer functioning directly in gene regulation. In transient tranfection experiments, Hoxa2 protein can exert an inhibitory activity in the absence of the Pbx, in addition the presence of Pbx did not significantly contribute to or inhibit this activity of Hoxa2 protein. Taken together with the fact that Hoxa2-VP16 protein can reverse the role of Hoxa2 protein from a transcriptional inhibitor to a transactivator, we speculate the involvement of other unknown cofactors that are likely to contribute to the specific target selection and regulatory function of Hoxa2 protein.

One thing worth noticing is that the GATGATG motif in the *HtrA3* fragment is very similar to the published Pbx/Hoxa2 consensus binding site [(T/A) GAT (T/G) GA (T/A) G] (Lampe et al., 2004). It has two overlapping GATG motif and also carries the half Hoxa2 protein binding site GATGA in the Pbx/Hoxa2 consensus binding site. A similar motif also exists in the *Dyrk4* gene and shown to bind to Hoxa2 protein (Chapter 3, Figure 3.6). Given the presence of multiple binding sites in *HtrA3* fragment, it raises the question that if Hoxa2 protein can exert its function

through only one site. Interestingly, preliminary evidence shows Hoxa2 protein binds to this motif but cannot initiate a transcription regulatory effect in COS-7 cells (Chapter 3, Figure 3.7). Therefore, in this study Hoxa2 protein might function as a transcription repressor in COS-7 cells through occupation of multiple monomer binding sites in *HtrA3* fragment. A similar mechanism for Hox protein has been observed previously (Galant et al., 2002; Stevens et al., 2004; McCabe and Innis, 2005), where Hox proteins can directly regulate target genes in the absence of the cofactor Exd/Pbx, presumably through an additive effect of Hox proteins binding to multiple monomer binding sites (Galant et al., 2002).

The Hox-VP16 fusion protein has been successfully used to isolate Hox protein targets (Friedman-Einat et al., 1996), and to investigate transcription activity of Hox protein on previously identified targets (Li et al., 1999; Nasiadka et al., 2000). One advantage is that the Hox-VP16 fusion protein can circumvent the needs of indispensable and unknown cofactors (Li and McGinnis, 1999; Li et al., 1999). The other advantage is that the target of the Hox-VP16 protein is limited by the binding specificity of Hox protein, therefore it mostly only affects known targets (in certain temporal and spatial circumstances) that Hox alone cannot affect (Li and McGinnis, 1999; Li et al., 1999; Nasiadka et al., 2000). The fact that the Hoxa2-VP16 protein overturned the inhibition of Hoxa2 protein on reporter gene to a transactivation, along with the mutation analysis demonstrates the interaction between Hoxa2 protein and GATG motifs in the *cis*-regulatory element. It is believed that the VP16 activation domain fulfills its ability to activate transcription via multiple mechanisms and at multiple levels (Nasiadka et al., 2000). One mechanism is that

the VP16 activation domain interacts directly with multiple factors in the transcription machinery (Matis et al., 2001). Therefore, with high concentration of VP16 protein transcription factors and many other components can be sequestrated and depleted with overexpression of the VP16 activation domain, a phenomenon known as "squelching". The Hoxa2-VP16 protein was reported to specifically inhibit the CMV and RSV promoter due to the squelching effect (Matis et al., 2001). We observed this phenomenon in our experiments when using pRL-CMV as the internal control plasmid and the variation in the levels of inhibition prevented our initial intention to utilize renilla luciferase to normalize transfection efficiency. Fortunately, this problem was solved by using the promoterless renilla plasmid, pRL-null, which apparently precluded the influence of VP16 domain on the CMV promoter.

The DNA fragment characterized in this study acts as a repressor of *HtrA3* gene expression. Since the *cis*-regulatory elements can extend several hundred kb from the transcription unit (Kleinjan and van Heyningen, 2005), it may also contribute to the regulation of neighboring genes. Interestingly, the specific genes flanking *HtrA3* are conserved in various species (chicken, rat and human). Also BLAST analysis shows that the target sequence from *HtrA3* is partially conserved within chicken, rat and human.

HtrA3 was discovered initially as a pregnancy-related serine protease that is up-regulated dramatically during mouse placental development (Nie et al., 2003b); *HtrA3* is selectively expressed at the maternal-fetal interface during placentation in the mouse (Nie et al., 2006b). Research findings show that *HtrA1* and *HtrA3* are

expressed mostly in the same embryonic organs but exhibit complementary expression patterns in various tissues (Tocharus et al., 2004). As *HtrA1* and *HtrA3* share a high degree of domain homologies, they also share a functional similarity (Nie et al., 2003a). Both HtrA1 and HtrA3 are inhibitors of transforming growth factor-β (TGF-β) signaling and bind to its various superfamily members (Oka et al., 2004; Tocharus et al., 2004). HtrA1 and HtrA3 also exhibit similar substrate specificity toward β-casein and certain extracellular matrix proteoglycans (Tocharus et al., 2004). Both *HtrA1* and *HtrA3* act as tumor suppressors. *HTRA1* and *HTRA3* mRNA and protein levels decrease with increasing grades of human endometrial cancer (Bowden et al., 2006). They are both upregulated in the arthritic cartilage (Tocharus et al., 2004). Interestingly, Hoxa2 protein regulatory binding element found in this study is partially conserved in both genes. However, whether the regulatory role of Hoxa2 protein on *HtrA3* is conserved in *HtrA1* remains to be shown.

The direct regulation of *HtrA3* expression by Hoxa2 is intriguing; although, the tissue-specific role this regulation plays *in vivo* is unclear and needs further investigation. There are, however, a few clues that show both factors may be involved in the same processes during development. For instance, during craniofacial development, neural crest cells migrate into the branchial arches to form the skeletogenic elements (Trainor and Krumlauf, 2001). Hoxa2 plays an important role in regulating morphogenesis of the head skeletal derivatives. Evidence shows that Hoxa2 promotes the formation of cartilage and prevents ossification during craniofacial development (Kanzler et al., 1998; Grammatopoulos et al., 2000; Plant

et al., 2000; Trainor and Krumlauf, 2001; Creuzet et al., 2002). These capacities of Hoxa2 match with the function of *HtrA1*. *HtrA1* is up-regulated in arthritis and is suggested to play a role in promoting degeneration of cartilage and contributing to ossification through its TGF-β inhibitory activity and protease activity (Tsuchiya et al., 2005). Given the striking structural and functional similarities between HtrA1 and HtrA3, Hoxa2 protein may contribute to cartilage formation and inhibit bone formation by down-regulating *HtrA3* or/and *HtrA1* gene. It would be interesting to investigate this as a possible mechanism occurring during the craniofacial development.

TGF signaling pathway is involved in determining the dorsoventral patterning in *Xenopus*, and the left-right symmetry within the murine system (Heasman, 1997; Whitman and Mercola, 2001). Several members of this superfamily have been implicated in determining neuronal phenotypes, neuronal proliferation and differentiation, as well as regulation of oligodendroglial differentiation and cell adhesion (Bottner et al., 2000). *Hoxa2* gene also has been implicated in specification of neuronal phenotypes (Hao et al., 1999; Ohnemus et al., 2001) and oligodendroglial differentiation (Nicolay et al., 2004). It is possible that Hoxa2 protein regulates the TGF signaling pathway via repression of *HtrA3* in specific cell types at later stages of development.

Using ChIP to identify Hoxa2-bound DNA and target genes has given insight into the nature of target sequences and mechanisms that are involved in regulation of gene expression by *Hoxa2* in the context of native chromatin. Using *HtrA3* as a direct target of Hoxa2 protein will allow us to thoroughly explore the domains of the

Hoxa2 protein that are necessary for its transcriptional effects, determine whether the addition of known cofactors will influence the ability of Hoxa2 protein to regulate expression via the *cis*-regulatory elements and explore the chromatin structure at a known direct target containing multiple requisite Hoxa2 protein binding sites. Moreover, the results of this study enhance our understanding of the regulatory pathway that may be controlled by Hoxa2 protein during development. Further studies will be needed to clearly define the regulatory role of Hoxa2 protein during embryo development.

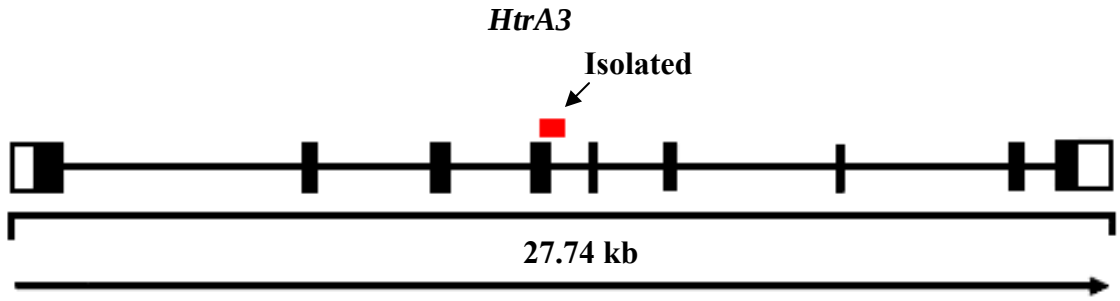
4.5 Figures and Table

Table 4.1 EMSA probes for *HtrA3*

Probe name	Sequence	Note
Phtra286-314	5′ GCGAGTTCGTGGTGGCCATCGGCAGCCCC 3′	HtrA3 wild-type
Phtram286-314	5′ GCGAGTTCGTGGTGGCCGTCGGCAGCCCC 3′	HtrA3 mutant
Phtra352-382	5′ GCACTGCCCAGCGGGATGGCAAGGAGCTGG 3′	HtrA3 wild-type
Phtram352-382	5′ GCACTGCCCAGCGGGCCGGCAAGGAGCTGG 3′	HtrA3 mutant
Phtra406-436	5′ TATCCAGACCGATGCCATCATCAATGTGAG 3′	HtrA3 wild-type
Phtram406-436	5′ TATCCAGACCGACGCCGTCAGCAATGTGAG 3′	HtrA3 mutant
Phtra488-508	5′ TATCATACAACACACCACCATCTAGGAAGG 3′	HtrA3 wild-type
Phtram488-508	5′ TATCATACAACACACCACCACCTGGGAAGG 3′	HtrA3 mutant

The altered sites in mutant probes and their corresponding sites in wild-type probes are underlined. The probes are designed based on the isolated *HtrA3* sequence (Figure 4.1)

A



B

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ACTAGACTCAAGGATATCAGAGGACAGACCCTGCCTGGATGCTCTGTACCTCCCCATGAC  60
AGACATTTCCGGCCTCCTCAGATGAGCCTTGGTTCTGTGGACTCCAGCCTTAGTCTCAGC 120
CAGCTGCTGGCCTGGCCTCGGGGCTGGGAATAGCCTCCTTCCTCTCCAGAAAGCAGCCTG 180
CCAGGATCCCTGTCTGGTCTGAGCCTCAGTCCTCTCTCACACCCTTCTCCTTCCAGAAAA 240
AGCTCCCTGTGTTGCTGCTGGGTCACTCAGCAGACCTGCGGCCTGGCGAGTTCGTGGTGG 300
CCATCGGCAGCCCCTTTGCCCTGCAGAACACCGTGACAACGGGCATTGTCAGCACTGCCC 360
AGCGGGATGGCAAGGAGCTGGGTCTCCGGGACTCAGACATGGACTATATCCAGACCGATG 420
CCATCATCAATGTGAGTGCTGTGGGGAAGGCTGACCTCGGCAACTTCGGACCAGCTTGTG 480
CCCTGTCCTATCATACAACACACCACCATCTAGGAAGGCCTTTGTTATCTCATGCAGAGC 540
ATCCACCCACCCAGTGACTGTCTCCAGGGATGGGCCATCTTGGACAGTTGCAGAGATGAA 600
GTGGCCCAGGCATACCCTTGGAAGGTCATAGCCTAACCTGCATAAGTTGTCACTTGACTA 660
AGATTAAGGTTATATTGGATGGCACCATGACATTCTCACATCTCCAAGCCTGGGTGCCTT 720
GGCATGTACCCCTATGCCTGCCATGACTGGTAGCCTGGTAGCTGGAGCCATTCATAGCTG 780
TAGAGATGACACTGCCTGTCTTGCAACTGCCTAGCAAC                        840
```

Figure 4.1. Schematic representation of the murine *HtrA3* gene. (A) 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 27.74 kb *HtrA3* genomic sequence present on mouse chromosome 5, direction of transcription is indicated by the black arrow. The isolated sequence displayed 99% identity with the 4th exonic and 5th intronic sequence (indicated by red box). (B) A 218 bp fragment was isolated by immunoprecipitation of Hoxa2 protein from chromatin preparations. The sequence of immunoprecipitated fragment which is present in the mouse *HtrA3* homolog is indicated in grey. (Akin Z, Ph.D Thesis, University of Saskatchewan, 2004)

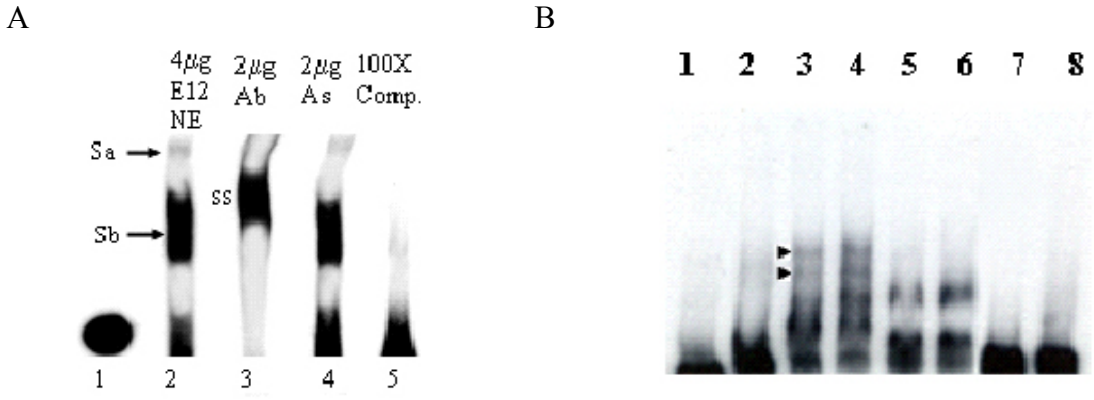


Figure 4.2. Electrophoretic mobility shift assay (EMSA) for the isolated *HtrA3* sequence (218 bp). (A) EMSA analysis using E12 hindbrain and spinal cord nuclear extracts. Binding reactions for target sequences were performed with 4 µg of E12 nuclear extracts (lane 2). Migration of unbound target sequences can be observed in lane 1. The addition of anti-Hoxa2 polyclonal antiserum resulted in the formation of a supershift band (lane 3). Incubation of binding reactions with non-immune rabbit serum did not affect shift formation (lane 4). Competition reactions using 100 X unlabelled probe resulted in inhibition of shift band formation (lane 5). (B) EMSA analysis using E18 hindbrain and spinal cord nuclear extracts. Binding reactions for the *HtrA3* fragment were performed with 0, 2 and 5 µg of nuclear extracts (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). (Akin Z, Ph.D Thesis, University of Saskatchewan, 2004)

A

> *HtrA3*

5’CGTGGTGGCCATCGGCAGCCCCTTTGCCCTGCA CCAGCG

GAACACCGTGACAACGGGTATTGTCAGCACTGCC

CAGCGGGATGGCAAGGAGCTGGGTCTCCGGGACT GGCCGG

CAGACATGGACTATATCCAGACCGATGCCATCAT CGACGCCGTCAGCA

CAATGTGAGTGCTGTGGGGAAGGCTGACCTCAGC

AACTTTGGACCAGCTTGTGCCCTGTCCTATCATAC

AACACACCACCATCTAGGAAGG 3’ CCAGCGAG

B

Consensus Pbx/Hoxa2 site:

T	G	A	T	T	G	A	T	G	G
A				G			A	C	T
									A

C

CGATGGCCACC

GGATGGCAAGG

TGATGATGGCATCG

CCTAGATGGT

Figure 4.3. Sequence analysis of the *HtrA3* fragment. All sites are shown in a 5′ to 3′ orientation. (A) Isolated *HtrA3* sequence. The putative Hoxa2 protein binding sites carrying the core GATG motif are underlined. The altered base pairs in the GATG motif in luciferase assays are highlighted in red (right). (B) Consensus Pbx/Hoxa2 site (Lampe et al., 2004) (C) Alignment of all the region with GATG sites. The highlighted sites are consistent with the half Hoxa2 protein binding site in B.

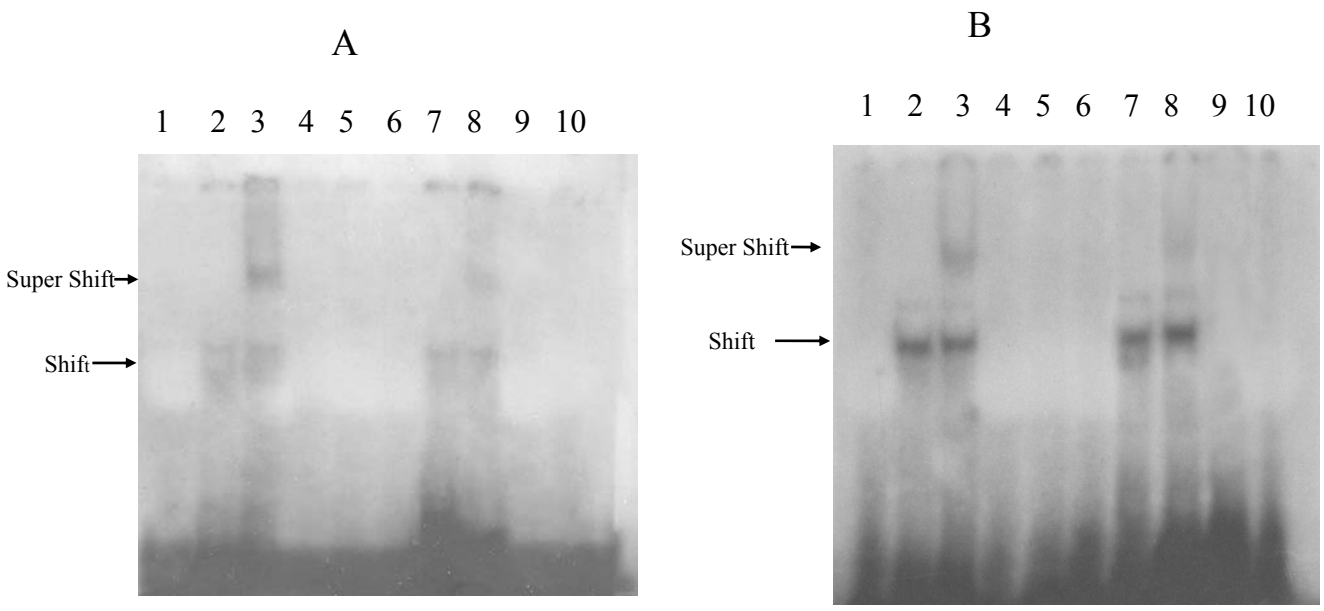
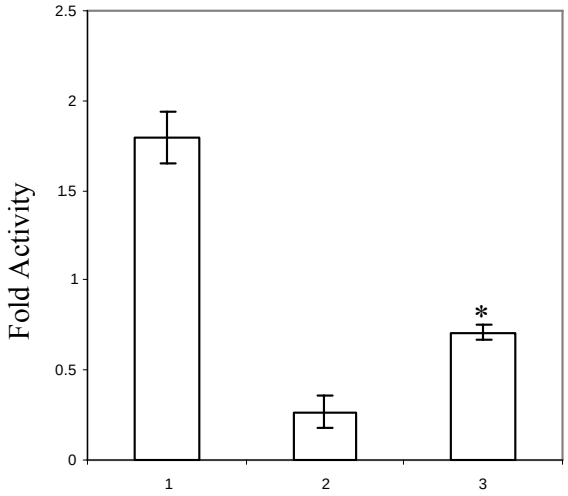


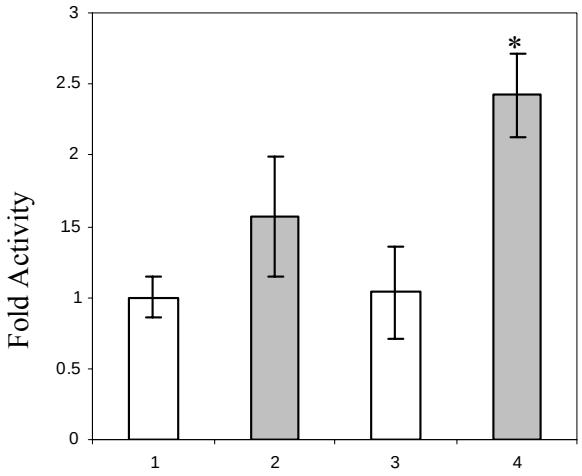
Figure 4.4. EMSA analysis of all the potential binding sites in *HtrA3* sequence. Sequences of the probes are shown in Table 1. (A) Lane 1, Free probe Phtra286-314; Lane 2, Phtra286-314 + recombinant Hoxa2 protein; Lane 3, Phtra286-314 + recombinant Hoxa2 protein + Hoxa2 antibody; Lane 4, Phtra286-314 + 100-fold unlabeled probe + recombinant Hoxa2 protein; Lane 5, Phtram286-314 + recombinant Hoxa2 protein; Lane 6, Free probe Phtra352-382; Lane 7, Phtra352-382 + recombinant Hoxa2 protein; Lane 8, Phtra352-382 + recombinant Hoxa2 protein + Hoxa2 antibody; Lane 9, Phtra352-382 + 100-fold unlabeled probe + recombinant Hoxa2 protein; Lane 10, Phtram352-382 + recombinant Hoxa2 protein. (B) Lane 1, Free probe Phtra406-436; Lane 2, Phtra406-436 + recombinant Hoxa2 protein; Lane 3, Phtra406-436 + recombinant Hoxa2 protein + Hoxa2 antibody; Lane 4, Phtra406-436 + 100-fold unlabeled probe + recombinant Hoxa2 protein; Lane 5, Phtram406-436 + recombinant Hoxa2 protein; Lane 6, Free probe Phtra488-508; Lane 7, Phtra488-508 + recombinant Hoxa2 protein; Lane 8, Phtra488-508 + recombinant Hoxa2 protein + Hoxa2 antibody; Lane 9, Phtra488-508 + 100-fold unlabeled probe + recombinant Hoxa2 protein; Lane 10, Phtram488-508 + recombinant Hoxa2 protein.

A



pRL-CMV	+	+	+
pRSV-Hoxa2	+	+	+
pGL3-Promoter	+	-	-
pGL3-HtrA3	-	+	-
pGL3-HtrA3mu	-	-	+

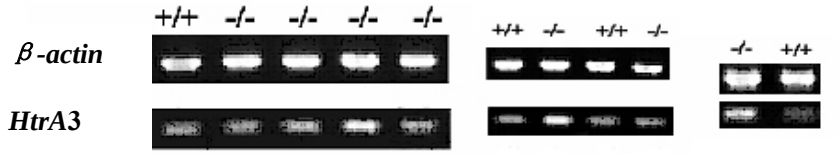
B



pRL-null	+	+	+	+
pRG-Hoxa2-VP16	-	+	-	+
pGL3-HtrA3	-	-	+	+
pGL3-HtrA3mu	+	+	-	-

Figure 4.5. Relative regulatory activity of Hoxa2 and the Hoxa2-VP16 proteins via Hoxa2 protein binding sites in *HtrA3*. (A) Relative luciferase activity assayed from COS-7 cells transfected with the *Hoxa2* gene expression vector and the reporter constructs as indicated. (B) Relative luciferase activity assayed from COS-7 cells transfected with the Hoxa2-VP16 expression vector and the reporter constructs as indicated. For each combination of reporter vector plasmids, the relative luciferase activity obtained in the absence of Hox expression vector is set at 1. Values are expressed as fold activation over transfection of the reporter plasmid alone. Bars indicate the standard deviation of at least four independent experiments. "∗" indicates that the difference between Hoxa2/Hoxa2-VP16 activity on wild-type and mutant *HtrA3* fragment was significant according to *t* test with $p < 0.05$.

A



B

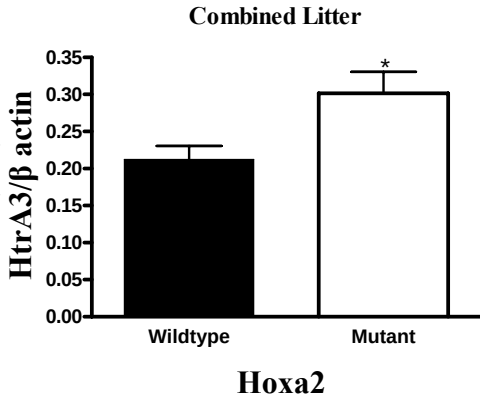


Figure 4.6. Hoxa2 protein inhibits *HtrA3* transcription. (A) semiquantitative RT-PCR analysis of *HtrA3* and control *β actin* mRNA from both *Hoxa2*$^{-/-}$ and wild-type hindbrain and spinal cord tissue. Three litters were analyzed by RT-PCR and combined for data analysis. (B) Quantification of A. Density measurements of *HtrA3* RT-PCR were normalized to *β actin* amplified with 0.2 μM *β actin* primer, corresponding to samples observed in A. A one-tailed t-test shows that expression of *HtrA3* in *Hoxa2* $^{-/-}$ mice is significantly higher (*$p < 0.05$) than in *Hoxa2* wild-type mice. Error bars represent SD, with an $n = 4$ for wild-type mice and n=7 for *Hoxa2*$^{-/-}$ mice. (Akin Z, Ph.D Thesis, University of Saskatchewan, 2005)

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5. DISCUSSION AND FUTURE DIRECTIONS

The homeobox genes were first identified in the fruit fly (*Drosophila melanogaster*) and have subsequently been identified in many other species, from insects to reptiles and mammals. Mutations to homeobox genes could easily result in visible phenotypic changes and it has been well recognized that Hox transcriptional factors control body segmentation along the anteroposterior body axis during development. However, it always has been difficult to demonstrate precisely how Hox proteins exert such function *in vivo*. One uncertainty is the discrepancy between the high developmental specificity and relatively loose DNA-binding specificity of Hox proteins. Two models so far have been proposed to explain how Hox proteins select the appropriate target genes; namely selective binding model and activity regulation model (Nasiadka et al., 2000). As mentioned ealier, in the selective binding model cofactors direct Hox proteins to their binding sites by increasing their DNA-binding specificity; while in the activity regulation model cofactors release the activation domain function of Hox proteins. These two different models are not mutually exclusive. Our results favors activity regulation model, however, no definite conclusion could be drawn regarding to model selection.

Our experimental results indicate that *HtrA3* is a direct downstream target of Hoxa2 protein. Since high structural and functional similarities are shared by *HtrA1* and *-3*, Hoxa2 protein might also play a role during the transcription of *HtrA1*. Both HtrA1 and

HtrA3 are found to inhibit TGF-β signaling and at least bind to TGF-β1, BMP-2 and BMP-4 (Oka et al., 2004; Tocharus et al., 2004). For example, *HtrA1* is found to be up-regulated in arthritis and is suggested to play a role in promoting degeneration of cartilage and contributing to ossification through its TGF-β inhibitory activity and protease activity (Tsuchiya et al., 2005). The TGF-β family members induce cellular responses mainly through Smad protein pathway, although they can also trigger MAPK signaling pathway (Nakamura et al., 1999; Hassel et al., 2003; Nohe et al., 2004). These findings indicate that there might be a connection between Hoxa2 protein and the TGF-β pathway, and that Hoxa2 protein might indirectly facilitate TGF-β signaling pathway by inhibiting *HtrA*. In fact, available literature indicates a linkage between Hox family members and TGF-β pathway. For instance, Hoxc8 (a member of the Hox family) was shown to repress BMP signaling pathway through interaction with Smad1 protein (Shi et al., 1999; Yang et al., 2000; Liu et al., 2004). Similar interaction exists between Hoxa9, Hoxa13, Hoxd13 and Smad proteins (Bai et al., 2000; Shi et al., 2001; Williams et al., 2005). All of these reports suggest that the connection between Hox and TGF-β pathway maybe universal and through multiple mechanisms.

Hox family genes govern the body patterning along the anteroposterior body axis during animal development. The mutation of some *Hox* genes results in homeotic transformation of skeletal tissues. For instance, *Hoxa2*$^{-/-}$ mice were found to contain multiple cranial facial skeletal defects, including a cleft secondary palate and duplicated ossification centers of the middle ear bones (Gendron-Maguire et al., 1993; Rijli et al., 1993). Skeletal elements defects can also be observed in *Hoxc8*, *Hoxa11*, *Hoxa13* and *Hoxd13* null mutant mice (Small and Potter, 1993; Akarsu et al., 1996; Mortlock et al.,

1996; Yueh et al., 1998). Extensive studies have also shown that BMPs (BMP2, -3, -4, -5, -6, -7) are important regulators during chondrogenesis and skeletogenesis throughout embryonic development (Nie et al., 2006c). A great deal of literature indicates that Hoxa2 protein and BMPs might be involved in the similar process (es).

The palate development is a complex process. Formation of mammalian secondary palate can be divided into multiple steps including mesenchymal cell proliferation, palatal shelf outgrowth, elevation and fusion. Interruption of any above process could results in a cleft secondary palate (Nie et al., 2006c). The most common mechanism of cleft is the insufficiency of mesenchymal proliferation or failure of epithelial fusion (Nie et al., 2006c). Research in our laboratory has demonstrated that Hoxa2 protein plays a direct role in palatogenesis (Xia, W., M.Sc. Thesis, 2005). Hoxa2 protein might interact with this BMPs pathway through the downregulation of *HtrA1/3* genes and mediate the palate shelves fusion, although further experiments are needed to verify this hypothesis. Considering the complexity of the vertebrate organism system, Hoxa2 protein might regulate palatogenesis through multiple pathways but it is also possible that only one of the many pathways contributes to palatogenesis. Similarly, the regulation of Hoxa2 protein on *HtrA1/-3* may also contribute to the development of other craniofacial elements such as ear, tooth, etc. There is evidence indicating the involvement of Hoxa2 protein and BMPs during such processes (Li and Cao, 2006; Nie et al., 2006c).

6. CONCLUSION

Hox genes are evolutionarily conserved genes encode transcription factors that control body segmentations along the anteroposterior axis during animal development. *Hoxa2* gene is expressed in the developing central nervous system in rhombomeres 2-7 and affects cellular differentiation. *Hoxa2* gene is involved in the patterning of the cranial neural crest cells (Trainor and Krumlauf, 2001; Tumpel et al., 2002b; Creuzet et al., 2005) and contributes to both anterior-posterior positioning as well as dorsal-ventral patterning (Hao et al., 1999). Disruption of *Hoxa2* gene causes 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., 1998; Davenne et al., 1999a; Barrow et al., 2000). Additionally, defects of the branchial arch derivatives, such as cleft palate and duplicated ossification centers of the middle ear bones (Gendron-Maguire et al., 1993; Barrow and Capecchi, 1999; Nazarali et al., 2000), are observed in *Hoxa2* mutant mice. This evidence suggests that *Hoxa2* gene is involved in the cell differentiation. *Hoxa2* gene is further shown to promote the formation of cartilage and prevent ossification during craniofacial development (Kanzler et al., 1998; Grammatopoulos et al., 2000; Plant et al., 2000; Trainor and Krumlauf, 2001; Creuzet et al., 2002). Although the importance of *Hoxa2* gene during development is well recognized, few target geness of Hoxa2 protein have been identified so far and mechanisms of how Hoxa2 transcription factor regulates developmental processes are still unclear.

In our group chromatin immunoprecipitation (ChIP) was utilized by a former graduate student (Akin Z, Ph.D Thesis, University of Saskatchewan, 2004) to isolate

downstream target genes. Seven clones were obtained and sequenced from E18 hindbrain and spinal cord chromatin. BLAST query of these sequences identified two potential target genes, the murine homolog of the human *dual specificity tyrosine kinase 4 (Dyrk4) gene* and *high temperature requirement factor A 3 (HtrA3) gene*. In this study, by using EMSA I have demonstrated that Hoxa2 protein binds to *HtrA3* and *Dyrk4* fragments through a core GATG motif. Using site-directed mutagenesis, transient transfection and luciferase assays I have shown that Hoxa2 protein inhibits the reporter expression through binding to multiple GATG motifs in the *cis*-regulatory element. A Hoxa2-VP16 fusion protein further confirmed this activity in transient transfection and luciferase assays. RT-PCR analysis revealed an *in vitro*/*in vivo* correlation of the inhibitory role of Hoxa2 protein on *HtrA3*.

HtrA3 is a member of the HtrA family, which belongs to a family of serine proteases. HtrA3 was discovered initially as a pregnancy-related serine protease that is up-regulated dramatically during mouse placental development (Nie et al., 2003b). As *HtrA1* and *HtrA3* share a high degree of domain homologies, they also share a functional similarity (Nie et al., 2003a). Both HtrA1 and HtrA3 are inhibitors of transforming growth factor-β (TGF-β) signaling and bind to various superfamily members (at least TGF-β1, BMP-2, BMP-4) (Oka et al., 2004; Tocharus et al., 2004). BMPs and Hoxa2 protein are involved in many processes during development simultaneously. The finding that Hoxa2 protein inhibits *HtrA3* gene expression implies that Hoxa2 protein might interact with BMP signaling pathway and control the development of craniofacial elements.

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