Role of the paired-like gene Pitx1 in Xenopus head development.

Wing Yean. Chang

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ROLE OF THE PAIRED-LIKE GENE PITX1 IN XENOPUS HEAD DEVELOPMENT

By

Wing Yean Chang

A Thesis
Submitted to Faculty of Graduate Studies and Research
Through the Department of Biological Sciences
In Partial Fulfillment of the Requirements for the
Degree of Master of Science at the
University of Windsor

Windsor, Ontario, Canada
2000

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Abstract

The process of head development is complex and with the exception of the hindbrain region, it is unlike trunk development in as much as it does not involve discrete segmentation. Recent evidence suggests that the paired-like family of genes play a role in regulating anterior development. To further understand the role that these genes possess, we have cloned the Xenopus homolog of Pitxl. XPitxl is present as a maternal transcript with expression observed as a dorsal streak during gastrulation. This streak restricts to a small circular domain underlying the centre of presumptive neural plate. Concomitantly, a crescent of expression is observed at the border of anterior neural ectoderm. Expression of XPitxl persists throughout the cement gland anlage during gastrulation. At the onset of organogenesis, XPitxl is expressed within the cement gland, eye, lateral plate mesoderm, and first branchial arch derivatives. Misexpression of XPitxl in whole embryos leads to the formation of enlarged or ectopic cement glands. In addition, variable posterior deficits are observed with extreme cases where the embryo exhibits no recognizable structures posterior to the cement gland. Expression of markers such as XCG-1, xOtx2, xPax6, and xTwist suggest that increases in cement gland and lower mandibular size are likely at the expense of other head tissues. Conversely, antagonization of XPitxl with a mutant form of the gene results in cement gland inhibition and head malformations. Animal cap assays show that XPitxl can directly induce cement gland formation and that induction requires DNA binding by XPitxl.
To my Wife, Parents, and my Brothers.
ACKNOWLEDGEMENTS

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Last but not least, I would like to express my thanks and love to my wife Nancy Yue, my parents, Yu-Ting and Lailin Chang, and my brothers, Peter, Richard, Robin, and Steven Chang.

“When the goin get tough, Leopards!”
-Frank-
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CHAPTER ONE
GENERAL INTRODUCTION

Chapter Summary

During formation of the vertebrate body plan, mesoderm forms at gastrulation and simultaneously the dorsal/ventral and anterior/posterior axes begin to differentiate. Curiously, the head and torso appear to be organized by different means. The tissues which gives rise to head pass through the same organizing centre as body tissues but the head requires additional developmental cues. The hypothesis that both head and trunk derive from separate organizers developed following results from early tissue recombination experiments. Current molecular techniques have allowed us to study these two organizing centres during the development of the head and trunk. A new family of transcription factors known as the paired-like proteins appears to be expressed within anterior domains during vertebrate development, and possibly to play a role in organizing the head once it has been induced.

Introduction

Understanding the complexity which underlies formation of the body plan has been the goal of many scientists, and the genes involved are being discovered rapidly due to the advancement of new technologies. Although the rate of gene isolation is phenomenal, the elucidation of the complete network by which genes function and interact remains a daunting task. A super-family of genes that has been shown to play important roles in patterning of organisms are the homeobox genes. Homeobox genes
were first described in *Drosophila* with the isolation of *Antennapedia* and *Ultrabithorax*. For example, mutations in either *Antennapedia* or *Ultrabithorax* lead to homeotic transformations (where one body part is transformed to another) of the antenna to legs and halteres to wings, respectively (Kaufman *et al.*, 1990). Homeobox genes encode a conserved 60 amino acid sequence called the homeodomain. This homeodomain binds to and alters the activity of target genes. In addition to the highly conserved homeobox, these genes are sometimes found clustered together within the genome. In *Drosophila*, the clustered genes are known as the antennapedia complex (ANT-C) and the bithorax complex (BX-C), and together they are called the homeotic complex (HOM-C). Interestingly, in vertebrates these two clusters are contiguous and have duplicated into several clusters. These clusters are now referred to as the *Hox* gene complex (for recent reviews see Veraksa *et al.*, 2000; Deschamps *et al.*, 1999). The function of the *Hox* gene cluster is to assist in specifying the anterior/posterior body axis. Interestingly, the genomic arrangement of these genes within the cluster is correlated with the order of their expression. Recently however, additional homeobox genes have been found which are not clustered together but are scattered individually throughout the genome. Understanding the role that these genes and their partners play may provide insight into the basis of molecular patterning.

To address the role of homeobox genes, one model organism that has been of great importance in these studies is the South African clawed frog, *Xenopus laevis*. *Xenopus* adult frogs are relatively simple to maintain while their embryos are easily accessible. The large size and robust nature of the *Xenopus* embryo endow it with characteristics which make it amenable to micromanipulation. Study of this embryo has
contributed to some of the most important advances in developmental science we know today. For the purposes of this study, the animal provides several key features which facilitate the study gene function. Gain- and loss-of-function assays can be performed simply through injecting capped mRNA into the embryo. Furthermore, specificity of gene function can be determined by animal cap assays. The animal cap is the upper (animal pole) ectodermal cell layer which is fated to differentiate epidermal tissue unless it is induced by underlying tissue to differentiate along different lines. Hence, misexpression of capped mRNA in this tissue subsequently may be used to induce a specific type of differentiation. These properties provide powerful tools to study gene function as it relates to the whole animal.

**Head Induction**

One question that developmental biologists have been trying to uncover is how the body axes of vertebrates are patterned. When the vertebrate body is formed, several axes are constructed as the organism develops from one cell (zygote) into the intricate workings of trillions of cells (the body). As the embryo develops there are initially three distinct cell types that are distinguishable within the embryological tissue. These different cell types are known as the three germinal layers: ectoderm, mesoderm, and endoderm. Each of these germ layers gives rise to distinct tissues and organs as development progresses. The mesoderm arises upon gastrulation when mass cell movements occur. It is this re-organization of cells, which initiates the formation of axes. During early gastrulation in amphibians (Fig. 1), cells at the marginal zone begin to involute and migrate anteriorly forming the mesodermal layer. The region of involution is called the
dorsal lip. Early microsurgical work by Spemann and Mangold (1924) on amphibians shed light upon the process of head development. When they grafted tissue from the upper dorsal blastopore lip onto a host embryo, it resulted in the formation of a secondary axis resembling an organized conjoined or siamese twin (Fig. 2). Subsequently, they showed that as gastrulation progressed, transplantation of the upper dorsal lip could not induce a head but rather could induce either a trunk and tail or a tail alone (Spemann, 1931). From these experiments they predicted the existence of specific head and trunk inducers which correlated with the chronological age of the dorsal lip. This small area of tissue is not a homogenous structure but has positionally specific inducing activities: the anterior portion of this region specifies head while posterior regions induce trunk characteristics (Zoltewicz and Gerhart, 1997). Although head and trunk structures are subject to some of the same developmental cues, head specification is less well understood. Trunk formation involves distinctly defined segments, the borders of which correspond to Hox gene expression domains. In contrast, head tissues display no overt signs of segmentation other than regions near and in the hindbrain. Moreover, in head development there is extensive migration of different cell types, often between what might otherwise be considered developmental compartments.

In addition to specifying the anterior/posterior axes, grafting experiments also reveal that this early lip tissue contributes to dorsal structures. As mesoderm begins to form there are two characteristic paths it can follow. It can adopt a dorsalizing character that gives rise to structures such as the notochord and somites, or it can adopt a ventral character and become blood and connective tissue. How then, does mesoderm acquire its dorsal or ventral character? Work by Pieter Nieuwkoop revealed the source of the signals
**FIG. 1. (A)** Amphibian gastrulation results in the formation of mesoderm and mass cell movements which result in the specification and formation of the body axis. Involution of tissue occurs at the marginal zone forming the dorsal lip and mesodermal germ layer. During gastrulation, the mesodermal layer migrates toward the presumptive anterior region of embryo while concurrently inducing overlying ectoderm to become neural tissue (Adapted from Gilbert S., 2000).

**FIG. 2. (B)** Transplantation experiment of the upper dorsal lip of one embryo into the blastocele of a host embryo results in conjoined twins. Early dorsal lip tissue can induce head structures while late dorsal lip tissue is capable of inducing trunk and tail structures (Adapted from Gilbert S., 2000)
involved in specifying the mesodermal germ layer. Experiments utilizing recombinant
grafts of naïve ectodermal caps and dorsal vegetal cells yield differentiated tissues which
exhibit both dorsal mesoderm and head endoderm characteristics: vegetal cells carry
some of the signals necessary to dorsal axis formation (Nieuwkoop and Ubbels, 1972).
This vegetal mass of cells is now referred to as the Nieuwkoop centre. In contrast,
recombinants using ventral vegetal cells tend to give rise to ventral mesoderm
(Boterenbrood and Nieuwkoop, 1973). Not all of the molecules involved in
differentiating the dorsal and ventral axis are yet known but several pathways involved in
mesoderm induction have been associated with dorsal/ventral patterning. Thus far, there
are four discrete pathways known to be involved in mesoderm induction and
specification. These four pathways include transforming growth factors-beta (TGF-β),
fibroblast growth factors (FGF), wingless (Wnt) pathway, and bone morphogenetic
proteins (BMP) (reviewed in Harland and Gerhart, 1997). Studies have shown the
complex individual functions of these pathways where TGF-β and FGF factors function
to induce mesoderm while Wnt and BMP factors then act to specify the axial attributes of
that mesoderm. Although the functions of these genes are specific, they are nonetheless
inextricably linked. Evidence of pathway crosstalk was presented by Nishita et al. (2000)
when they showed interaction between the Wnt and TGF-β signal transduction pathways.

This structure which gives rise to the mesoderm while simultaneously specifying
the anterior/posterior and dorsal/ventral axes is now known as Spemann’s organizer in
amphibians. In other organisms, there are structures equivalent to Spemann’s organizer
such as the shield in fish and Hensen’s node in mouse and chick embryos (Shih and
Fraser, 1996; Beddington, 1994; Storey et al., 1992).
**Organizer Activity**

As predicted by Hans Spemann, the dorsal mesoderm carries the signals necessary for head induction. The homologs of the *Drosophila wingless* pathway have been implicated as key players in Spemann’s organizer as mesoderm dorsalization agents (for review, see Dierick and Bejsovec, 1999; Wodarz and Nusse, 1998). Overexpression of *xWnt-8* or *xWnt-1* results in a duplication of axes that has been attributed to the dorsalization of ventral mesoderm (Smith and Harland, 1991; Sokol *et al.*, 1991). The *Wnt* pathway likely plays at least two roles. First, it has an early function which is to create the Nieuwkoop signalling centre and secondly, it has a later role in maintaining the dorsal/ventral axis. Disruption of the components in Wnt pathway leads to the build-up of *β-catenin* and will result in axial duplication of the embryo (Heasman, 1997). The disruption of any number of the down-stream molecules involved in the Wnt pathway will lead to the induction of secondary axes such that supernumerary head structures can be formed. For example, overexpression of other downstream targets of Wnt signaling such as the *frizzled-like* receptor or *dishevelled* will result in dorsalization whereas the overexpression of *GSK3* (which phosphorylates *β-catenin* and results in its degradation) ventralizes embryos (for review, see Heasman, 1997; Wodarz and Nusse, 1998). Interestingly, Wnt proteins are not expressed within the dorsal mesoderm but are expressed in the lateral and ventral mesoderm (Smith and Harland, 1991; Sokol *et al.*, 1991).

As a consequence of these and other experiments, the Wnt pathway initially seemed a likely candidate for a head-inducer, but work by Christian and Moon (1993) revealed that ubiquitous expression of *xWnt-8* resulted in head truncations. Moreover,
dorsal expression of a dominant-negative $x^{Wnt-8}$ resulted in enlarged head structures and reduced trunk structures (Hoppler et al., 1996). More recently, transgenic techniques have also shown that transient expression of $x^{Wnt-8}$ during early neurulation represses head induction (Wheeler et al., 2000). This suggests a later role for Wnts in maintaining the dorsal/ventral axis instead of specifying head structures although head structures are induced when ventrally misexpressed at earlier stages. The induction of head structures is likely a secondary effect induced by the presence of a new dorsal axis. This raises the question of what specifies the ventral axis if Wnts are capable of dorsalizing ventral mesoderm?

One candidate for specifying ventral mesoderm is the family of bone morphogenetic proteins (BMP), which are capable of ventralizing *Xenopus* embryos (Koster et al., 1991; Dale et al., 1992; Jones et al., 1992). This ventral-inducing role is further supported by the ability of BMPs to induce ventral mesoderm in animal cap assays. Thus far, there have been a number of BMPs isolated each of which is possessed of different roles. Currently only three BMPs, BMP2, BMP4, and BMP7 have been shown to play roles in early *Xenopus* development such as specifying ventral mesoderm. Interestingly, overexpression of either one of these BMPs results in a ventral phenotype whereby the embryos lack any head structures. If one antagonizes BMP function with a dominant-negative receptor of BMP2 and BMP4, a partial dorsal axis is induced ventrally suggesting that BMPs are critical for maintaining ventral mesoderm character by actively suppressing formation of head (Graff et al., 1994; Suzuki et al., 1994).

Misexpression of either Wnt or BMP yields head truncations. This led Glinka et al. (1997) to hypothesize that head induction may be the result of the inhibition of both
Wnt and BMP signaling. These investigators showed that if one co-injects Xenopus embryos with dominant-negative forms of the \(xWnt-8\) and the \(BMP\ 2.4\) receptor, it results in the formation of a complete supernumerary body axis including head structures. This data supports the notion that at the molecular level dorsal/ventral axis maintenance may be separate from that of head induction. In order for head patterning to occur, however, dorsal and ventral cues must be carefully regulated so that head-inducing genes can exert their effect.

Two candidates for head-inducing genes have been recently isolated in Xenopus namely \(cerberus\) (Bouwmeester et al., 1996) and \(dickkopf-1\) (Glinka et al., 1998). Both \(dickkopf-1\) and \(cerberus\) exhibit head inducing activity whereby misexpression of either gene is capable of inducing head structures (Bouwmeester et al., 1996; Glinka et al., 1998). In addition to head inducing activity, both genes appear to inhibit Wnt signalling (Glinka et al., 1998; Piccolo et al., 1999) while \(cerberus\) has been also shown to antagonize signalling pathways of BMP and Nodal (a member of the TGF-\(\beta\) superfamily involved in left-right asymmetry) (Piccolo et al., 1999). The suppressor activities of these head-inducing genes correlate with the observation that with inhibition of BMP and Wnt signalling results in head induction (Glinka et al., 1997). More recently, another homeobox gene, \(Hex\), has been also associated with head development. The Xenopus homolog \(xHex\) is expressed within the meso-endodermal region of the organizer, however overexpression of \(xHex\) does not induce head structures. Alternatively, animal cap assays revealed the induction of \(cerberus\) in \(Hex\) expressing cells (Jones et al., 1999). Brickman et al. (2000) showed that \(xHex\) functions as a transcriptional repressor and possible targets could be organizer genes such as goosecoid. Inhibition of \(xHex\) function results in
head truncations without concomitant perturbations of trunk development (Brickman et al., 2000). *Hex* is clearly necessary but insufficient to direct head development.

Evidence of an anterior signalling centre has also been reported in mouse. Mouse knock-out experiments of genes such as *Lim-1* (Shawlot and Behringer, 1995) and *Otx2* (Ang et al., 1994; Matsuo et al., 1995; Acampora et al., 1999) have resulted in mice with complete head truncations although trunk development remains unaffected. It has been suggested that this anterior signalling centre may reside in the anterior visceral endoderm (AVE) where *Lim-1* and *Otx2* expression is observed. In *Xenopus* an analogous structure would be the anterior most endodermal region of Spemann’s organizer. However, in *Xenopus*, removal of these cells during development does not affect head development but instead impairs heart development (Schneider and Mercola, 1999). These results suggest that an anterior signalling centre analogous to that in mouse may not exist in frogs. In contrast to this, recombination experiments with ectoderm and anterior endoderm containing *xHex* expression result in the formation of cement glands, the most anterior structure of the embryo (Jones et al., 1999). The development of the cement gland suggests an anterior specifying capacity within this ectodermal/endodermal region.

So how are the genes involved in patterning turned on in the right place and at the right time? Many of these genes play several roles in development and can pattern different things at different times. Therefore, gene regulation is also an important aspect of development. One interesting coincidence is that a growing group of homeobox factors belonging to the *paired-like* gene family are being demonstrated to express in anterior regions, possibly suggesting an important regulatory role for these genes in anterior development.


**Paired-Like Genes**

The *paired* class of homeobox genes are related to the *Drosophila* gene *paired* (*prd*). These proteins contain a characteristic amino acid residue at position 50 of the homeodomain, which distinguishes them from other homeobox genes (Schneitz *et al.*, 1993). This residue lies within the 3\textsuperscript{rd} helix of the homeodomain which is the region that contacts cognate DNA sequence. In other words, this residue likely plays a critical role in determining DNA binding site specificity (Treisman *et al.*, 1989; Pellizari *et al.*, 1997). The *paired* class of homeobox genes consists of three subgroups determined by which amino acid residue is encoded at position 50 of the homeodomain. The subgroup known as the *prd-type* or *Pax* genes contain a serine residue at position 50 and another DNA-binding domain called the paired domain near the C-terminal (Bopp *et al.*, 1986; Frigerio *et al.*, 1986). The two other related subgroups lack the paired-domain and are characterized by either a lysine (K50) or glutamine (Q50) residue at position 50 of the homeodomain (Miller *et al.*, 1992; White *et al.*, 1992; Schneitz *et al.*, 1993). These two subgroups are called the paired-like (*prd-like*) transcription factors. Within the group of paired-like transcription factors, phylogenetic analysis has revealed 18 distinct families to date: a) 12 Q50 Prd-like genes (*aristaless, Ceh-10, Rx, Unc-4, Cart1, Otp, Arix, Prx, Og12, Anf, Mix, siamois*); b) 3 Pax-type (*Pax-3, Pax-7, Pax-6, Pax-4*); and c) 3 K50 Prd-like (*Otx, Ptx, goosecoid, bicoid*) (for review, see Galliot *et al.*, 1999).

The focus of the continuing sections will be to examine the K50 Prd-like family of transcription factors, and address some of the possible functions of these gene families. Thus far, members of this family of transcription factors, which includes the *goosecoid*, *Otx*, and *Pitx* families, have exhibited different roles in anterior development. The first
paired-like transcription factor was isolated in *Drosophila* and is called *bicoid* (Driever and Nusslein-Volhard, 1988). *Bicoid* functions as an anterior patterning gene during early development of the *Drosophila* embryo. In this organism it is essential to formation of the head (Driever *et al*., 1990). However, since no homolog of *bicoid* has been found in the vertebrates we will not address this gene in further detail.

**Goosecoid**

*Goosecoid* has been isolated in a number of organisms such as frog, zebrafish, chick, and mouse (Blumberg *et al*., 1991; Stachel *et al*., 1993; Shulte-Merker *et al*., 1994; Izpisua-Belmonte *et al*., 1993; Blum *et al*., 1992). It is the second member of the K50 family of transcriptions to be isolated following the discovery of *bicoid* (Driever and Nusslein-Volhard, 1988). *Goosecoid*, initially isolated in *Xenopus*, is expressed in the deep cells of the organizer region fated to become head mesoderm (Cho *et al*., 1991). Expression persists within the head mesoderm underlying prospective forebrain regions (Steinbeisser and De Robertis, 1993). During mouse organogenesis, transcripts have also been detected within the developing heart (Conway, 1999). Misexpression of *goosecoid* in *Xenopus* leads to duplication of axes containing trunk and occasionally complete head structures (Cho *et al*., 1991). Ferreiro *et al.* (1998) showed that inhibiting *goosecoid* function elicits malformations in head and trunk development. Loss of function experiments using antisense mRNA also results in minor head defects in *Xenopus* (Steinbeisser *et al*., 1995). Similar to the activity of *xHex*, *goosecoid* is also a transcriptional repressor (Ferreiro *et al*., 1998). The involvement of *goosecoid* in head development is further supported by the recent findings that *goosecoid* is a downstream
target of several other paired-like factors such as *Lim-1* and *Otx2* (Mochizuki *et al.*, 2000). Recall that both *Lim-1* and *Otx2* have been associated with head development and that null mutants of either gene in mice results head truncation. In addition to being a downstream target of head inducing genes, *goosecoid* also functions to inhibit *brachyury*, a mesodermal gene involved in trunk organization (Latinkic and Smith, 1999).

In mouse, mutation of the *goosecoid* gene result in craniofacial and rib defects as well as neonatal death (Rivera-Perez *et al.*, 1995; Yamada *et al.*, 1995; Boucher *et al.*, 2000). Craniofacial abnormalities include aplastic nasal cavities, hypoplasia of the lower jaw, and abnormalities in the musculature of the lower jaw. Interestingly, given the axis irregularities in goosecoid misexpressing frogs, gastrulation defects were not observed in the developing mutant mouse embryo.

*Otx family*

The vertebrate *Otx* family of transcription factors belongs to the K50 group of paired-like genes and is related to the *Drosophila orthodenticle*. *Orthodenticle* is expressed at the anterior pole and plays a critical role in specifying head segments during *Drosophila* development (Finkelstein *et al.*, 1990a, Finkelstein *et al.*, 1990b). Several vertebrate *Otx* genes have been isolated and play anterior-specifying roles similar to their *Drosophila* counterparts. Murine *Otx1* is critical in the proper development of anterior structures: it is expressed within the olfactory placode, otic and optic vesical, and the anterior neuroectoderm corresponding to the fore and mid-brain regions (Simeone *et al.*, 1993). Homozygous null mutant mice (*Otx1 -/-*) display reduced brain weight and size as well as eye and inner ear abnormalities (Acampora *et al.*, 1996). In addition to
morphological anomalies, behavioural attributes are observed with mice exhibiting high speed turning behavior and epileptic behavior (Acampora et al., 1996, Acampora et al., 1999). In *Xenopus*, *xOtx1* is initially observed as a maternal transcript, however it is then observed in the fore and mid-brain regions much as seen in mouse (Kablar et al., 1996; Andreazzoli et al., 1997; Pannese et al., 2000). Gain-of-function experiments in *Xenopus* reveal that *xOtx1* is capable of inhibiting tail-inducing activity of the organizer (Andreazzoli et al., 1997).

In addition to *Otx1*, *Otx2*, has been isolated in several different vertebrates such mouse (Simeone et al., 1992), chick, zebrafish (Li et al., 1994; Mori et al., 1994; Mercier et al., 1995) and frog (Pannese et al., 1995; Kablar et al., 1996). Mice deficient of *Otx2* display gastrulation defects and severe head deformities suggestive of a role for this gene in directing head induction (Ang et al., 1994; Matsuo et al., 1995; Acampora et al., 1999). Furthermore, *Otx2* is expressed within the anterior visceral endoderm (AVE), a region that has been shown to possess head-inducing properties (for review, see Knoetgen et al., 1999). Overexpression of *xOtx2* in *Xenopus* results in phenotypes ranging from axis duplication to supernumerary cement gland induction (Pannese et al., 1995; Blitz and Cho, 1995). However, ectopic expression of *xOtx2* did not induce full head structures when supernumerary axes were formed. More recently, another member, *xOtx5* was cloned by Kuroda et al. (2000) which also displayed phenotypes similar to *xOtx2*. Ectopic expression of *xOtx5* induces enlarged and supernumerary cement glands. The *Otx* family of genes appears to be crucial for the proper development of head structures, but whether it acts to provide cues in a head-inducing center remains to be seen. In loss-of-function experiments, mice develop without heads suggesting a critical
role for the Otx genes in head development, yet, gain of function experiments in Xenopus do not result in the complete induction of supernumerary head structures. In contrast to Otx2, Xenopus cerberus is capable of inducing head structures when ectopically expressed as mentioned above (Bouwmeester et al., 1996) whereas cerberus -/- mice displayed normal head development (Belo et al., 2000; Shawlot et al., 2000). However, in both organisms, cerberus expression leads to the expression of Otx2 in explant assays suggesting that Otx2 may be a downstream target of head inducers (Belo et al., 2000; Shawlot et al., 2000). Redundant pathways may be involved in specifying head structures with one such candidate being lefty-1 which can also activate Otx2 (Shawlot et al., 2000). Another important candidate for head induction, dickkopf-1 (dkk-1), is capable of inducing head structures in Xenopus embryos while in animal cap assays it shows the ability to induce Otx2 (Glinka et al., 1998). Loss-of-function assays have not been reported for dickkopf-1 in mice but should reveal some interesting attributes for anterior patterning, particularly with respect to effects upon Otx expression domains.

**Pitx Family**

The Pitx family of genes is the most recent addition to the K50 group of prd-like transcription factors. To date, there have only been three members isolated; Pitx1 (Lamonerie et al., 1996; Muccielli et al., 1996; Szeto et al., 1996; Shang et al., 1997; Vorbruggen et al., 1997; Hollemann and Pieler, 1999; Chang et al., unpublished), Pitx2 (Muccielli et al., 1996; Semina et al., 1996; Gage and Camper, 1997; Campione et al., 1999; Schweickert et al., 2000), and Pitx3 (Semina et al., 1997; Smidt et al., 1997;
Khosrowshahian et al., unpublished) amongst animals ranging from Drosophila to zebrafish, frogs, chick, mouse, and human.

*Pitx1* (Pituitary homeobox 1) was first isolated based upon its ability to activate transcription of the pro-opiomelanocortin (POMC) gene within pituitary corticotrope cells (Lamonerie et al., 1996). *Pitx1* is expressed in all cell lineages of the pituitary gland in mice and is important for the proper development of the pituitary (for review, see Drouin et al., 1998). Besides playing a role in pituitary development, the early expression pattern of *Pitx1* suggested a role in anterior patterning as well as in hindlimb development. *Pitx1* is initially expressed within the stomodeum and in the mesenchyme of the first branchial arch from which anterior structures such as the tongue, palate, teeth, olfactory system, and Rathke’s pouch are formed (Lanctot et al., 1997; Shang et al., 1997). In support of *Pitx1* as a key regulator in the development of particular facial attributes, Crawford et al. (1997) mapped the human and murine *Pitx1* to a chromosomal region associated with Treacher Collins Syndrome. Treacher Collins Syndrome (Fazan et al., 1967) is an autosomal dominant disorder which results in craniofacial dysmorphologies such as hypoplasia of the malar bones and mandible, cleft palate, malformations of the auricle, hearing loss, lower eyelid coloboma, and anti-mongoloid slant. Interestingly, transcripts are also present in the posterior lateral plate mesoderm and ultimately expression is detected within the hindlimb (Lanctot et al., 1997; Shang et al., 1997). *Pitx1* is not observed in the forelimb, which makes it one of only three genes known to be differentially expressed in the fore and hind limb. The other two genes belong to the *T-box* family.
Consistent with the linkage between Treacher Collins and Pitx1, Pitx1 --/-- mice exhibit malformation within the mandible region: severe micrognathia, cleft plate, and a bifurcated tongue can result. Other bone deformities included bone depositions around Meckel’s cartilage, absent gonial bone, and reduction in the tympanic bone size (Lanctot et al., 1999). Oddly, tooth development is not affected since Pitx1 is expressed in both the epithelium and mesenchyme encompassing the tooth-forming region of the mandible (St. Amand et al., 2000). However, this may be due to a redundant pathway initiated by the overlapping expression of Pitx2, a related family member expressed in the same tissue. Null mutants exhibit severe hindlimb deformities including foreshortened femur, shorter tibia, and absent patella. (Lanctot et al., 1999; Szeto et al., 1999). In a set of elegant experiments, Szeto et al. (1999) utilized retroviral techniques to misexpress cPitx1 within the chick wing bud. Their results indicate that Pitx1 is a specific inducer of hindlimb patterning. The wild-type skeletal pattern of the chick wing is characterized with digit IV being the longest and II the shortest. In the leg, there are extra digits positioned toward the back of the foot while digits II, III, and IV are similar in length. Misexpression of Pitx1 in wing leads to an increase in digit II size and also to the development of an extra short digit. More striking is a reduction of distal feathers in the wing where the integument of wing transformed to the scaled skin morphology typical of the feet.

The discovery of Pitx1 in hindlimb development has giving us a glimpse of hope in elucidating the genetic basis of fore vs hindlimb patterning. Recent evidence has linked Pitx1 and the T-box genes in specifying limb identity. In the chick, Tbx5 and Tbx4 exhibit differential expression within the wing and hindlimb, respectively (Ohuchi et al.,
1998). Logan and Tabin (1999) have shown that Pitx1 is an upstream regulator of Tbx4 and misexpression of Pitx1 in the wing-bud correlates with ectopic expression of Tbx4.

A related family member isolated around the same time as Pitx1, termed Pitx2 (also known as RIEG), not only plays a role in craniofacial development, but is a key regulator in left-right asymmetry. The gene appears to influence the process that governs the rightward looping of our heart, asymmetric pattern of our gut, and the orientation of organs such as the liver and gall bladder. Pitx2, like Pitx1, plays several roles in early patterning: it is expressed anteriorly in the first branchial arch, the eye, brain, pituitary gland, mandible, heart, limbs, and left lateral plate mesoderm (Seminà et al., 1996; Muccielli et al., 1996; Gage and Camper, 1997, Kitamura et al., 1997; Arakawa et al., 1998). Mutations of PITX2 in humans cause an autosomal dominant disorder, Rieger Syndrome Type I (Seminà et al., 1996) which exhibits phenotypes such as iris dysplasia, glaucoma, maxillary hypoplasia, abnormal ear, teeth malformations, and cardiac defects (Jorgenson et al., 1978). Gage et al. (1999) showed that Pitx2 +/− heterozygous mice displayed severe Rieger syndrome phenotypes such small body size, eye and tooth defects. Moreover, Pitx2 −/− mice exhibit severe internal organ malformations of the lung, heart, and stomach (Gage et al., 1999; Lin et al., 1999; Lu et al., 1999). During organogenesis in mice and chick, Pitx2 transcripts are detected only on the left side of the developing heart and gastrointestinal track. It is always a question whether the presence of transcripts also represents functional protein. Only recently has Hjalt et al. (2000) showed that the Pitx2 protein is present in these tissues providing confirming evidence that Pitx2 plays a role in the left side of the developing heart and gastrointestinal track. Misexpression of Pitx2 on the right side of the body often leads to laterality defects.
where heart and gut looping is reversed. At lower frequency more severe phenotypes were observed where these organs displayed partial *situs inversus* (Ryan *et al.*, 1998; Campione *et al.*, 1999). Studies in Zebrafish (Essner *et al.*, 2000) and *Xenopus* (Schweickert *et al.*, 2000) have associated left-right asymmetry to distinct isoforms of *Pitx2*. There have also been other proteins found to have asymmetric expression namely the activin and nodal-related factors, which belong to the super-family of transforming growth factors (TGFs). *Pitx2* appears to function as a downstream regulator within the TGF-β signalling pathway. *Pitx2* can be activated by factors such as activin and nodal (Ryan *et al.*, 1998; Campione *et al.*, 1999). Blocking activin also blocks the expression of the *Pitx2* (Ryan *et al.*, 1998).

The last family member isolated is *Pitx3*, however it has not been as well studied as the previous two members. Initially isolated by Semina *et al.* (1997), *Pitx3* is expressed primarily in the lens and the gene maps to a chromosomal location associated with aphakia in mouse. A deletion in the promoter altering *Pitx3* expression is believed to be the cause of aphakia in mouse (Seminal *et al.*, 2000). Semina *et al.* (1998) also showed that human PITX3 is mutated in patients with anterior segment mesenchymal dysgenesis (ASMD) and congenital cataracts. Rat (Smidt *et al.*, 1997) and frog (Khosrowshahian *et al.*, unpublished) *Pitx3* genes have also been isolated. In the rat, expression of *Pitx3* is confined to mesencephalic dopaminergic system. However, in frog, expression is conserved in both lens and brain regions (Khosrowshahian *et al.*, unpublished). In the last four years, the study of the *Pitx* family of transcription factors has yielded substantial insight into mechanisms governing anterior patterning, limb identity, and left-right asymmetry.
Description of Project

The main objective of this thesis was to gain further insight into the process of anterior development utilizing the South African clawed frog (Xenopus laevis) as a model. In order to accomplish this, genes belonging to the Pitx family of transcription factors were cloned and characterized. Since murine Pitx1 revealed an important function in anterior development, we attempted to further characterize the function of the gene by studying it in Xenopus. An advantage of using Xenopus is the facility the animal affords to perform gain-of-function, grafting, and explant experiments.

The murine Pitx1 and a PCR fragment containing conserved regions of the Xenopus Pitx family of genes were used to isolate the full-length clone of Pitx1 from a λ phage library. The expression pattern and function of xPitx1 is described in Chapter 2 utilizing whole mount in situ and overexpression techniques in Xenopus. The results describe interesting aspects of a possible anterior signalling centre residing in or near the cement gland. Proper patterning of the cement gland appears to function as a critical determinant during head development.
CHAPTER TWO

*XPitx1* Plays a Role in Specifying Cement Gland and Head During Early *Xenopus* Development

Chapter Summary

*Xenopus Pitx1* is a homeobox gene whose family members are structurally and functionally conserved in organisms as diverse as *Drosophila*, chick, mouse, human, and frog. Present as a maternal transcript, the gene is zygotically expressed during gastrulation in a dorsal streak of cells. This streak restricts to a small circular domain underlying the centre of presumptive neural plate. Shortly thereafter, a crescent of expression develops at the border of anterior neural ectoderm, and as the central plate domain diminishes, the crescent coalesces to define the presumptive cement gland. Expression remains high throughout cement gland development, and subsequently expands to include ectodermal cells involved in stomodeal invagination. During early organogenesis, expression ensues in developing eye, posterior lateral mesoderm, and first branchial arch derivatives. Ectopic expression of *xPitx1* causes head deformities including enlarged cement gland, ectopic cement glands, and posterior deformities or, in extreme cases, inhibition of recognizable structures posterior to the cement gland. Expression of markers such as *XCG-1*, *xOtx2*, *xPax6*, *neuralβ-tubulin* and *xTwist* suggest that increases in cement gland and lower mandibular size are likely at the expense of other head tissues. Paradoxically, over-expression is sufficient to partially rescue embryos which are axially perturbed by ultraviolet irradiation or retinoic acid administration. Ectopic expression of *xPitx1* in ectodermal explants directly promotes
cement gland development since there was no evidence that neural tissue was present in explants.

**Introduction**

The paired-like class of homeodomain proteins are a family of transcription factors that play a critical role in the early development of flies and vertebrates. They include factors such as bicoid, goosecoid, and members of the Otx, and the Pitx families. Recently, three Pitx (pituitary homeobox) family members have been characterized which possess high sequence conservation among chicks, frogs, mice and humans (Crawford et al., 1997; Gage and Camper, 1997; Lamonerie et al., 1996; Lanctot et al., 1997; Semina et al., 1996; Semina et al., 1997). Moreover, in these disparate species they express in similar structures and at similar times throughout development. A related gene, Ptx1, is expressed during Drosophila development (Vorbruggen et al., 1997). The genes play a role in body patterning: in Xenopus, xPitx2 is involved in regulating left-right asymmetry of internal organs such as heart and gut (Ryan et al., 1998; Campione et al., 1999). Pitx2 knock-out mice however, reveal normal looping of the heart but possess defective cardiac positioning as well as defects in tooth morphogenesis, lung asymmetry, mandibular and maxillary facial prominences (Gage et al., 1999; Lin et al., 1999; Lu et al., 1999). Mutations at this locus are responsible for Rieger's syndrome in human (Semina et al., 1996). Another family member, Pitx3 expresses in the lens and in dopaminergic neurons in mouse, and is involved in other human developmental mutations (Semina et al., 1998; Semina et al., 1997; Smidt et al., 1997).

Recent work on Pitx1 has similarly revealed an important role for this gene in body patterning. Murine Pitx1 is expressed in derivatives of the first branchial arch and
also in the posterior lateral mesoderm and hindlimb (Lanctot et al., 1997; Shang et al., 1997). Mice mutated at the Pitxl locus develop severe mandibular deformities as well as pituitary developmental arrest (Lanctot et al., 1999; Szeto et al., 1999). Anomalies in limb development suggest that the gene plays a role in differentiating fore- from hind-limb identities. This perspective has been consolidated in ectopic expression studies in chick wing buds where leg-like structures have emerged following localized introduction of Pitxl (Szeto et al., 1999). Finally, the human homolog, PTX1, has been situated in the chromosomal region associated with Treacher Collins Syndrome, a human developmental mutation which results in craniofacial anomalies and sometimes foot and thumb abnormalities (Crawford et al., 1997 and references therein).

The early expression patterns of Pitxl and the mandibular deformities which arise in knock-out mice suggest that the gene specifies the anterior-most “segment” of the developing head. Several other of the bicoid-related genes in Xenopus such as goosecoid and Otx are both required for proper head development. For example, goosecoid is expressed primarily in the organizer region and overexpression leads to the formation of an ectopic body axis sometimes even with the duplication of head structures (Cho et al., 1991). The expression pattern of xOtx2 partially overlaps that of goosecoid, and xOtx2 over-expression similarly leads to the development of partial secondary axes, additional cement glands, and bent axes (Blitz and Cho, 1995; Pannese et al., 1995). Moreover, Gammill and Sive (1997) have shown that the cement gland marker genes XCG and XAG are targets of xOtx2.

Here we report the cloning of the Xenopus laevis Pitxl homolog which has also been isolated recently by Hollemann and Pieler (1999). xPitxl is expressed in a
conserved manner consistent with a role for the gene in specifying anterior craniofacial
development. \(xPitx1\) is expressed early during gastrulation in presumptive head regions
and becomes localized to the prospective cement gland anlage. It is then transiently
expressed within the optic cup and olfactory epithelium. Throughout development \(xPitx1\)
remains strongly expressed in the cement gland. In ectodermal cap explant experiments,
we show that \(xPitx1\) plays a role in cement gland induction. Finally, since recent work on
the ectopic expression of \(xPitx1\) has been limited to chick wing bud, we sought to study
the effects of \(xPitx1\) over-expression in specification of anterior structures during early
embryogenesis.

Materials and Methods

Library Screening, cloning, and mutation

A \(\lambda\)ZAP II cDNA head library prepared from stage 28-30 \(Xenopus\) tailbud
tadpoles with a titer of \(2.5 \times 10^{10}\) pfu (Hemmati-Brivanlou \textit{et al.}, 1991) was screened at
moderate stringency with a murine \(Pitx1\) probe. Positive plaques were isolated and the
cDNA subcloned into Bluescript\textsuperscript{®} II SK using ExAssist\textsuperscript{™} Interference-Resistant Helper
Phage (Stratagene). Sequence analysis was performed by dideoxy chain termination
(Sanger \textit{et al.}, 1977) using an ABI 377 robotic sequencing apparatus (York University,
Canada). Both DNA strands were analyzed using primers designed to provide nested and
overlapping data sets (Appendix A). Several independent clones were isolated, two of
which overlapped by 371 nucleotides. These latter two were spliced together at a \(PaeI\)
site to render a contiguous sequence comprising the open reading frame, and portions of
both the 5' and 3' untranslated regions.

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Expression constructs were derived using Vent polymerase (New England Biolabs) and primers which bracketed the open reading frame and possessed restriction sites for EcoRI and XbaI at the 5’ and 3’ ends respectively, and which facilitated insertion into pCS2- (Rupp et al., 1994).

**Embryos**

Embryos were fertilized, dejellied in 2% cysteine and cultured as previously described (Drysdale and Elinson, 1991). Developmental staging was according to Nieuwkoop and Faber (1967). Axial perturbations using lithium, ultraviolet irradiation (UV), and retinoic acid (RA) were performed using previously described protocols (Drysdale and Elinson, 1991; Kao and Elinson, 1988; Kao et al., 1986).

**Whole-mount in situ hybridization and histology**

Whole-mount in situ hybridization was performed according to Harland (Harland, 1991). RNA probes were made from linearized cDNA of the 5’, 3’, and coding regions respectively of xPitx1. Probes were labeled with digoxigenin (Roche) and revealed using anti-digoxigenin antibodies conjugated to alkaline phosphatase (detected with NBT/BCIP). Additional riboprobes for XCG-L, xOtx2, xPax6, neuralβ-tubulin and xTwist were synthesized in similar fashion. Histological sections were prepared according to Fagotto and Gumbiner (1994). Embryological sections of 30 um were prepared with a cryostat.
Microinjection

The coding region of *xPitx1* was inserted into the *XbaI* and *EcoRI* cloning sites of the pCS2+ vector. Synthetic capped mRNA of *xPitx1* was made from linearized template using mMessage Machine (Ambion) driven by a SP6 promoter. Capped mRNA was resuspended in water and injected into embryos with a Drummond nanoinjector. Injections were made into the animal pole of embryos at either the 1-cell or 2-cell stages. Concentrations of the capped mRNA injected ranged from 60 pg to 1.2 ng. Injection volumes never exceeded 9.2 nl. Injected embryos were cultured in 0.1 X Marc's Modified Ringers (MMR) (Ubbels et al., 1983), 50ug/ml gentamicin, and 2% Ficoll-400 (Sigma) at 13 °C for at least 1 hr to allow healing before being removed and allowed to develop at room temperature. At stage 10-10.5 the solution was changed to 0.1 X MMR supplemented with 50ug/ml gentamicin.

Ectodermal cap culture

Stage 8-9 embryos were removed to 1 X MMR containing 50ug/ml gentamicin and 2% Ficoll. Ectodermal explants were removed and cultured overnight at room temperature in Petri dishes the bottom of which had been coated with a thin layer of 1% agarose in 1xMMR. Explants were then removed to 0.1xMMR and cultivated until they reached the stage at which sibling intact control embryos had developed cement glands (stage 25 or later). Explants were then fixed and processed for *in situ* hybridization as described.
RESULTS

Sequence of \textit{xPitx1}

The sequence of \textit{xPitx1} (Genbank accession AF217647) contains an open reading frame which encodes a conceptual protein of 305 amino acids. The homeodomain and other regions share high amino acid identity with \textit{Pitx} family members and in particular with \textit{Pitx1} homologs from other species (Fig. 1A). Like other \textit{Pitx} sequences, \textit{xPitx1} encodes a carboxy-terminal motif produced by other homeobox genes which are thought to play a role in craniofacial development. This 14 amino acid motif is encoded in genes such as \textit{Pitx2}, \textit{Cart1}, \textit{Chx10}, \textit{Prx}, \textit{Otp}, and \textit{Drg11} (Semina et al., 1996).

Sequence comparison of the recently cloned \textit{xPitx1} by Hollemann and Pieler (1999) revealed only minor differences both at the nucleotide and amino acid level (Fig. 1B). Discrepancies are likely due to the PCR-based approach used by these investigators to isolate the coding region and/or to allelic variation.

Spatial and temporal expression of \textit{xPitx1} in \textit{Xenopus}

\textit{Xenopus Pitx1} expression was examined by whole mount \textit{in situ} hybridization. In order to determine the spatial distribution of \textit{xPitx1} during early development, whole mount \textit{in situ} hybridization was performed using three different digoxigenin-labeled antisense fragments: one possessed 5’ untranslated and coding regions including the homeodomain, one contained 3’ regions excluding the homeodomain, the last encoded the complete coding region. Expression patterns for each of the three riboprobes were identical. \textit{XPitx1} is expressed in a rapidly changing and dynamic manner. It is first detected during early gastrulation as a faint dorsal streak. As gastrulation progresses, a
**FIG. 1.** (A) Amino acid comparison of *Xenopus Pitx1* with other *Pitx* genes in mouse, chick, and human. *xPitx1* shares greatest sequence similarity with chick *Pitx1*, however all *Pitx1* members encode a highly conserved homeodomain (light grey) and 100% identity within the C-terminal “face domain” motif (white).

(B) Comparison of *xPitx1* cDNA sequence with *xPitx1* fragment previously published (Holleman and Pieler, 1999). For convenience, only portions of the open reading frame are shown: the complete open reading frame, 3', and 5' untranslated sequences are available under accession number AF217647. An asterix indicates nucleotide identity. Where nucleotide sequences diverge, the conceptual amino acid sequence is given.

**FIG. 2.** Expression of *xPitx1* detected by whole mount *in situ* hybridization. All gastrula to early neurula stage embryos are positioned with the yolk plug to the rear with the presumptive anterior regions facing up. (A) Expression is detected in the presumptive anterior ectoderm as a band. An earlier forming dot of expression sits in the central region of the presumptive head plate out of view on the top of this stage 12 specimen. (B) Late gastrula/early 12.5 neurula expression of *xPitx1* is detected as a dot in the center of the neural plate as the anterior band forms a crescent shape. (C) High expression of *xPitx1* occurs in the cement gland anlage at stage 16. (D-F) Expression of *xPitx1* continues within the cement gland and stomodeum as transient expression commences in the optic cup (arrow in E) and olfactory epithelia during organogenesis through to tailbud stages.
### Table A

<table>
<thead>
<tr>
<th>Protein</th>
<th>mPitx1</th>
<th>hPitx1</th>
<th>chickPitx1</th>
<th>xPitx2</th>
<th>xPitx3</th>
</tr>
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<tr>
<td>% Identity</td>
<td>98%</td>
<td>98%</td>
<td>96%</td>
<td>98%</td>
<td>98%</td>
</tr>
<tr>
<td>% Similarity</td>
<td>76%</td>
<td>79%</td>
<td>89%</td>
<td>92%</td>
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</table>

### Table B

<table>
<thead>
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<th>Technique</th>
<th>Holleman-PCR</th>
<th>xPitx1-cDNA</th>
</tr>
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<tr>
<td>1 ATG6ATTCTTCTTCAAAGGTTGGAAATGAAATTTGCCCTGAGAGTTAAGACCCCAAG...</td>
<td>ATG6ATTCTTCTTAAAGGAGCTATGAAATTTGCCCTGAGAGTTAAGACCCCAAG...</td>
<td></td>
</tr>
<tr>
<td>M D S F K G M</td>
<td>M D S F K G A M</td>
<td></td>
</tr>
<tr>
<td>2 ATG6ATTCTTCTTCAAAGGTTGGAAATGAAATTTGCCCTGAGAGTTAAGACCCCAAG...</td>
<td>ATG6ATTCTTCTTAAAGGAGCTATGAAATTTGCCCTGAGAGTTAAGACCCCAAG...</td>
<td></td>
</tr>
<tr>
<td>L Q S P A S L N A</td>
<td>L Q S P A S L N A</td>
<td></td>
</tr>
<tr>
<td>3 ATG6ATTCTTCTTCAAAGGTTGGAAATGAAATTTGCCCTGAGAGTTAAGACCCCAAG...</td>
<td>ATG6ATTCTTCTTAAAGGAGCTATGAAATTTGCCCTGAGAGTTAAGACCCCAAG...</td>
<td></td>
</tr>
<tr>
<td>C Q Y N S</td>
<td>C Q Y N S</td>
<td></td>
</tr>
</tbody>
</table>
band of expression appears in the presumptive anterior region (Fig. 2A). A transient dot in the centre of the presumptive anterior neural plate forms, where it remains until the onset of neural fold development (Fig. 2B). The dot at the center of the neural plate disappears as neural fold closure begins and stronger expression is observed within the anterior band, which presumably defines the cement gland anlage (Fig. 2C). As development progresses, strong expression continues within the cement gland. In addition, expression is also detected within the invaginating stomodeum, optic eminence, and lens placode (Fig. 2D-F).

Expression of \textit{xPitx1} in embryos subjected to anteriorizing and posteriorizing agents

Treatment of embryos with either ultraviolet light (UV) or retinoic acid (RA) inhibits development of anterior structures such as the cement gland, eye and other head structures and leads to the differentiation of posteriorized or truncated embryos. The reverse is commonly induced by treatment with lithium chloride: severely affected embryos may lack posterior ventral structures altogether and form radially symmetrical cement gland and eye bands. We investigated the expression of \textit{xPitx1} following treatment with these agents. Lithium chloride administration resulted in a broadening of \textit{xPitx1} expression to the extent that it would sometimes circumscribe the gastrulating and neurulating embryo (Figs. 3A and A' respectively). Whole mount \textit{in situ} hybridization of UV-treated embryos did not reveal any expression of \textit{xPitx1} at late gastrula (stages 12-12.5; fig. 3B) or during neuralation (stage 19; fig. B') when it is normally otherwise observed in the cement gland anlage as a crescent anterior to the neural plate. Later stages of UV treated embryos similarly failed to reveal \textit{xPitx1} expression (data not
shown). Consistent with the effect of UV-treated embryos, specimens which were anteriorly inhibited by treatment with 1 μM RA (from the 2-cell stage until blastula) failed to express detectable \textit{xPitx1} during gastrulation or neurulation (Fig. 3C and C' respectively).

**Microinjection of \textit{xPitx1} mRNA**

To determine the effects of \textit{xPitx1} over-expression, synthetic capped \textit{xPitx1} RNA was injected into 1-cell embryos at different concentrations near the animal pole. The amount of injected RNA ranged from 60 pg to 1.2 ng. At higher doses (300 to 600 pg mRNA), injected \textit{xPitx1} resulted in several classes of cement gland phenotypes, sometimes yielding some form of head and axis deformity. At low mRNA concentrations, 60 and 120 pg, defects within the head region yield abnormal head structures and cycloptic features. These features always attend a cement gland/lower mandibular region that protrudes prominently. In addition, posterior defects are observed including bent axes, trunk and tail deformities (Table 1). The phenotypes observed appear to be dose-related: concentrations of 60 and 120 pg do not cause the formation of supernumerary cement glands - these are only obtained with injections of 300 pg or higher. At low concentrations enlarged cement glands do not extend across the body flank, however, supernumerary cement glands do, and they usually display as pigmented but somewhat dispersed patches (figure 4A and B display ectopic flank cement glands, and 4B also retains an enlarged anterior cement gland). Some early-stage head malformations are hard to interpret and could only be resolved when tadpoles were analyzed at much later stages. For example, when \textit{xPitx1}-injected embryos with subtly
diminished optic protrusions are reared into swimming tadpoles, a small percentage of them develop severe eye deformities (data not shown). Furthermore, a low number of dwarfed tadpoles develop densely pigmented skin.

Two phenotypes that particularly stand out are enlarged cement glands and supernumerary cement glands on the body flank (Fig. 4A, B), which are outlined in Table 1. Although, the primary phenotypes observed appear to be cement gland related, other phenotypes include prominent cement gland/lower face, reduced trunk and tail structures (Fig. 4C-i), bent tail (Fig. 4C-ii), and severe axes, trunk and tail deformities (Fig. 4C-iii). At a much lower frequency, a grossly enlarged cement gland forms which is attendant with severe anterior and posterior defects (Fig. 4C-iv). Very rarely (only twice during the course of our experiments) embryos lacking identifiable body parts save a giant cement gland were observed (not shown). The morphological and functional status of ectopic patches of cement gland tissue was confirmed using a cement gland-specific marker XCG-1 (Sive et al., 1989) (Fig. 4D). In section, XCG-1 is seen confined to the cement gland ectoderm (Fig. 4E). A more common early effect of over-expressing xPitx1 is anomalous blastopore closure (identical to xOtx2 phenotype seen in Blitz and Cho, 1995). In addition to the phenotypes described above, rare phenotypes arise such as axes duplications, enlarged and supernumerary eyes.

**Over-expression of xPitx1 in UV and RA treated embryos**

The effect of xPitx1 in promoting cement gland development and in some cases, development of enlarged or supernumerary eye structures led us to determine whether xPitx1 played a specific role in specifying anterior structures. Both ultraviolet light and
retinoic acid treatment perturb the proper development of anterior structures, however by different means. Early irradiation of the vegetal pole at the 1-cell stage inhibits anterior development via inhibition of cortical rotation. The result of UV irradiation at its most severe results in a completely vegetalized embryo, with a DAI (Dorso-Anterior Index) of 0 (Kao and Elinson, 1988). By injecting synthetic \( xPitx1 \) mRNA into UV treated embryos we hoped to ascertain its ability to promote dorso-anterior structures. Exposure to UV light for 7 minutes results in 73 % of embryos displaying a DAI of 0 and only 9 % of the embryos exhibiting development of eyes (Fig. 5A-iv; Table 2). \( xPitx1 \) injection (300-1200pg) into UV-treated embryos did not appear to fully rescue ventralized phenotypes, however the number of DAI 0 embryos declines as much as three quarters with \( xPitx1 \) injection. More infrequently, \( xPitx1 \) could induce cement glands and eyes in the irradiated embryos (Fig 5A-i to iv). The morphologies in these rare examples are difficult to interpret and might devolve from one of two scenarios. Either ectopic \( xPitx1 \) induces eyes and cement gland in embryos that would otherwise be classified as DAI 0-2, or ectopic transcript is posteriorly truncating DAI 3-4 embryos to make them resemble DAI 0-2 specimens at the gross morphological level. The numbers of “DAI 0-2” embryos with eyes and cement glands seems to resemble the number of DAI 3-4 embryos in the uninjected but UV irradiated cohort. Therefore, the second alternative appears the most likely. Shown in figure 5A is the extent of anterior re-establishment with \( xPitx1 \) over-expression in UV treated embryos.

The teratogenic effects of RA include severe truncation of anterior structures from the mid-brain forward. Embryos were injected with of \( xPitx1 \) mRNA (300-1200pg) at the 1-cell stage and treated with 1uM RA at the 2-cell stage through to blastula.
FIG. 3. Changed *xPitx1* expression in axially manipulated embryos. Expression of *xPitx1* was detected by riboprobe whole mount *in situ* hybridization in stages 12-12.5 (dorso-anterior aspect) and 19 (dorsal view, anterior oriented to bottom, except A’ which exogastrulated) embryos. (A, B, C and A’, B’, C’ respectively). Lithium chloride (A and A’) treated embryos revealed a band of expression circumscribing the embryo. Embryos which were vegetally irradiated with ultraviolet light (UV) (B and B’) or treated with retinoic acid (RA) (C and C’) failed to express *xPitx1*. As no superficial staining was detected in the UV and RA treated embryos they were cleared prior to photographing. Slight bluish tinges are an artifact of light diffraction and disappear when the embryos are rotated.

FIG. 4. Gain-of-function experiments using *xPitx1* results in severely anteriorized embryos. Embryos injected with 600 pg of *xPitx1* form many deformed phenotypes. Stage 33 embryos reveal enlarged or ectopic cement gland-like structures. (A) Elongated cement glands often fuse the mandibular region to the body flank. (B) Ectopic cement glands frequently develop along the side of the body. (C) Other phenotypes observed exhibited axial, trunk, and tail deformities. Cement glands induced on the flank express the gland-specific marker, XCG-1 (D, and in section E). Solid arrows – ectopic cement gland; CG – anterior cement gland

FIG. 5. Ectopic *xPitx1* partially preserves dorso-anterior structures in axially perturbed embryos. The ability of *xPitx1* to rescue embryos (A) vegetally irradiated with ultraviolet light or (B) treated with retinoic acid. Controls are presented on the bottom for purposes of comparison. In uninjected UV treated embryos, 73% of embryos exhibited a DAI of zero (Ai), and the rest demonstrated other indices of perturbation. In uninjected retinoic acid-treated embryos, 100% were anteriorly truncated (Bi).
Table 1. Percentage of phenotypes observed in embryos injected with wild-type *xPitx1*.

<table>
<thead>
<tr>
<th>RNA injected (pg)</th>
<th>N</th>
<th>site</th>
<th>Normal</th>
<th>Extra Cement Glands</th>
<th>Enlarged Cement Gland</th>
<th>Other Deformities</th>
</tr>
</thead>
<tbody>
<tr>
<td>1200</td>
<td>54</td>
<td>animal</td>
<td>15%</td>
<td>24%</td>
<td>35%</td>
<td>5.5% (cement gland only)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5.5% (reduced trunk and tail)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>15% (bent axis, trunk and tail)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>42% (blastopore closure)</td>
</tr>
<tr>
<td>600</td>
<td>66</td>
<td>animal</td>
<td>21%</td>
<td>14%</td>
<td>44%</td>
<td>1% (cement gland only)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>6% (reduced trunk and tail)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>14% (bent axis, trunk and tail)</td>
</tr>
<tr>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td>32% (blastopore closure)</td>
</tr>
<tr>
<td>300</td>
<td>56</td>
<td>animal</td>
<td>14%</td>
<td>16%</td>
<td>41%</td>
<td>4% (cement gland only)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>18% (reduced trunk and tail)</td>
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<td>7% (bent axis, trunk and tail)</td>
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<td></td>
<td>20% (blastopore closure)</td>
</tr>
<tr>
<td>120</td>
<td>73</td>
<td>animal</td>
<td>44%</td>
<td>4%</td>
<td>4%</td>
<td>42% (bent axis, trunk and tail)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5% (extension of mandible)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>27% (blastopore closure)</td>
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<tr>
<td>60</td>
<td>69</td>
<td>animal</td>
<td>43%</td>
<td>0%</td>
<td>3%</td>
<td>49% (bent axis, trunk and tail)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1% (reduced trunk and tail)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3% (extension of mandible)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>33% (blastopore closure)</td>
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<tr>
<td>uninjected</td>
<td>164</td>
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<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>600pg beta-Gal</td>
<td>52</td>
<td>animal</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

Note: Phenotypes outlined as Other Deformities are shown in figure 4: cement gland only (Fig. 4C-iv), reduced trunk and tail (Fig. 4C-i), bent axis, trunk and tail deformities (Fig. 4C-ii to iii). As deformities were sometimes compound, row percentages will not sum to 100.
Injection into RA-treated embryos failed to rescue anterior structures completely however, ectopic cement glands (Fig. 5B-ii and iii) and development of anterior structures such as the eye were observed (Fig. 5B-ii to iv; Table 2). Embryos treated with 1μM RA resulted in 100% of the embryos with head truncations whereas 75% of embryos injected with xPitx1 and then RA treated developed ectopic cement glands, eye structures, or both (Table 2). The range of phenotypes exhibiting head structures such as cement gland and eyes are shown in figure 5B. Ectopic cement gland identity was confirmed by hybridization with XCG-1.

**xPitx1 induces misexpression of anterior marker genes**

To ascertain the effects of xPitx1 overexpression on other genes, one blastomere of the two-cell staged embryos was injected with xPitx1 and co-injected with capped green fluorescent protein (GFP) mRNA as a tracer. As the embryos develop, one side (left or right) serves as a control for the other. Embryos displaying a high degree of fluorescence within the anterior region of the embryos (on one-side) were isolated and probed with several anterior markers xOtx2 (Blitz and Cho, 1995), xPax6 (Hirsh and Harris, 1997), neuralβ-tubulin (Richter et al., 1988), and xTwist (Hopwood et al., 1989). Ectopic expression of xPitx1 diminished expression of several markers of brain and face development (Fig 6A-D, A’-D’). Specifically xOtx2 is diminished in the optic cup and brain, whereas the uninjected side displayed the normal highly specific expression of xOtx2 within these regions (Fig. 6A, A’). xPax6 expression is also perturbed in xPitx1 injected embryos. xPax6 expression appears to be reduced on the injected side of embryos (compare the expression of Pax6 on the uninjected and injected sides figures 6B
and B' respectively). Expression of the neural crest marker \(xTwist\) is also diminished on the side of \(Pitx1\) injection (compare uninjected fig. 6C with injected 6C'). Neural development as revealed by staining with a probe for \(neural\beta\text{-tubulin}\) demonstrated a similar dispersion and diminution of activity on the \(xPitx1\)-injected side (Fig. 6D and D'). All embryos exhibiting a concentration of anterior GFP fluorescence (and presumably also of co-injected ectopic \(xPitx1\)) in the head showed perturbations of these anterior markers.

In contrast to the effects of ectopic \(xPitx\) in anterior head, effects differed in other regions. For example, under certain circumstances ectopic \(xPitx1\)-induced posterior defects can be associated with increases in \(xOtx2\) and \(neural\beta\text{-tubulin}\) expression. For example, induced supernumerary flank cement gland always expresses \(xOtx2\) both within and around the gland (Fig 7A). Like cement gland in the head, no \(neural\beta\text{-tubulin}\) expresses in the ectopic organ itself (Fig 7B), however in some cases an additional body axis forms, and a neural tract appears to develop which expresses this marker (Figs. 7C,D).

**\(xPitx1\) and cement gland formation in ectoderm cap explants**

To further investigate the role of \(xPitx1\) in specifying cement gland formation, embryos were injected with \(xPitx1\) or control mRNA near the animal pole of the 1-cell embryo. At blastula, the animal caps were removed and cultured for 2-days. Uninjected caps (Fig. 8A), or animal caps injected with \(\beta\text{-galactosidase}\) mRNA fail to form cement gland as assessed either by anatomical features or by hybridization with the cement gland-specific riboprobe for \(XCG-1\). When animal caps are derived from embryos first
FIG. 6. Ectopic xPitx1 diminishes expression of several anterior head markers. The effects of xPitx1 overexpression on anterior and neural crest markers were assayed by injection of 600pg of xPitx1 into a single blastomere at the 2-cell stage followed by whole-mount in situ hybrization with xOtx2 (A -control side, A’ - xPitx1 injected), xPax6 (B, B’), xTwist (C, C’), and neural β-tubulin (D, D’). Black arrows - diminished xOtx2 expression in eye; white arrow - diminished expression of neural β-tubulin in dorsal neural structures.

FIG. 7. Ectopic xPitx1-induced cement glands reveal xOtx2 expression, and posterior axis duplications demonstrate the presence of neural tissue. xPitx1-induced cement gland displays induction of xOtx2 (A) expression but not of neural β-tubulin (B). Supernumerary body axes sometimes form which demonstrate differentiated neural tissue as indicated by the expression of neural β-tubulin in two parallel axis dorsally (C), and one not quite coalesced more ventrally in the same specimen (D). Dark arrows - ectopic cement gland; white arrows - expression of marker gene.

FIG. 8. Animal cap explants can be induced to form cement gland by injection of xPitx1 transcript. (A) Control explants (uninjected); (B) Animal caps isolated from embryos that have been injected with xPitx1 develop sticky cement gland-like structures which hybridize with the cement gland-specific marker XCG-1; (C) neural β-tubulin staining demonstrates the absence of induced neural tissue in the explants even though cement gland is induced following xPitx1 injection.
Table 2. Effect of *xPtx1* overexpression in Retinoic Acid and Ultraviolet light treated embryos.

<table>
<thead>
<tr>
<th>RNA injected (pg)</th>
<th>N</th>
<th>site</th>
<th>Normal</th>
<th>Posteriorized</th>
<th>cement gland and eye</th>
<th>ectopic cement gland</th>
<th>other phenotypes*</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA treatment alone</td>
<td>49</td>
<td>N/A</td>
<td>0%</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>300+RA treated</td>
<td>105</td>
<td>animal</td>
<td>0%</td>
<td>1%</td>
<td>17%</td>
<td>61%</td>
<td>21% (posterior defects)</td>
</tr>
<tr>
<td>600+RA treated</td>
<td>110</td>
<td>animal</td>
<td>1%</td>
<td>1%</td>
<td>23%</td>
<td>50%</td>
<td>25% (posterior defects)</td>
</tr>
<tr>
<td>1200+RA treated</td>
<td>63</td>
<td>animal</td>
<td>0%</td>
<td>0%</td>
<td>10%</td>
<td>79%</td>
<td>11% (posterior defects)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>DAI 3-5</td>
<td>DAI 0-2 (No cement gland)</td>
<td>DAI 0-2 (cement gland and eye)</td>
<td></td>
</tr>
<tr>
<td>UV treatment alone</td>
<td>114</td>
<td>N/A</td>
<td>4% (DAI 3-4)</td>
<td>18% (DAI 1-2)</td>
<td>73% (DAI 0)</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>300+UV treated</td>
<td>78</td>
<td>animal</td>
<td>1%</td>
<td>5% (DAI 1)</td>
<td>3%</td>
<td>1% (DAI 1)</td>
<td>12% (DAI 0)</td>
</tr>
<tr>
<td>600+UV treated</td>
<td>54</td>
<td>animal</td>
<td>0%</td>
<td>4% (DAI 1)</td>
<td>6%</td>
<td>13% (DAI 1)</td>
<td>11% (DAI 0)</td>
</tr>
<tr>
<td>1200+UV treated</td>
<td>66</td>
<td>animal</td>
<td>0%</td>
<td>3% (DAI 1)</td>
<td>3%</td>
<td>1% (DAI 1)</td>
<td>8% (DAI 0)</td>
</tr>
<tr>
<td>no treatment</td>
<td>74</td>
<td>N/A</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

*Note: Posterior defects include a variation of axial deformities including bent axes, trunk and tail which, though present are abnormal. Tail defects often resemble those seen in an *xOtx2* study (Pannese et al., 1995).
injected with \( xPitx1 \) mRNA, cement glands develop: sticky, thickened epithelial patches form which hybridize \( XCG-1 \) (Figs 8B). Against the eventuality that \( xPitx1 \) induction of cement gland was indirect, we assessed injected caps for the presence of differentiated neural tissue using a probe for \( neural\beta\)-tubulin (8C). Clearly no neural tissue was present.

**Discussion**

We have cloned the *Xenopus* homolog of *Pitx1*. The cDNA sequence encodes a conceptual protein of 305 amino acids which contains a homeodomain with high homology to previously described *Pitx* sequences (Campione *et al.*, 1999; Lamonerie *et al.*, 1996; Semina *et al.*, 1998; Semina *et al.*, 1996). Like other homeoproteins of the Pitx group, \( xPitx1 \) possesses a conserved 14 amino acid C-terminal motif that is found in several other homeodomain proteins which are expressed during craniofacial development. Presumably, this domain plays a role in interactions with other head-specific gene products of developmental significance (Semina *et al.*, 1996). A *Pitx1* fragment containing the conceptual open reading frame was recently published which is possessed of some sequence differences, particularly at the 3’ and 5’ ends, and which encodes a slightly different amino acid sequence at the N-terminus (Hollemann and Pieler, 1999). While some of the differences might be attributable to allelic variation, we assume that the differences at the 5’ and 3’ ends of the open reading frame reflect the PCR strategy employed by these authors to amplify \( xPitx1 \), and that the sequence extracted from our cDNA library is a closer reflection of the actual transcript.
Expression of the gene is similar to patterns described for other vertebrates: cement gland is the anterior-most structure to develop in frogs and occupies a topographical location similar to the primordia from which the Pitx1-expressing first branchial arch derives in chick and in mouse (Lanctot et al., 1997). In all three organisms, this anterior expression domain is displaced ventrally as the cranial vault expands, so that by the time facial structures have developed, the gland and the mouth no longer have the appearance of being the most extreme anterior "segment" of the head. Expression of xPitx1 in the cement gland supports the hypothesis that the amphibian cement gland has a developmental homolog in the buccopharyngeal region of amniotes (Sive and Bradley, 1996). Just as the cement gland primordia is the first head organ to display Pitx1 expression in frog, the ectoderm of the buccal face of the first branchial arch is the first to express transcript in mice (Lanctot et al., 1997). In all three organisms the regions are innervated by the trigeminal nerve, and in both mice and frog the tissues are sites of Otx2 expression (Sive and Bradley, 1996). In frog, Otx5 and xOtx2 are related head-specific paired-like homeobox genes which appear to play a role during cement gland formation, however unlike xPitx1, their expression is first detected within the organizer region during the onset of gastrulation (Blitz and Cho, 1995; Pannese et al., 1995; Kuroda et al., 1999). The expression of xPitx1 in the dorsal axis during gastrulation is quite faint and whether this suggests a role in patterning the dorsal axis and an interaction with specific organizer genes such xOtx, goosecoid, noggin, or chordin remains to be seen. With regard to this early and faint period of expression, and also with respect to the transient expression of xPitx1 observed in the centre of the early neural
plate, our findings augment those of Hollemann and Pieler (1999). In all other respects, the expression patterns appear identical.

*XPitx1* expression within the most anterior region of the developing embryo led us to investigate whether it could be inhibited by posteriorizing manipulations such as treatment with ultraviolet light and retinoic acid. UV and RA both abolish expression of *xPitx1* during gastrulation and neurulation. Conversely, anteriorizing treatment using lithium chloride enhances expression of *xPitx1*. Not only do the expression patterns of *xPitx1* suggest a role for the gene in the regulation of head development, but mutated *Pitx* gene family members have been associated with anomalous craniofacial development in humans (Semia *et al.*, 1998; Semina *et al.*, 1996). There is also a possibility that PITX1 is linked to Treacher Collins Syndrome (Crawford *et al.*, 1997). We sought to test the effects of enhanced and inhibited activity of this gene during craniofacial development in frogs. Paradoxically, ectopic expression appears at first glance to have contradictory effects: injected embryos often suffer axial truncations, particularly in the face and head, whereas *xPitx1* injection of RA and UV treated embryos appears to achieve quite the opposite. In this latter case axial deficits in the head are partially rescued.

**Over-expression of *xPitx1***

Over-expression of *xPitx1* induces the formation of enlarged or ectopic cement glands and often results in embryos with posterior defects in which trunk and tail structures could be severely reduced (Fig 4A-E). The most severe phenotypes display the appearance of an embryo transformed into a cement gland-like structure with both severe
head and tail deformities (Fig 4C-iv). Occasionally during gastrulation in ectopically expressing embryos, there is anomalous blastopore closure reminiscent of defects seen in experiments where \(xOtx2\) has been over-expressed (Blitz and Cho, 1995; Pannese et al., 1995). The mildest phenotypes were seen following injection with low quantities of \(xPitx1\) mRNA (60-120 pg): while enlarged or ectopic cement glands head phenotypes appear subtle, some deformities arose where the mandibular region appeared enlarged relative to forehead structures. The variability of effect seen when ectopic \(xPitx1\) perturbs marker gene expression likely reflects variability in synthetic mRNA dispersion and local concentration. Indeed, when we monitored injection dispersal with co-injected FITC-dextran or \textit{in vitro} transcribed mRNA for GFP, distribution of fluorescent material always correlated well with the \(xPitx1\) induced phenotype.

When \textit{in vitro} transcribed capped \(xPitx1\) mRNA was injected into either the left or right side of a 2-cell embryo, we were able to monitor the effects that ectopic expression had upon endogenous gene products such as the transcripts from \(xOtx2\), \(xPax6\), and \(xTwist\), \(XCG-1\), and \textit{neural\(\beta\)-tubulin}. When \(xPitx1\) is introduced, cement gland marker gene expression increases, while markers for other head regions decline. For example, \(xOtx2\), a gene associated with cement gland development increased expression when that organ was forming ectopically, while \(xOtx2\) expression in eye and brain, and \(Pax6\), \(xTwist\), and \textit{neural\(\beta\)-tubulin} expression domains were dissipated or abrogated. Possibly, ectopic \(xPitx1\) recruits cells from the presumptive ectoderm/neurectodermal margin to form cement gland, and the axial and craniofacial defects which arise are due to an insufficient supply of competent precursor cells. A common phenotype which was consistent with this interpretation occurred when embryos reflexed inwards towards the
injection site as if less tissue was being distributed axially on that side, and more was being co-opted to the formation of cement gland(s). *XPitx1* appears to induce cement gland directly: Neural markers such as *neuralβ-tubulin* were not found in association with either enlarged or ectopic cement glands. However, in cases where *XPitx1* induced ectopic cement glands, ectopic expression of *xOtx2* was induced. This is consistent with a role for *XPitx1* acting upstream of *xOtx2* assuming that one or the other is not phenocopying the cement gland induction pathway under the artificial conditions of ectopic expression assays. With regard to this last caveat, in our hands *XPitx1* is a more potent inducer of cement gland than either *xOtx2* or *xOtx5*.

Alternatively, ectopic *XPitx1* homeoprotein might bind factors or sites normally bound by other *bicoid*-related transcription factors such as *goosecoid* or members of the *xOtx* family. It is known that the Pitx proteins interact directly with certain basic helix-loop-helix factors to activate gene transcription in a manner distinct from the Otx and goosecoid proteins (Poulin et al., 2000). The reverse might also be true, in which case ectopic *XPitx* protein could be binding cognate sequence without appropriate heterodimeric partners thereby acting as a dominant negative during head development. As a consequence, *Pax, twist, Otx*, and *neuralβ-tubulin* expression domains might be perturbed.

Posterior defects are relatively common, and often entail a truncation of tail structures, however supernumerary axes can also form. These axes stain positively with probe for *neuralβ-tubulin*, and arise posterior to an ectopic cement gland on the body flank. We cannot determine on the basis of these experiments if the presence of a *XPitx1*-induced ectopic cement gland is causative, or whether a supernumerary axis is generated.
as a secondary consequence of the aforementioned gastrulation anomalies. Possibly this consequence of overexpression indicates a prominent role for xPitx during gastrulation.

**Over-expression in axially impaired embryos preserves some dorso-anterior attributes**

Since xPitx1 apparently has the capacity to direct development of the cement gland and mandible, and since the endogenous gene appears to be down-regulated in dorso-anteriorly impaired embryos (UV irradiated or RA treated), we decided to test the efficacy of xPitx1 to alter ventralized phenotypes. Rescue experiments with ectopic xPitx1 in UV-treated embryos did not result in the complete rescue of dorsal axis, however they did prohibit the formation of severely vegetalized DAI 0 embryos. Another, more infrequent phenotype was hard to interpret: 3-6% of injected DAI 0-2 embryos possessed eyes and cement glands. This phenotype might represent either DAI 3-4 embryos which have been axially retarded by xPitx1, or DAI 0-2 embryos which have acquired anterior attributes. Nonetheless, the relative promotion of dorsal attributes by xPitx1 is unambiguous given the diminished proportion of DAI 0 embryos observed in xPitx1 injected cohorts. This corroborates similar findings seen in UV-treated embryos administered xOtx2 transcript (Pannese et al., 1995).

Retinoic acid inhibits the development of structures anterior to the hind-brain (Durston et al, 1989) and rescue experiments with over-expressed xPitx1 in RA-treated embryos also failed to fully rescue head structures. Remarkably, although head structures could not be fully rescued, xPitx1 over-expression nevertheless induced the formation of cement glands and even of eyes and other recognizable anterior head elements in 75% of
embryos where none might otherwise have been expected. At the very least, data observed with UV and RA rescue experiments implicates a direct role for \textit{xPitx1} in initiating the pathway towards cement gland formation, and possibly in patterning of the head.

It is noteworthy that cement gland normally develops in a mesoderm-free zone (Hausen and Riebesell, 1991). The developmental inhibition of craniofacial regions in the context of enlarged cement glands suggests one of two possibilities. Firstly, since \textit{Pitx1} enjoys a period of expression in the cement gland anlage, ectopic transcript might render presumptive head ectoderm insensitive to mesodermal cues thereby expanding the domain of naïve ectoderm which perceives an anterior mesoderm-free zone. The result of this impaired competence might be that fewer cells differentiate into neurectoderm, and consequently that the inductive interactions which normally occur between neurectoderm, neural crest, mesoderm, and ectoderm then fall into disarray. This explanation seems unlikely, however, since the partial rescue of anterior attributes in RA and UV treated embryos is at odds with the inhibitory activity that ectopic \textit{xPitx1} demonstrates upon head marker gene expression in 1 and 2-cell injected axially “intact” embryos.

Alternatively, \textit{xPitx1} might normally express in the presumptive cement gland ectoderm subsequent to induction by direct contact with underlying endoderm. Consistent with this interpretation is the finding that the dorso-anterior endoderm possesses a potent cement gland-inducing activity at mid-gastrulation (Bradley \textit{et al.}, 1996). Possibly ectopic \textit{xPitx1} mimics a late step in this induction pathway with the result that cement gland forms where it should not, and often at the expense of structures which require
greater participation from the diverted ectoderm. This explanation might also satisfy the paradox embodied in the head-preserving function of xPitx1 in axially truncated embryos. Early stage UV irradiation or retinoic acid treatment will have repressed the steps necessary to normal anterior dorsal development, whereas ectopic xPitx1, a later participant in the induction pathway, could overcome this patterning deficit and elicit a partial recovery by acting upon still-competent anterior ectoderm. It is worth noting that both heart and craniofacial development likely require the participation of pharyngeal endoderm, and both regions are adversely affected by retinoic acid treatment. Furthermore, the endoderm of the second, third, and fourth pharyngeal arches requires a careful modulation of retinoid signal pathways in order for neural crest and mesoderm to pattern normally in mice (Wendling et al., 2000). Finally, one might speculate that intercalary regulation would ensue where an xPitx1-induced cement gland forms in close juxtaposition to an abnormally posterior (i.e.; hindbrain-level RA-truncated) tissue. If the response were intercalary, moreover, the ectopic axis which occasionally arises behind flank-situated cement glands in xPitx1-injected embryos should manifest signs of other head structures. This may occur in some instances (Fig. 7C and D). If intercalary regulation occurs in xPitx1 injected/RA-treated embryos or behind flank-localized cement gland tissues, then either this form of regulation must supercede the inhibitory effects of xPitx1 extant in head, or flank and hindbrain-level tissues must possess the capacity to locally suppress the effects of ectopic xPitx1. For example, xPitx1 might induce Otx2 in flank and posterior head and then exert a patterning effect because heterodimeric partners for one or the other exist there and not in more anterior regions.
Within this context we note that ectopic \(xOtx2\) elicits a secondary axis at low frequency (4%) (Pannese et al., 1995).

**Over-expression in animal cap explants supports a direct role for \(xPitx1\) in cement gland differentiation**

To study possible direct effects of \(xPitx1\) in specifying cement gland, animal caps of injected embryos were removed at stage 9 and cultured. Ectodermal caps which over-express \(xPitx1\) develop a pigmented sticky patch of cells which resembles a cement gland. Hybridization of animal caps with the cement gland-specific marker \(XCG-1\) confirmed that it was indeed a cement gland and that \(xPitx1\) along with \(xOtx2\) and \(xOtx5\) may be upstream regulators of \(XCG-1\). The relationship between \(xPitx1\) and the \(xOtx\) genes is unknown, however both factors induce ectopic cement glands. As in \(xPitx\)-injected embryos, injected animal cap explants differentiate cement gland in the absence of differentiated neural tissue. This confirms the hypothesis that \(xPitx\) acts upon ectoderm to specify and differentiate cement gland in a direct manner.

Although \(xPitx1\) induces ectopic cement glands, it is clearly not sufficient to induce formation of a complete head. The extent to which the gene is required for other aspects of anterior development remains to be defined, however it seems clear that the context necessary to support cement gland formation also actively precludes specification to other anterior phenotypes in the head of otherwise unperturbed embryos.
CHAPTER THREE

Overexpression of an antagonistic xPitx1 results in head malformations suggestive of a role for gene in anterior specification

Chapter Summary

We report here that the development of the cement gland requires the DNA binding activity of xPitx1. Antagonization of xPitx1 during early development affects the proper patterning of the cement gland and head during early development in Xenopus laevis. Overexpression of a mutant form of xPitx1, namely xPitx1-δPL, in which DNA binding activity is impaired results in the down-regulation of the anterior markers xOtx2, xPax6, xTwist, and XCG-1. Furthermore, defects do not appear to be directly related to dimerization with endogenous xPitx1 or a related member xOtx5, since preliminary assays on animal caps revealed that xPitx1-δPL does not impair their function. We speculate that xPitx1 may be involved in specifying anterior head regions and that high concentrations of xPitx1-δPL may inhibit embryo development by titering essential transcription co-factors in the developing head.

Introduction

Transcription factors regulate the expression of proteins by binding to specific DNA binding elements and/or by interacting with other transcription factors by heterodimerization. The proper regulation of transcription factors requires a complex network of interactions. During embryogenesis, vertebrate body patterning requires cell-specific transcription to activate, then to specify, and finally to induce differentiation.
The homeobox transcription factors are more promiscuous in recognizing the consensus DNA sequence ATTA than other transcription factors (Mann, 1995). This is likely due to the high conservation of regions encoding the DNA binding domains. Therefore, it is the temporal and spatial restriction of expression as well as the interactions which occur between transcription factors and cofactors within a given context which lend these factors their specificity.

Our study focuses on the Pitx family of transcription factors. Pitx1 was first isolated based upon its ability to activate transcription of the pro-opiomelanocortin (POMC) gene (Lamonerie et al., 1996). Pitx1 has been shown to activate genes in a cell-specific manner during pituitary development and function by interacting with factors such as Pit1, NeuroD1/Pan1, SF-1, and Egr1 (Szeto et al., 1996; Poulin et al., 1997; Tremblay et al., 1998; Tremblay and Drouin, 1999). More recently, Pitx1 has been shown to physically interact with SF-1 (Tremblay et al., 1999) and Pan1 (Poulin et al., 2000). Interactions with helix-loop-helix factors such as Pan1 suggest an important role for Pitx1 in regulating transcription during early development.

One way of interfering with transcriptional regulation is to generate mutations within the DNA binding domain of a transcription factor that will inhibit DNA binding activity while retaining the protein's ability to bind its homo/heterodimeric and/or accessory partners. Several groups have utilized this strategy to perform loss-of-function assays. Inhibitory effects have been observed with several homeobox genes when a critical leucine residue between the second and third helix of the homeodomain is mutated to a proline (Grow and Krieg, 1998; Mead et al., 1996). We report here that a similar mutation within the homeodomain of xPitx1 is capable of inhibiting cement gland
formation as well as of eliciting head deformities. The resulting head malformations likely indicate that endogenous Pitx1 interacts with other anteriorly-specified gene products.

Materials and Methods

Embryos and Whole-mount in situ hybridization

Embryos were fertilized, dejellied in 2% cysteine and cultured as previously described (Drysdale and Elinson, 1991). Developmental staging was according to Nieuwkoop and Faber (1967). Whole-mount in situ hybridization was performed according to Harland (Harland, 1991). RNA probes were made from linearized cDNA of the 5’, 3’, and coding regions respectively of xPitx1. Probes were labeled with digoxigenin (Roche) and revealed using antibodies conjugated to alkaline phosphatase. Additional riboprobes for XCG-1, xOtx2, xPax6, and xTwist were synthesized in similar fashion.

Constructs and Injection of xPitx-8PL

Vent polymerase and the reverse-strand primer 5’-CCTGGCTTCAGTTGATTTGTCCATACAGCAATC- 3’ were employed to derive a construct that contained two mutations, one of which was silent but removed an AvaII site for diagnostic purposes. The second mutation altered the codon for amino acid residue 117 from one encoding a leucine to a proline. Constructs were verified by nucleotide sequence analysis (York University, Canada) and sequence analyses were performed using Lasergene (DNASTAR, fifth edition, Madison, Wisconsin).
Chapter Three

Synthetic capped mRNA of *xPitx1* was made from linearized template using mMessage Machine (Ambion) driven by a SP6 promoter. Capped mRNA was resuspended in water and injected into embryos with a Drummond nanoinjector. Injections were made into the animal pole of embryos at either the 1-cell or 2-cell stages. Concentrations of the capped mRNA injected ranged from 300 pg to 1.2 ng. Injection volumes never exceeded 9.2 nl. Injected embryos were cultured in 0.1 X MMR, 50ug/ml gentamicin, and 2% Ficoll-400 (Sigma) at 13 °C for at least 1 hr to allow healing before being removed and allowed to develop at room temperature. At stage 10-10.5 the solution was changed to 0.1 X MMR supplemented with 50ug/ml gentamicin.

**Ectodermal cap culture**

Stage 8-9 embryos were removed to 1 X MMR containing 50ug/ml gentamicin and 2% ficol. Ectodermal explants were removed and cultured overnight at room temperature in Petri dishes the bottom of which had been coated with a thin layer of 1% agarose in 1X MMR. Explants were then removed to 0.1X MMR and cultivated until they reached the stage at which sibling intact control embryos had developed cement glands (stage 25 or later). Explants were then fixed and processed for *in situ* hybridization as described.

**Results**

**XPitx1 antagonist**

Site directed mutagenesis was performed via Polymerase Chain Reaction (PCR) which mutated the sequence encoding a crucial leucine residue in the homeodomain of *xPitx1*. The mutation was confirmed by sequence analysis. Figure 1 shows the amino
FIG. 1. Amino acid comparison of the wild-type $xPitx1$ to that of the mutant construct, $xPitx1-\delta PL$. The highly conserved leucine residue in the homeodomain (blue) has been substituted with a proline (red).
Wt Ptx1 MDSFKGAMNLERL PESLRPQPS HDMA TSFH LQRSSE ARDP MDNSA SESS
PL Ptx1 MDSFKGAMNLERL PESLRPQPS HDMA TSFH LQRSSE ARDP MDNSA SESS

Helix 1
Wt Ptx1 DTEIAEKERTGEPKGEDNGDDPSKKKKQRRQRTHTSQQLQELEATFQ
PL Ptx1 DTEIAEKERTGEPKGEDNGDDPSKKKKQRRQRTHTSQQLQELEATFQ

Helix 2
Wt Ptx1 RNRYPDMSMREELIAVWTNLTEARVRVWFKNRRAKWRKRERNQQMDLCKN
PL Ptx1 RNRYPDMSMREELIAVWNPTEARVRVWFKNRRAKWRKRERNQQMDLCKN

Helix 3
Wt Ptx1 GYVPQFSGLMQPYDEMYAGXYPNNXXTKSLTPAPLSTKSFTFFNSMSPL
PL Ptx1 GYVPQFSGLMQPYDEMYAGYPNNWATKSLTPAPLSTKSFTFFNSMSPL

Wt Ptx1 SSQSMFSGPSSISSMSMPSMGSAVPMANSSLNNINLNNISGSSLN
PL Ptx1 SSQSMFSGPSSISSMSMPSMGSAVPMANSSLNNINLNNISGSSLN

Wt Ptx1 SAMSSTGCPYGPPGSPXTVYRDTCNSSLASRLKSKQHSXFGXSSLQSP
PL Ptx1 SAMSSTGCPYGPPGSPYT VYRDTCNSSLASRLKSKQHSTFGYSSLQSP

Wt Ptx1 ASSLNACQYN
PL Ptx1 ASSLNACQYN
acid alignment between the wild-type sequence and the mutant form. The homeodomain is highlighted in blue and the mutation is in red.

Inhibition of xPitx1

The phenotypes observed following over-expression of xPitx1 in Xenopus embryos appear to be dose-dependent. At higher doses of injected wildtype xPitx1 RNA, it is possible to induce ectopic development of cement glands, whereas low doses appear to affect head development by augmenting the lower mandibular regions at the expense of other more dorsal and posterior structures. This led us to ponder the importance of xPitx1 in cement gland development and whether its immediate downstream targets were also crucial for proper head development. We sought to test whether inhibition of xPitx1 activity has an inhibitory effect upon cement gland formation. Substitution of a proline for the conserved leucine in the hinge region between helices two and three of the homeodomain has been shown to have inhibitory effects upon the DNA binding activity of homeoproteins (Grow and Krieg, 1998; Mead, 1996). We employed a similar mutant derived from xPitx1, xPitx1-ΔPL, to test inhibition at concentrations ranging from 300pg to 1.2ng. Injected embryos were cultured to stage 25-26 and observed (Fig 2). Compared to controls (Fig. 2A), a proportion of the injected embryos developed reduced or inhibited cement glands and heads (Figs 2B, C, D; Table 1). Several of the embryos exhibiting defects at stage 25-26 were reared to later stages. By stage 36, embryos that lack a cement gland nevertheless appear to contain other head structures such as eyes, however, in some cases where head anomalies are severe, embryos fail to form any recognizably anterior anatomical features. In addition to the two extreme phenotypes
Table 1. Percentage of phenotypes observed in embryos injected with xPttx1-Δ PL.

<table>
<thead>
<tr>
<th>RNA injected (pg)</th>
<th>N</th>
<th>site</th>
<th>Normal</th>
<th>Extra or Enlarged Cement Glands</th>
<th>Head Deformities</th>
<th>Other deformities</th>
</tr>
</thead>
<tbody>
<tr>
<td>1200</td>
<td>50</td>
<td>animal</td>
<td>64%</td>
<td>0%</td>
<td>16% total</td>
<td>20% (axial, trunk and tail)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(4% suppressed head)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(6% head truncation)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(6% absent cement gland)</td>
<td></td>
</tr>
<tr>
<td>600</td>
<td>85</td>
<td>animal</td>
<td>79%</td>
<td>0%</td>
<td>12% total</td>
<td>11% (axial, trunk and tail)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(4% suppressed head)</td>
<td>1% (duplication of axis)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(4% head truncation)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(4% absent cement gland)</td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>99</td>
<td>animal</td>
<td>66%</td>
<td>0%</td>
<td>12% total</td>
<td>10% (axial, trunk and tail)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(4% suppressed head)</td>
<td>1% (duplication of axis)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(6% head truncation)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(2% absent cement gland)</td>
<td></td>
</tr>
<tr>
<td>uninjected</td>
<td>95</td>
<td>N/A</td>
<td>98%</td>
<td>0%</td>
<td>2% total</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(1% extended forehead)</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(1% head truncation)</td>
<td></td>
</tr>
<tr>
<td>300 pg ΔPL +300 pg WT</td>
<td>183</td>
<td>animal</td>
<td>-</td>
<td>27%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Other deformities are similar to those shown in wild-type xPttx1 mis-expression. Refer to figure 6 for phenotypes regarding head deformities. *Other phenotypes such bent axes, trunk and tail, and head deformities were observed at high frequency that may have been due to additive effects of both transcripts (these phenotypes were not scored).
FIG. 2. Antagonization of *xPitx1* by *xPitx1-δPL* results in variable head malformations in whole embryos. (A-B, D) Extreme head deformities are observed where larvae develop without heads. (C) At a lower frequency, head development occurs in the absence of the cement gland. (E-H) Variable head size development in *xPitx1-δPL* injected embryos, (E) control, (F-G) reduced head size, and (H) absence of noticeable head structures. (I-I') Co-injection of *xPitx1-δPL* with Dextran-FITC show a possible correlation between the distribution lineage marker and capped mRNA.
(absent cement glands or complete head suppression), intermediate phenotypes are observed where head and cement gland size also varied from xPitx1-δPL injected embryos. These larvae display a reduction in head size while retaining a normal trunk. Figure 2E displays the size of a normal head and cement gland and variable head and cement gland size are shown in Figure 2F and G. Ultimately, extreme cases demonstrate an absence of cement gland (Fig. 2H).

Since the response to mutant mRNA was variable for a given injection concentration, xPitx1-δPL was coinjected in some instances with a FITC-dextran lineage marker (Fig 9I), and in others with mRNA for green fluorescent protein (GFP). Cement gland size varies in proportion to intensity and distribution of the lineage markers. Presumably, the lineage markers reflect the variability of dispersion of injected mutant mRNA. The varied craniofacial phenotypes may be attributed to the uneven distribution of the injected mutant RNA encoding xPitx1-δPL. In figure 2I, both embryos were injected with the same concentration of xPitx1-δPL plus a lineage marker, however the cement gland size as well as the head appear diminished in the lower of the two embryos. In the top embryo, lineage tracer did not distribute evenly to the head tissue, presumably reflecting a lack of inhibitory mRNA in that region where a more normal progression of head development ensued.

xPitx1-δPL overexpression perturbs anterior markers

To study the changes in anterior development caused by the over-expression of xPitx1-δPL, whole mount in situ hybridizations with XCG-1, xOtx2, xPax6, and xTwist were performed. Embryos at the 2-cell stage were injected with 600pg of xPitx1-δPL
into one blastomere with GFP capped mRNA as a lineage marker. Embryos exhibiting unilateral GFP expression in the head were selected for in situ hybridization. At stage 18, the cement gland marker XCG-I expression is reduced 16% (n=43) of the time on the side of injection (Fig. 3A). Furthermore, figure 3B reveals a reduction of the eye and brain marker xPax6 (black arrow) expression (27%, n=22) on the side of injection. In addition to xPax6 expression, the morphological field of the cement gland is also reduced (Fig. 3C, white arrow). Perturbation of another neural crest marker xTwist was also observed at low frequency (14%, n=14) as shown in figure I (injected side). The control side (uninjected) resembled the wild-type expression of xTwist (Fig. I'). XOtx2 expression was assayed and a slight diminution (17%, n=17) was observed within the developing eye (Fig. 3D, arrow), however, expression within the brain region appeared normal as compared to the uninjected side.

**Specificity of xPitx1-δPL**

The specificity of xPitx1-δPL was determined by animal cap assays. Injecting xPitx1-δPL RNA into animal caps in a number of experiments did not induce the formation of cement glands (Fig. 4A). The absence of cement gland tissue was confirmed morphologically and through whole mount in situ hybridization demonstrating the absence of the cement gland marker XCG-I (Fig. 4B). However, the most recent animal cap assay data set presented in Table 2 revealed a small number of animal caps developing cement glands. The most likely explanation may be the result of contaminating mesoderm or possibly endoderm during microdissection which possess some of the signals capable of inducing cement gland. One of the major technical issues
FIG. 3. Perturbations of anterior markers by $xPitx1-\delta PL$ injection. $xPitx1-\delta PL$ was injected into one-blastomere of the two-cell embryo and assayed by whole mount \textit{in situ} hybridization. A and B show the perturbed expression of $XCG-1$ and $xPax6$, respectively (dashed line bisects midline). At stage 28, expression of $xTwist$ (C) and $xOtx2$ (D) are diminished compared to the uninjected side (C' and D').
associated with animal cap experiments is the ability to isolate naïve ectoderm without contaminating tissue resulting in false positives. Therefore, data analysis of animal cap experiments may sometimes be difficult.

In order to determine a possible role for the inhibitory effects of \( xPitx1-\delta PL \) rescue experiments were performed. Co-injection experiments in animal caps and whole embryos were utilized to determine the specificity of \( xPitx1-\delta PL \). Does \( xPitx1-\delta PL \) have an inhibitory effect upon endogenous \( xPitx1 \) activity? Varied concentrations of wild-type \( Pitx1 \) were co-injected with a standard concentration of \( xPitx1-\delta PL \). Injected animal caps were cultured overnight and assayed for cement gland induction based on morphology. At all concentrations (300, 600, and 1200 pg) of wild-type \( Pitx1 \), 1200 pg of \( xPitx1-\delta PL \) failed to antagonize wild-type \( Pitx1 \) in animal cap assays: cement gland induction was observed with all concentrations and combinations (Fig. 4C-E; Table 2, Expt #1). When one compares animal caps injected with 600 pg of wild-type \( xPitx1 \) with those co-injected with both 600 pg of wild-type \( xPitx1 \) and 1200 pg of \( xPitx1-\delta PL \), there is no significant difference. Increasing the concentration of wild-type \( xPitx1 \) from 600 pg to 1200 pg dramatically increased the percentage of cement glands induced from 69% to 90% indicating the inability of \( xPitx1-\delta PL \) to inhibit \( xPitx1 \). Furthermore, it suggests that localization and diffusion of synthetic RNA transcript is another factor associated with variable induction. Conversely, decreasing the concentration of \( xPitx1-\delta PL \) did not increase the ability of wild-type \( xPitx1 \) to induce cement glands in caps (Table 2, Expt #2). Alternatively, we assessed the ability of \( xPitx1-\delta PL \) to antagonize the function of wild-type \( xPitx \) to induce enlarged and ectopic cement glands in whole embryos. Co-injection experiments utilizing wild-type \( xPitx1 \) and \( xPitx1-\delta PL \) at a 1:1 ratio were
Table 2. Co-injection of *xPitx1-dPL* with wild-type *Pitx1* and other related factors into animal caps to determine specificity of *Pitx1* function.

<table>
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<tr>
<th>RNA injected</th>
<th>N</th>
<th>Percent Cement Gland Induction</th>
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<tr>
<td>Control</td>
<td>26</td>
<td>0%</td>
</tr>
<tr>
<td>Wild-type 600 pg</td>
<td>11</td>
<td>64%</td>
</tr>
<tr>
<td>xPitx1-dPL 600 pg</td>
<td>24</td>
<td>13%</td>
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<tr>
<td><strong>Experiment #1</strong></td>
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<td></td>
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<tr>
<td>Wild-type:*xPitx1-dPL 300:1200 pg</td>
<td>9</td>
<td>56%</td>
</tr>
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<td>69%</td>
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<tr>
<td><strong>Experiment #2</strong></td>
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<tr>
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<td>28</td>
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</tr>
<tr>
<td>Otx2 600 pg</td>
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<td>Otx2:*xPitx1-dPL 600:1200 pg</td>
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FIG. 4. *XPitxl-δPL* is unable to fully inhibit the ability of wild-type *XPitxl* to induce cement gland formation in animal caps. (A) Injection of *XPitxl-δPL* does not induce cement glands in animal caps. (B) *XCG-1* whole mount *in situ* hybridization confirmed the absence of cement gland induction. Cement gland induction (arrow) is present in animal caps co-injected of wild-type *XPitxl* and *XPitxl-δPL* at a 1:4 (C), 1:2 (D), and 1:1 (E) ratio, respectively.
performed in whole embryos. Although head and trunk defects were ultimately observed, the number of enlarged and supernumerary cement glands were reduced. Wild-type \textit{xPitx1} injection of 600 pg results in approximately 57\% (Table 1 in Chapter 2) of the embryos growing enlarged and supernumerary cement glands however, if co-injected with \textit{xPitx1-δPL}, this portion is reduced to 27\% (Table 1). This suggests that cement gland formation \textit{in vivo} is highly regulated by other factors with which \textit{xPitx1-δPL} may be interfering.

In light of the above data, we sought to test other related members involved in cement gland formation, namely \textit{xOtx2} and \textit{xOtx5}. \textit{XOtx5} co-injected into animal caps with \textit{xPitx1-δPL} showed no diminution in frequency of cement gland formation compared to injection of \textit{xOtx5} alone (Table 2, Expt. #2). Surprisingly, \textit{xOtx2} alone did not induce cement glands in animal caps hence, we could not evaluate the ability of \textit{xPitx1-δPL} to affects its function.

**Discussion**

**Inhibition of cement gland and head by \textit{xPitx1-δPL}**

To better elucidate \textit{xPitx1} function, the DNA binding domain of \textit{xPitx1 (Pitx1-δPL)} was mutated: a highly conserved leucine in the hinge domain between homeodomain helices two and three was switched to a proline residue. This substitution has been shown by others to inhibit the DNA binding activity of several homeobox genes without inhibiting the ability of the gene to bind to itself or to other proteins (Grow and Krieg, 1998; Mead \textit{et. al.}, 1996). Injection of \textit{xPitx1-δPL} into the 1-cell embryo in either the animal or vegetal pole results in a variety of phenotypes ranging from head
truncations to minor head deformities where cement gland and head structures are diminished in size. Coinjection of \textit{xPitx1-δPL} with a lineage marker revealed that the varied phenotypes observed were likely the cause of uneven distribution of \textit{xPitx1-δPL} transcript in head tissue. With specific regard to the formation of cement gland, \textit{xPitx1-δPL} acts as a dominant negative mutant, and this is supported by the ability that the construct has to effectively decrease the ability of co-injected wildtype \textit{xPitx} to induce ectopic or enlarged cement glands. Injection of \textit{xPitx1-δPL} mRNA into 2-cell embryos confirmed at a molecular level what was apparent at an anatomical level. Namely, that unilateral injections at the 2-cell stage have a local inhibitory effect upon cement gland-associated markers such as \textit{xOtx2} and \textit{XCG}, while simultaneously depressing \textit{xPax6} and \textit{xTwist}. While depression of cement gland development is expected, the depressed expression of \textit{xPax6} and \textit{xTwist} is not: this latter was also the effect of ectopic expression of wild-type transcript.

The observed effects in \textit{xPitx1-δPL}'s ability to attenuate wild-type \textit{xPitx1} are not based on its capacity to titrate wild-type \textit{xPitx1}. This is supported by observations that \textit{xPitx1-δPL} is not capable of inhibiting wild-type \textit{xPitx1} in animal cap assays: other factors must be involved in cement gland induction and must heterodimerize with \textit{xPitx1} in order to facilitate activation of target genes in whole embryos despite the ability of \textit{xPitx1} to bind to its cognate sequence and to activate cement gland formation in animal caps. These results suggest two features of \textit{xPitx1} activity: \textit{xPitx1} does not form homodimers with itself to activate cement gland induction and other factors are involved in cement gland development but are not necessarily \textit{required} to direct cement gland formation.
How then does xPitx1-δPL affect head development? While it appears that proper cement gland formation may require the DNA binding activity of xPitx1, interpretation of the head phenotypes derived in whole embryos suggest that in all xPitx1 mRNA-injected embryos, ectopically translated protein might exert its effect indirectly by interacting with (and impairing normal function of) other proteins which are capable of heterodimerizing. Therefore, head defects observed with xPitx1-δPL overexpression may be attributed to ectopic xPitx1 heterodimerization with other cofactors that are needed to activate or repress head specific genes. Presumably, DNA binding activity is not necessary for xPitx1 to mediate these other effects upon craniofacial development, and heterodimeric partners are playing a substantial role in the patterning of these domains.

**Specificity of xPitx1-δPL**

What are the heterodimeric proteins involved with xPitx1 in patterning the head? xPitx1-δPL does not appear to inhibit cement gland development by interfering with endogenous Pitx1 (discussed above) or with other paired-like genes such as xOtx5. The ability of xPitx1-δPL to inhibit xOtx2 was also tested but could not be determined due to inability of xOtx2 to induce cement glands in animal caps in our hands. Other possible partners that xPitx1-δPL maybe removing from active circulation, which we did not test, are xPitx2 or xPitx3. However, xPitx3 is not expressed in the cement gland and its overexpression does not result in ectopic cement glands (unpublished observation). xPitx2 is also expressed in the cement gland but earlier reports of its overexpression did not describe an ability in *Xenopus* to induce ectopic cement glands (Campione et al., 1999; Ryan et al., 1998). More recently however, xPitx2 overexpression has been shown
to induce the development of ectopic cement glands (Schweickert et al., unpublished). In this report, histological preparations did not reveal patterns identical to wholemounts raising the possibility that these investigators have mixed probes. In addition to its function in cement gland induction, Schweickert et al. (unpublished) showed that coinjection of both \( xPitx1 \) and \( xPitx2 \) into animal caps revealed, by RT-PCR, a down-regulation of anterior and cement gland markers. From the apparent down regulation of \( XCG, xPitx2 \) may be a likely candidate to attribute the resulting head deformities induced by \( xPitx1-\delta PL \) injections, since both wild-type forms inhibit each other's function. Finally, given the apparent link between cement gland size and degree of subsequent head development, it is tempting to speculate that this terminal-most structure plays a critical polarizing and growth stimulatory role during craniofacial morphogenesis.
CHAPTER FOUR
GENERAL DISCUSSION

Chapter Summary

Several lines of evidence suggest that anterior patterning may be directed by an agency separate from that of the organizer region. Inhibitory assays in *Xenopus* suggest an important role for the cement gland during head development, since inhibition of this structure often leads to perturbation of other head structures. In this chapter, we speculate that the proper patterning of the cement gland is crucial for proper head development and that the anterior organizing centre might require a feedback signal from the cement gland. Possible regions for this anterior signalling centre reside in the anterior endoderm, which has been shown to express genes associated with head development.

Discussion

The cement gland is the most anterior structure to develop during *Xenopus* embryogenesis. Not only does it function to adhere the developing larvae to its environmental substrates, but at an anatomical level it serves as a boundary for dorsal/ventral and anterior/posterior patterning. Understanding the mechanisms by which the cement gland is patterned may provide us with insights into axis formation and head patterning. The cement gland is derived from ectodermal tissue at the most dorsal anterior region of the embryo and can be identified by several molecular markers. It is considered the first organ to develop during embryogenesis. Studies have shown that this organ is regulated by at least three different regions of the embryo such as the deep ectoderm,
prechordal mesoderm, and anterior endoderm (Sive and Bradley, 1996). Positive signals from these regions appear to induce the formation of the cement gland, while negative signals arise from ventral ectoderm to restrict cement gland formation (Bradley et al., 1996). Thus far, several genes have been isolated in Xenopus which play a putative role in specifying cement gland. Through gain of function experiments, several genes have been implicated in cement gland formation, xOtx2 (Blitz and Cho, 1995; Pannese et al., 1995), xOtx5 (Kuroda et al., 2000), xOtx5b (Vignali et al., 2000), XAG-2 (Aberger et al., 1998), xpitx1 (Chang et al., unpublished; Schweickert et al., unpublished), and xpitx2 (Schweickert et al., unpublished). Under certain circumstances all of these genes induce ectopic cement glands in embryos which are accompanied by the expression of a cement gland marker XCG-1. An interesting observation made by Gammill and Sive (1997) was that xOtx2 could only induce cement gland formation in a ventrolateral permissive region of the embryo. The authors proposed that the ectodermal tissue in which the cement gland derives must manifest both dorsal and ventral character. If xOtx2 provides the dorsal character, then what provides the ventral character needed for cement gland formation? One candidate they show may be BMP4 whose expression overlaps that of xOtx2 (Gammill and Sive, 2000). However, BMP4 is a potent inhibitor of cement gland induction (Fainsod et al., 1994; Schmidt et al., 1995). More recently, induction of cement gland appears to require the cooperation of both xOtx2 and BMP4. BMP4 signalling is capable of modulating xOtx2 activity (Gammill and Sive, 2000). One hypothesis, which Gammill and Sive (2000) propose is that there must be the presence of a BMP gradient and that high concentrations of BMP initiate an epidermal fate while, low doses contribute to cement gland identity. Ultimately, very low concentrations of BMPs
versus high doses of Otx results in a neuralizing fate. Figure 1 outlines the current view of cement gland induction proposed by Gammill and Sive (2000).

**Involvement of xPitx1 in cement gland and head formation**

Our studies show that cement gland formation requires another key player, the *Xenopus* homolog of Pitx1. Phylogenetic analysis confirms that xPitx1 is indeed the *Xenopus* homolog to previously described vertebrate genes (Fig. 2). It is most similar to that of chick Pitx1 according to a phylogenetic tree analysis. As described in chapter one, Pitx1 in other vertebrates tends to play an important function during anterior development as well during limb differentiation. From our expression studies, we show that as in other vertebrates, *Xenopus* Pitx1 is expressed during anterior development primarily in the cement gland, stomodeum, pituitary, derivatives of the buccopharyngeal region, and also later in the optic eminence and hindlimb (data not shown). Posteriorizing agents such as Retinoic acid (RA) and ultraviolet light (UV) down regulate xPitx1 and, moreover, its expression is increased by an anteriorizing agent, namely lithium chloride.

Gain-of-function experiments have revealed that xPitx1 is capable of inducing either enlarged or ectopic cement glands with concomitant expression of the cement gland marker XCG-1 in whole embryos and animal caps. Furthermore, cement gland induction relies on the ability of xPitx1 to bind to its DNA binding element. The induction of cement glands in naïve animal caps suggests that Pitx1 lies within the signalling pathway of cement gland induction with XCG as a possible downstream target. XCG has already been shown to be a direct downstream target of xOtx2 (Gammill and Sive, 1997). The relationship between xPitx1 and xOtx2 is not known, but both appear to
FIG. 1. Model proposed by Gammill and Sive (2000) outlines the involvement of $Otx2$ and BMP in cement gland induction. Simultaneous expression of $Otx2$ and BMP result in the induction of the cement gland while $Otx2$ and low expression of BMP signaling leads to neural induction. BMP signaling alone ultimately leads to the development of epidermal tissue.
FIG. 2. Phylogenetic analysis of the *xPitxl* amino acid sequence with other related *paired-like* genes. *XPitxl* (Red) clusters within the clade of other vertebrate *Pitxl* sequences.
induce cement glands. However, overexpression of either wild-type \textit{xPitx1} or the mutant form of \textit{Pitx1}, \textit{xPitx1-δPL}, leads to the down-regulation \textit{xOtx2} in the face region suggesting that \textit{xPitx1} likely inhibits the expression of \textit{Otx} genes. Moreover, down regulation is not limited to only \textit{xOtx2}; other genes expressed in the head such as \textit{xPax6} and \textit{xTwist} are both down-regulated. In contrast to the down-regulation of \textit{xOtx2} in the regions where it is normally highly expressed, \textit{Pitx1}-induced ectopic cement glands exhibited expression of \textit{xOtx2} surrounding the ectopic structure. From our studies, we know that \textit{xPitx1} does not directly interact with \textit{xOtx5} (nor likely \textit{Otx2}) because coinjection experiments revealed that \textit{xPitx1-δPL} did not alter its cement gland inducing ability in animal caps. One reason for the decrease in expression may be that \textit{xPitx1} and \textit{xPitx2} provide a possible negative feedback loop, whereby \textit{xOtx2} is down-regulated in certain regions (Schweickert et al., unpublished). However, these authors also observed the induction of \textit{xOtx2} in ectopically derived cement glands. It would seem that \textit{Otx2} is regulated at several different levels and our data is most consistent with the hypothesis that \textit{xPitx1} regulates \textit{xOtx2} and other genes in a context-specific manner of which is dependent upon the presence of heterodimerizing partners.

A possible reason for the down regulation of head genes is that \textit{xPitx1} has expanded the competence of anterior ectoderm to become cement gland at the expense of other head structures such as the eye and brain. Significantly, \textit{xPitx1} is capable of inducing head structures in severely posteriorized embryos treated with retinoic acid. Overexpression of \textit{xPitx1} in retinoic acid treated embryos gives rise to head structures such as eyes and cement glands in \textit{75\%} of the embryos where none might otherwise be expected to form. It may seem contradictory that overexpression of \textit{xPitx1} causes
enlarged cement glands at the expense of head structures while on the other it is capable of rescuing head structures in RA treated embryos. One possibility, mentioned earlier in Chapter 2, may be the result of an intercalary effect. A second possibility is that Pitx1 plays an important role in head development generally, however, its role in head development does not supercede its role in cement gland development. Its role in head development may be highly regulated through association with other head inducing factors. For example, the related member Otx2 can interact with Lim-1 through its homeodomain to enhance transcription of its target genes. Furthermore, Otx2 can also interact with HNF-3beta (forkhead transcription factor) through both the homeodomain and C-terminal to repress its target genes (Nakano et al., 2000). Evidence for the highly interactive role of Pitx1 comes from recent studies by other groups (Tremblay et al., 1998; Poulin et al., 2000) and our own study (refer to Chapter 3). Inhibition of the DNA binding activity of xPitx1 is unlikely to affect its ability to associate with other transcriptional partners either through its homeodomain or N and C-terminals. Overexpression of a mutant form of xPitx1, xPitx1-ΔPL, results in head malformations ranging from reduced head structures to head truncations. The antagonistic effects of xPitx1-ΔPL inhibit cement gland development but also suggest additional roles in head development. Another important feature presented by Schweickert et al. (unpublished) is that xPitx1 may autoregulate itself, therefore overexpression of xPitx1-ΔPL could ultimately be down-regulating endogenous xPitx1 by binding a specific co-factor required for xPitx1 auto-induction.

The simplest explanation for a reduction in head size could be explained by the down-regulation of Otx genes. In other words, if Otx genes are critical for specifying
head then its altered expression may lead to head malformations such as head reduction. Another possibility for the involvement of Pitx1 in head development may be related to interaction of the cement gland with the anterior endoderm. The anterior endoderm has gained more attention recently due to the discovery of several anterior specifying genes expressed in this region. Some of the genes discussed in Chapter One such as xHex and cerberus are involved in head induction (Jones et al., 1999; Bouwmeester et al., 1996). The anterior endoderm itself does not appear to be critical to head formation since ablation of this tissue does not result in head malformations (Schneider and Mercola, 1999). However, if the genes expressed in this region such as xHex are inhibited, head malformations occur (Brickman et al., 2000). Anterior endoderm may function to regulate the size of the head as opposed to organizing it. This might explain why overexpression of xPitx1-δPL induces the varying head sizes while the trunk and tail remain unaffected. That trunk and tail formation is not affected suggests that the areas where dorsal mesoderm is present are likely not affected. In other words, deficiency of mesodermal populations is not the cause contributing to head diminishment. Therefore the other region that may be affected is the anterior endoderm which is in direct contact with the cement gland anlage throughout gastrulation. The effects caused by xPitx1-δPL may both be cell autonomous and non-autonomous affecting ectoderm and endoderm, respectively.

It is tempting to take this one step further and suggest that the anterior visceral endoderm in mouse, which is believed to be analogous to the anterior endoderm in frog, may possibly play a similar role in regulating head size. Nonetheless, homozygous Otx2-/- mutants develop without heads, however, heterozygous mutants develop with
phenotypes reminiscent of otocephaly (Matsuo et al., 1995): a craniofacial syndrome characterized by a symmetrically deficient development of the first branchial arch (Shermak and Dufresne, 1996). A non-lethal form of otocephaly, Aglossia-Adactyilia is also characterized by severe diminishment of the mandible and head as well as limb deformities (Buttien and Fryns, 1986). One possible function of the anterior visceral endoderm could be to provide the embryo with developmental cues which communicate the extent of head growth rather than actually inducing head structures. Perhaps the resulting head truncations displayed in Otx2 -/- mouse embryos are attributed to a deficient molecular boundary. By eliminating a boundary cue by which the head can normally develop Otx2 null mutants are possibly rendered incapable of specifying head regions even though the head field has been produced by other factors.

Does size matter? Well, apparently in some cases it does. If there are indeed two separate signalling centres, then how do organisms keep the dimensions of the head and trunk proportionate to one another? Perhaps by understanding the molecular constraints within which the head develops, we may understand why the head is so susceptible to developmental and teratogenic effects. I propose a new model (Fig. 3) that incorporates and expands the previous models proposed by Gammill and Sive (2000) and Bradley et al. (1996) exhibiting a role for xPitx1 in the development of the cement gland. As previously proposed, cement gland induction requires signals from both mesoderm and endoderm (Bradley et al., 1996). In addition, Otx2 and a gradient of BMPs are involved in specifying ectoderm to become cement gland (Gammill and Sive, 2000). I propose that xPitx1 assists cement gland development within the ectodermal region. Moreover, proper patterning of head may be regulated by a feedback signal from the developing
FIG. 3. Cement gland and head patterning require *xPitx1*. Cement gland induction is directly related to *xPitx1* while we hypothesize that head induction requires the involvement of *xPitx1* with other co-factors. We speculate that head formation may require a feedback signal from the cement gland anlage to that of the anterior endoderm regulating head specification.
cement gland. Regulation of head development by *xPitx1* may be directed through the anterior endoderm, since the cement gland is in direct contact with the anterior endoderm for a period of time during gastrulation.

**Future Endeavors**

We have only begun to understand the developmental genes involved in cement gland specification. To our knowledge, we are the first to show that cement gland specification provides critical cues that affect head development. In order to gain a better idea as to the multiple functions of *Pitx1*, further experiments need to be performed. Since cement gland induction appears to require the binding of Pitx1 to its cognate sequence, one approach may be to out compete endogenous *Pitx1* with either the homeodomain alone or a fusion construct of *Pitx1* containing a strong repressor domain. The role that Pitx1 plays with other transcriptional partners may require more extensive studies utilizing techniques such as the yeast two-hybrid assay which may provide further insight into its role in head development.
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References Cited


References Cited


References Cited

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References Cited


**APPENDIX A**

Table 1. Primers used for sequence analysis of *Xenopus Pitx1*.

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FIG. 1. Position of oligos used for sequence analysis of *Xenopus Pitx1*.
VITA AUCTORIS

WING YEAN CHANG

Place of Birth: Windsor, Ontario, Canada  
Date of Birth: October 11, 1972  
Languages spoken: English and Cantonese  
Citizenship: Canadian

EDUCATION:

September, 2000  
Master of Science, Department of Biological Science, University of Windsor  
Thesis: Cloning and Characterization of the Xenopus laevis homolog xPitx1. Supervisor: Dr. Michael J. Crawford

June, 1998  
Honours Bachelor of Science (Molecular Biology Program), University of Toronto  
Thesis: High resolution mapping of transcriptionally active loci on Drosophila polytene chromosomes.  
Supervisor: Dr. J. Timothy Westwood

PUBLICATIONS:


ABSTRACTS/POSTERS:


OTHER PRESENTATIONS:


AWARDS AND SCHOLARSHIPS:

2000  David Berks Memorial Scholarship
2000  Conference Travel Award, University of Windsor
1999  Conference Travel Award, University of Windsor

TEACHING EXPERIENCE:

Sept.-Dec. 98  Teaching Assistant
   Course:  Animal Cells and Tissue (3rd Year Biology Course)
Jan.-Apr. 99  Teaching Assistant
   Course:  Cell Biology (1st Year Biology Course)
Sept.-Dec. 99  Teaching Assistant
   Course:  Animal Cells and Tissue (3rd Year Biology Course)
Jan.-Apr. 00  Teaching Assistant
   Course:  Cell Biology (1st Year Biology Course)

VOLUNTEER WORK:

1997  Volunteer in a research laboratory under the supervision of Dr. Paul Horgan, Botany, University of Toronto.

1995-1997  Volunteer at the Credit Valley Hospital, Mississauga, in the Emergency Department.

GENBANK SUBMISSIONS:

Accession AF217647
Accession AF265671