Early steps in *Xenopus* neural determination: Cloning and analysis of *opl*

by

John Shu-Shin Kuo

A.B. in Biology
Harvard College, 1989

SUBMITTED TO THE DEPARTMENT OF BIOLOGY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN BIOLOGY
AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

JUNE 1998

Signature of author:_____
Department of Biology
May 27, 1998

Certified by:_____
Hazel L. Sive
Associate Professor of Biology
Thesis Advisor

Accepted by:_____
Frank Solomon
Professor of Biology
Chair, Committee on Graduate Studies

Early steps in *Xenopus* neural determination: Cloning and analysis of *opl*

by
John Shu-Shin Kuo

Submitted to the Department of Biology on
May 27, 1998 in Partial Fulfillment of the
Requirements for the Degree of
Doctor of Philosophy in Biology

Abstract

The early molecular events in vertebrate neural development and the genes that mediate these events during embryogenesis are not well understood. To identify genes expressed during early neural patterning in *Xenopus*, we performed a PCR-based subtractive cloning screen using micro-dissected gastrula ectoderm. In a non-saturating screen, we isolated over forty genes that are highly expressed in dorsal ectoderm.. One very interesting neural gene isolated is *opl*, an early marker of the neurectoderm. *opl* resembles the *Drosophila* pair-rule gene *odd-paired* (*odd-paired*-like) and encodes a zinc finger protein that is a member of the vertebrate *zic* gene family. When gastrulation starts, *opl* is expressed throughout the presumptive neural plate, indicating that neural determination has begun by this stage and correlating with the increased neural competence of the dorsal ectoderm. From early neurula, *opl* expression is restricted to the dorsal neural tube and neural crest. *opl* encodes a transcriptional activator with a carboxy terminal regulatory domain whose removal increases *opl* activity. In animal cap assays, *opl* strongly increases the response of ectoderm to the neural inducer *noggin*, suggesting that *opl* regulates neural competence. *opl* also alters the spectrum of genes induced by noggin, activating the midbrain neural marker *engrailed*. Consistent with its later dorsal neural expression, the activated form of *opl* is able to induce neural crest and dorsal neural tube markers in animal caps and in whole embryos. *opl* expression in the ventrolateral ectoderm of whole embryos leads to the formation of large loose aggregates that may indicate early commitment to neural crest lineages. These aggregates do not express an epidermis-specific marker gene, indicating that *opl* also suppresses ventral fates. Together, these data suggest that *opl* may mediate neural competence, and be involved in activation of midbrain, dorsal neural and neural crest fates.

Thesis advisor: Hazel L. Sive
Title: Associate Professor of Biology

Dedication

To the ones I love:

Linda Chi-Fen Juan

my parents

and my brothers.

Acknowledgments

I thank Hazel Sive for her consistent mentoring and insistence on a high scientific standard, and for serving as a role model: as a scientist and a teacher. She provided the scientific and intellectual environment that made this work possible.

The enthusiasm, curiosity, sense of humor and camaraderie of all of my past and present Sive lab colleagues has made working and studying science an enjoyable learning experience. Thanks especially to the Opl group: Mukesh Patel, Josh Gamse, Vladimir Apekin, Dave Willison; to the fellow 'senior' graduate students: Laura Gammill, Peggy Kolm; to postdocs: Ben Sun, Jenya Grinblat, Leila Bradley, Mary Ellen Lane, Charles Sagerstrom, Bertha Kao, Christa Merzdorf, Jacqueline Hoyle; to 'newer' graduate students Dan Wainstock, Sara Bush, Elizabeth Hick. I also benefited greatly from the members and resources of the Lodish, Jaenisch, Lander and Young labs here at the Whitehead Institute. The resources of the Whitehead Institute was a key part of making this thesis work possible, especially help from the technical, library and facility staff.

I appreciate the input of present and past thesis committee members: Profs. Richard Hynes, Rudolf Jaenisch, Andrew Chess, Nancy Hopkins and Leonard Zon.

Thanks also to the Harvard-MIT Division of Health Sciences and Technology (especially Dr. Lee Gehrke, Keiko Oh, and Patricia Cunningham), the Harvard MD-PhD Program (especially Linda Burnley) for their support in my studies at both Harvard Medical School and MIT. Funding from the Medical Scientist Training Program and the Whitehead Cancer Research Training Grant was appreciated.

I am always grateful to my family and to Linda's family for their love, support, encouragement and incredible patience.

Dearest Linda, most of all, I wish to thank you for always knowing what to say to cheer me up, for being a most willing and sympathetic listener, and for offering constant and unwavering encouragement. You are extremely wise in the ways of gently nudging me to realize fresh perspectives, and so patient when I am slow to reach such epiphanies. You strive always for new horizons, and have such zest and enthusiasm for learning and life that I am moved to stretch myself too. Thank you for going through the many ups and downs with me, and for your sense of humor, unending patience, special love and sensitive affection. My life continues to be immensely, magically delightful with you as an integral part of it. I look forward to many, many more years of sharing a life of wonder and happiness with you.

Linda, toi et nul autre.

Early steps in *Xenopus* neural determination: Cloning and analysis of *opl*

Table of Contents

Abstract 2
Dedication 3
Acknowledgments 4
Table of Contents 5

Chapter 1. Early neural determination in *Xenopus* 10
1.1 Introduction 11
1.2A Establishing the D/V axis during early *Xenopus* development 12
1.2A Cortical rotation establishes the D/V axis 12
1.2B β-catenin/Xtcf3 mediates the dorsal determinant activity 13
1.2C Mesoderm induction and determination 14
1.2D The dorsal organizer: pattern and function 14
1.2E Dorsalization of the early embryo by organizer signals 16
1.3 Neural determination in the gastrula ectoderm 17
1.3A Enhanced ectodermal competence for neural induction 17
1.3B Dorsalization of the ectoderm: neural induction 18
1.3C Initial A/P patterning of the neurectoderm 19
1.3D Posterior neural inducing molecules 21
1.3E Initial neurectodermal patterning along the future D/V neural tube axis 22
1.3F Later D/V patterning of the neural tube 23
1.4 What genes are expressed in the gastrula dorsal ectoderm? 25
1.4A A growing number of neural competence factors 25
1.4B Genes that determine and pattern neurectoderm 26
1.4C Vertebrate "odd-paired-like" genes 27

Chapter 2. Identification of early neural-specific genes in *Xenopus* by subtractive cloning 36
2.1 Preface 37
2.2 Summary 38
2.3 Introduction 39
2.4 Results 41
2.4A Subtractive cloning strategy 41
2.4A Monitoring subtractive enrichment 41
2.4B Screening the subtracted gastrula dorsal ectoderm library 42
2.4B1 Characterizing the subtracted dorsal ectoderm library 43
2.4B2 In situ analysis of dorsal-specific clones 43

2.4C Interesting clones 44
2.4C1 DD135 45
2.4C2 DB9 45
2.343 fkh5 45
2.4C4 opl 46
2.4C5 otx2 46
2.5 Discussion 47
2.5A Identification of candidate early neural patterning genes 47
2.5B Advantages of subtractive cloning 47
2.5C Improvements on the current subtraction 48
2.5D Analysis of interesting clones 49
2.5D1 Identified clones are early markers of prospective neurectoderm 49
2.5D2 Early neural-specific genes may regulate early neural determination 49
2.6 Acknowledgments 51

Chapter 3. Analysis of *opl*: a gene with multiple roles in early neural development 70
3.1 Preface 71
3.2 Summary 72
3.3 Introduction 73
3.4 Results 75
3.4A *opl* encodes a zinc finger protein with similarity to the *Zic* family 75
3.4B Temporal and spatial characterization of *opl* expression 75
3.4C opl is a nuclear protein whose carboxy terminal encodes a regulatory domain 77
3.4D oplΔC activates neural crest and dorsal neural tube markers 78
3.4E oplΔC sensitizes the ectoderm to induction by noggin 79
3.4F In conjunction with noggin, opl activates posterior and dorsoventral neural markers 80
3.4G opl induces cellular aggregates in the absence of DNA synthesis 81
3.4H opl activates expression of neural crest and dorsal neural tube markers and represses epidermal gene expression in whole embryos 83
3.5 Discussion 85
3.5A opl expression defines an early neurectodermal domain 85
3.5B opl can modulate neural competence 86
3.5C opl can activate engrailed 86
3.5D opl as an activator of dorsal neural tube and neural crest fates 87
3.5E opl activity is modulated by synergizing factors 87
3.5F opl modulates cell adhesion 88

3.5G opl and inhibition of epidermal fates 89
3.5H A model for opl function 89
3.6 Acknowledgments 91

Chapter 4. Future Directions 118
4.1 Summary 119
4.2 Future directions 120
4.2A What regulates *opl* expression? 120
4.2A1 What activates early *opl* expression? 121
4.2A2 How is *opl* expression regulated after the gastrula stage? 121
4.2B What are opl's functional roles in neural development? 122
4.2B1 Ablation of opl function in vivo 122
4.2B2 What factors interact with opl to mediate its functions? 122
4.2C What downstream target genes are regulated by opl? 123

Appendix I. Materials and Methods 125
IA Growth, dissection and culture of embryos and explants 126
IB.Subtractive cloning of opl 126
IC opl constructs 127
ID Microinjection 128
IE In vitro transcription 129
IF In situ hybridization and sectioning 129
IG Isolation of RNA and Northern analysis 130
IH Relative quantitative RT-PCR 130
IIWestern analysis and immunocytochemistry 131
IJ Transient transfection reporter assays 131

Appendix II. References 132

Appendix III. Translational inhibition by 5' polycytidine tracts in *Xenopus* embryos and in vitro 153
C.1 Preface 154
C.2 Abstract 155
C.3 Introduction 155
C.4 Experimental Design and Discussion 156
C.3A Poly(C) leader sequence depresses translation in Xenopus embryos 156
C.3B A poly(C) leader sequence depresses translation in vitro 157
C.5 Acknowledgments 160
C.5 References 171

Figures

Figure 1.1 Early stages of Xenopus development 30
Figure 1.2 Establishing the D/V axis during early development 32
Figure 1.3 Neural induction and early patterning occurs during gastrulation 34

Figure 2.1. Tissues used in subtraction. 52
Figure 2.2. Subtraction scheme. 54
Figure 2.3. General removal of tracer by subtraction 56
Figure 2.4. Specific enrichment and removal of genes by subtraction. 58
Figure 2.5. Isolated dorsal-specific clones are expressed in the tailbud nervous system. 60
Figure 2.6. Five interesting clones identified in the subtraction screen. 63

Figure 3.1. opl protein sequence alignments. 92
Figure 3.2. Temporal and spatial characteristics of opl expression. 94
Figure 3.3. opl constructs 98
Figure 3.4. opl is a nuclear protein with a regulatory domain in the COOH-terminal. 100
Figure 3.5. oplΔC can activate neural crest and dorsal neural tube marker genes in animal caps. 102
Figure 3.6. oplΔC sensitizes the ectoderm to induction by noggin. 104
Figure 3.7. opl synergizes with noggin to activate more posterior neural markers in animal caps. 106
Figure 3.8. opl induces cellular aggregates without cell division. 108
Figure 3.9. opl induces ectopic slug and pax3 and inhibits XK81 expression in embryos. 110
Figure 3.10. Model of the roles opl plays in early neural determination. 112

Figure C.1. CAT reporter constructs 161
Figure C.2. Similar stability in Xenopus embryos of RNAs with various 5' leaders 163
Figure C.3. Similar sizes of CAT RNAs transcribed by different polymerases 165

Tables

Table 2.1. Summary of screen 65
Table 2.2. Expression pattern of isolated gastrula dorsal ectodermal cDNAs by in situ hybridization analysis 66

Table 3.1. Frequency of ectopic cellular aggregates in injected embryos. 114
Table 3.2. Induction of cellular aggregates depends on time of addition and duration of dex treatment in oplGR-injected embryos. 115
Table 3.3. Frequency of ectopic slug and pax3 expression in oplGR-injected embryos. 116
Table 3.4. Frequency of XK81 inhibition in oplΔC-injected embryos. 117

Table C.1. Relative CAT activity in Xenopus embryos 167
Table C.2. Relative CAT activity in vitro 169

Chapter 1. Early neural determination in *Xenopus*

1.1 Introduction

To understand the early steps in vertebrate neural development, I studied how the embryonic ectoderm is determined and patterned to start forming a mature *Xenopus* central nervous system. Neural determination is the process of committing multipotent embryonic cells to the neural lineage. This is followed by neural differentiation, the process where cells complete biochemical and morphological changes to become functional neurons. Neural determination and differentiation are the early and late phases of neurogenesis, the developmental pathway of a cell turning into a neuron. The other key part of elaborating a nervous system is appropriate patterning of the developing neurectoderm. Neural precursor cells must be properly organized along the antero-posterior (A/P) and dorso-ventral (D/V) axes of the future nervous system to correctly form a mature brain.

Xenopus is a useful experimental organism to study early neural development due to the following advantages. External fertilization allows one to obtain many embryos at early stages (Figure 1.1). Development occurs rapidly in these large embryos, which are amenable to experimental manipulations such as microinjection and dissection. There is also a long history of experimental data accumulated by embryologists that form a basis for the present molecular biological approach to studying development.

Embryologists discovered and defined a dorsal mesoderm tissue, the organizer, as the source of signal(s) that induced and patterned neural tissue (reviewed in Gilbert, et al., 1993). When I started this project, there was great excitement over the identification of candidate neural inducer molecules. Two organizer-derived peptides, noggin and follistatin, were found to directly induce ectoderm to form neural tissue, the major dorsal ectodermal fate (Hemmati-Brivanlou, et al., 1994; Lamb, et al., 1993; Smith, et al., 1992, 1993). Ectodermal cells that do not receive neural induction will adopt the ventral ectodermal fate, epidermis. It was commonly accepted that the blastula ectoderm (animal caps) was a 'naive' tissue that was either induced at gastrula stage to become neural tissue, or otherwise developed into epidermis. It was also thought that many ectodermal patterning events defining the A/P and D/V organization of the nervous system occurred during gastrulation, many hours before neural differentiation. Since there was a dearth of known markers expressed in the gastrula dorsal ectoderm, such patterning events in the induced neurectoderm were inferred from

specification assays. Explants of gastrula ectoderm were assayed using differentiation markers or histological criteria after incubation in neutral saline. There was a notable gap in the knowledge of genes and mechanisms that act in the gastrula dorsal ectoderm after neural induction, many hours before neural differentiation.

Many interesting questions were raised as promising avenues of inquiry. What genes are activated in the gastrula dorsal ectoderm in response to neural induction? What are the functional roles of such genes? Are they involved in neural determination? Are they involved in establishing A/P or D/V pattern in the induced neurectoderm? Using subtractive cloning, we set out to identify and generate a repertoire of genes expressed in the mid-gastrula dorsal ectoderm. During the course of this work, other gastrula dorsal ectodermal genes were also reported (otx2: (Blitz, et al., 1994; Pannese, et al., 1995); pax3: (Espeseth, et al., 1995); eIF4AII: (Morgan, et al., 1997); XANF: (Mathers, et al., 1995; Zaraisky, et al., 1992, 1995); neurogenin: (Ma, et al., 1996)). Coupled with data from the genes we isolated, these early dorsal ectodermal markers revealed that a complex molecular pattern exists in the gastrula dorsal ectoderm. These genes also mediate many functions in neural determination in the gastrula ectoderm. Since neural tissue is induced by dorsal mesoderm, and dorsal mesoderm determination results from the fertilization and early cleavage stage events, I will first introduce early development in *Xenopus*.

1.2A Establishing the D/V axis during early *Xenopus* development

1.2A Cortical rotation establishes the D/V axis

The sites of action in neural development are on the dorsal side of the embryo. The dorsal ectoderm gives rise to the neural tissue, and neural inducer signals originate from the organizer in the dorsal mesoderm. How is the 'dorsal' side initially established in the zygote?

Xenopus eggs are radially symmetric, composed of the darkly pigmented animal hemisphere and the yolky vegetal hemisphere (Figure 1.2). The animal hemisphere gives rise to the presumptive ectoderm, the equatorial regions give rise to mesoderm, and the vegetal hemisphere gives rise to endoderm. Yolk is concentrated in the vegetal hemisphere, and cytoplasm and germinal vesicles are located in the animal hemisphere (reviewed by Gerhart, et al., 1986)). Maternal RNAs have been found in both

hemispheres, and factors localized in the vegetal pole are implicated in mesoderm determination (Rebagliati, et al., 1985). It is also plausible to imagine that similar maternal factors in the animal hemisphere play a role in ectodermal determination.

The dorso-ventral asymmetry of the embryo begins at fertilization. At fertilization, the sperm enters an egg in the animal hemisphere and stimulates cortical rotation, which moves dorsal determinants along a microtubule array underlying the plasma membrane (Figure 1.2) (Elinson, et al., 1988; Rowning, et al., 1997). The dorsal axis-inducing activity moves 30° from the vegetal pole to one side, thereby defining the zygote's dorsal side (roughly 180° across from the sperm entry point; (Fujisue, et al., 1993), and forming the dorsovegetal Nieuwkoop center. Disruption of the microtubule array (by treatment with ultraviolet light, or with depolymerizing agents) prevents cortical rotation, and results in the absence of a Nieuwkoop center, which leads to a completely ventralized embryo (Elinson, et al., 1989; Scharf, et al., 1983). Without cortical rotation, dorsal determinants remain in the vegetal pole and can activate ectopic dorsal-specific gene expression there (Darras, et al., 1997).

1.2B β-catenin/Xtcf3 mediates the dorsal determinant activity

The *wnt* genes were first proposed as candidates for the initial dorsalizing activity. But inhibition of upstream components of the *wnt* signaling pathway did not block dorsal axis formation (Hoppler, et al., 1996; Sokol, 1996), which suggested that a wnt ligand was not involved. What is now accepted is that cortical rotation leads to nuclear localization and activation of the cytoskeletal protein β-catenin, normally a wnt-activated factor, on the dorsal side (Harland, et al., 1997; Larabell, et al., 1997; Schneider, et al., 1996; Yost, et al., 1996). Activated β-catenin binds with another factor, Xtcf3, to translocate as a complex into dorsal embryonic cell nuclei and activate the dorsal-specific transcriptional program (Brannon, et al., 1997; Molenaar, et al., 1996). The requirement for β-catenin for dorsal determination was shown when antisense-mediated depletion of maternal β–catenin RNAs resulted in ventralized embryos (Heasman, et al., 1994).

In addition to the dorsal vegetal cells, β-catenin is also found in dorsal animal cells at early cleavage stages (Larabell, et al., 1997), and both isolated ectoderm (animal caps) and vegetal explants contain a β-catenin-dependent

activity for dorsalizing mesoderm (Wylie, et al., 1996). The β-catenin-dorsalizing activity is less in animal vs. vegetal pole cells (Wylie, et al., 1996), but may be sufficient in combination with unknown animal pole-localized determinants to determine dorsal animal blastomeres. In the 8-cell embryo, dorsal animal blastomeres are less specified to express a ventral epidermal marker (London, et al., 1988). This suggests that ectodermal determination is affected by cortical rotation, which mobilized dorsalizing β-catenin activity into the dorsal ectodermal precursors.

1.2C Mesoderm induction and determination

At the 32-cell stage, mesoderm is induced in the equatorial marginal zone between the animal and vegetal hemispheres (Figure 1.2) (Dale, et al., 1987b; Jones, et al., 1987). Mesoderm determination is now known to result from both localized cytoplasmic determinants (Lemaire, et al., 1995) and animal-vegetal signaling by TGF-β family members (Nieuwkoop, 1969). The intracellular activity that contributes to mesoderm determination may be partly ascribed to a localized T-box transcription factor, known as Xombi (Lustig, et al., 1996) or Veg-T (Zhang, et al., 1996), or Antipodean (Stennard, et al., 1996) or Brat (Horb, et al., 1997). Signaling molecules of the TGF-β superfamily including activin, processed Vg-1, nodal related factors (Xnr-1, -2) and derrière, a recently isolated novel TGF-β factor (Sun, et al., 1998) are all capable of inducing dorsal mesoderm. Another TGF-β factor, BMP4, ventralizes mesoderm (rev. in Harland and Gerhart, 1997). FGF is considered a competence or maintenance factor for mesoderm induction because ablation of FGF signaling blocks all mesoderm formation (Amaya, et al., 1993; Cornell, et al., 1994a; LaBonne, et al., 1994).

By blastula stage, mesoderm has formed as an equatorial ring around the embryo, and the organizer is formed from the overlap of the dorsal-most mesoderm with β-catenin activity. Signals from the dorsal organizer interact with ventral signals to pattern the mesoderm and ectoderm (Figure 1.2).

1.2D The dorsal organizer: pattern and function

The organizer is a group of cells in the dorsal marginal zone that plays a crucial role in ectodermal and mesodermal patterning. The dorsal organizer was first discovered by Mangold and Spemann in classical embryological experiments using the amphibian *Triturus*. Via

transplantation experiments, they defined a region in the dorsal marginal zone that could induce an ectopic embryonic axis in recipient embryos. This organizer tissue ultimately forms mostly dorsal mesodermal structures, but the induced ectopic neural axis is formed from recipient ectoderm (Spemann's original experiments reviewed in Gilbert and Saxen, 1993); in *Xenopus*: Jacobson, 1984).

The *Xenopus* organizer consists of a 60° wide arc centered on the dorsal midline marginal zone (Gimlich, et al., 1983; Stewart, et al., 1990; Zoltewicz, et al., 1997). It is formed by the combined activity of the dorsalizing β-catenin signal and mesoderm-inducing factors (rev. in Harland and Gerhart, 1997). The dorsalizing activity, β-catenin, activates molecular markers expressed in the dorsal organizer. The homeobox gene *siamois* and the nodal-related factor xnr-3 both contain β-catenin/Xtcf3 sites in their promoters, and are expressed in the regions containing dorsal determinants (anterior mesoderm and endoderm) (Brannon, et al., 1997; Lemaire, et al., 1995; McKendry, et al., 1997). In turn, *siamois* activates the homeobox gene *goosecoid* and the secreted factors noggin and chordin in mesoderm (Carnac, et al., 1996; Darras, et al., 1997). Combined with noggin and chordin activity from the mesoderm, expression of *siamois* in the endoderm activates the secreted factor cerberus in the deep anterior endoderm (Bouwmeester, et al., 1996; Darras, et al., 1997). Two newly described secreted factors, dickkopf and frzb-1 are also expressed in the deep endoderm (Glinka, et al., 1998; Leyns, et al., 1997; Wang, et al., 1997).

The A/P axis of the organizer is inverted relative to the future A/P axis of the embryo. This is also reflected in the different inductive abilities of the organizer subdomains. The anterior dorsal marginal zone (towards the vegetal pole) is defined as the "'head organizer", and activates only anterior markers when conjugated with uninduced ectoderm. The posterior dorsal marginal zone (towards the animal pole) is the "trunk-tail" organizer induces both anterior and posterior markers in conjugates (Doniach, et al., 1995; Zoltewicz and Gerhart, 1997). Before gastrulation movements, the restricted expression of transcription factors in the organizer reflect an A/P pattern. *Xbra* and *Xnot2* are expressed in the posterior dorsal mesoderm (notochord precursor) (Smith, et al., 1991; von Dassow, et al., 1993; Zoltewicz and Gerhart, 1997), *otx2*, *Xlim1*, and *goosecoid* in the anterior dorsal mesoderm (prechordal plate precursor) (Blitz and Cho, 1994; Pannese, et al., 1995;

Taira, et al., 1994; Zoltewicz and Gerhart, 1997), and *otx2* and *Xlim1* in anterior endoderm. The expression of secreted factors noggin, chordin, follistatin, frzb1 is throughout the organizer, with cerberus located in the endoderm, and xnr3 in the outer layer of the organizer. Many of these molecules are involved in mediating two critical organizer activites: neural induction in ectoderm and dorsalization of mesoderm.

1.2E Dorsalization of the early embryo by organizer signals

The current view is that mesoderm patterning results from the interaction between dorsalizing, organizer-derived factors (noggin, chordin, xnr3) and ventralizing signals (BMP4, Xwnt8) from ventral mesoderm. In the absence of an organizer or in ventralized embryos, the marginal zone adopts ventral mesodermal fates (Dale and Slack, 1987b; Heasman, et al., 1994; Stewart and Gerhart, 1990). Dorsalization is the process where the opposing signals from dorsal organizer and ventral mesoderm determine intermediate mesoderm fates (Figure 1.2) (Hansen, et al., 1997; Sasai, et al., 1994; Smith, et al., 1993). Dorsalization also occurs in the other two embryonic layers. In the endoderm it leads to cerberus and dickkopf expression and specification of liver and pancreas (Bouwmeester, et al., 1996; Sasai, et al., 1996). Neural induction is the equivalent of dorsalization of ectoderm by organizer signals (Lamb, et al., 1993; Sasai, et al., 1995). In all cases, dorsalization occurs via repression of ventral signals. The ventralizing signals, BMP4 and xwnt8, disrupt normal mesoderm patterning if ectopically expressed in dorsal mesoderm (Christian, et al., 1993; Dale, et al., 1992; Jones, et al., 1992). Another mechanism by which the marginal zone may be patterned is via different doses or types of inducers at the time of mesoderm induction. When applied in different concentrations, activin induces differential expression of dorsal and ventral mesodermal markers (Green, et al., 1992).

Furthermore, there are probably determinants within the tissues responding to dorsalization that cooperate in patterning decisions. For example, in the marginal zone, eFGF expression is restricted only to the posterior mesoderm (Isaacs, et al., 1992), and may contribute to the formation of the embryonic A/P axis. Animal caps are induced by noggin to form neural tissue (Lamb, et al., 1993), but ventral marginal zone is induced to express dorsal mesoderm genes instead (Smith and Harland, 1992). The same

molecule, noggin, acting via the same mechanism (described below), results in different readouts when added to different responding tissue. Pre-existing determinants in the responding tissue affect the outcome of dorsalization by organizer-derived signals.

1.3 Neural determination in the gastrula ectoderm

1.3A Enhanced ectodermal competence for neural induction

A general increase in competence of the entire ectoderm to respond to induction is observed during gastrula stages. This was discovered using transplantation experiments, and by exposing isolated ectoderm of various stages to purified factors or mesoderm conjugates (Jones and Woodland, 1987; Kintner, et al., 1991; Knecht, et al., 1997; Lamb, et al., 1993; Nieuwkoop, 1985; Servetnick, et al., 1991; Sive, et al., 1989). This ectodermal competence is progressively lost by the end of gastrulation, as uninduced ectoderm differentiates into epidermis (Albers, 1987). Recent studies show that there exist at least two pan-ectodermal molecular markers at late blastula: *BMP4* (ventral ectodermal marker) and *opl* (a neural marker) are expressed throughout the entire late blastula ectoderm (Kuo, et al., 1998; Wilson, et al., 1995). Two intriguing possibilities are that unknown maternal animal pole determinants activate expression of these ectodermal markers, or that their expression results from the general activation of zygotic genes at the mid-blastula transition.

In addition, the gastrula dorsal ectoderm is biased towards dorsal fates and is more competent for neural induction than ventral ectoderm. By early gastrula, isolated dorsal ectoderm fails to be specified to express epidermal markers that are characteristic of ventral ectodermal fates (Savage, et al., 1989). Sharpe and colleagues showed that dorsal ectoderm conjugated to mesoderm expresses a spinal cord marker (*HoxB9)*, while ventral ectoderm similarly conjugated did not express *HoxB9* (Sharpe, et al., 1987) A reasonable prediction is that this enhanced dorsal competence for neural induction results from differential protein activity or gene expression. Otte and colleagues found that protein kinase C (PKC) signaling pathways may contribute to enhanced dorsal ectodermal competence. They showed that the PKC-α isozyme is localized in the dorsal ectoderm, and that its activation or overexpression in ventral ectoderm confers increased competence for neural induction (Otte, 1992a; Otte, et al., 1991). The RNA-helicase translation

initiation factor, eIF4AII, was recently shown to increase ectodermal competence by sensitizing ectoderm to lower levels of the neural inducer noggin (Morgan and Sargent, 1997). eIF4AII and PKC-α genes activate each other in an autocatalytic loop ((Morgan and Sargent, 1997), that could be a molecular mechanism contributing to dorsal ectodermal competence. Another pathway that may enhance dorsal ectodermal competence is Notch signaling. Overexpression of a constitutively active *Xenopus* Notch (Xotch) homolog in animal caps enhanced and extended ectodermal competence (Coffman, et al., 1993). Xotch is postulated to act by delaying differentiation and maintaining cells in a competent state to respond to inducing signals.

1.3B Dorsalization of the ectoderm: neural induction

Analogous to mesodermal patterning, neural induction in the ectoderm occurs during gastrulation by the opposing actions of organizer-derived signals and ventral signals (Figure 1.2). At the start of gastrulation, bottle cells invaginate and a dorsal blastopore lip is observed on the dorso-vegetal side of the embryo (Nieuwkoop, et al., 1994). As gastrulation proceeds, mesoderm involutes and migrates under the ectoderm to form the archenteron roof. During gastrulation, signaling occurs via secreted factors between the dorsal mesoderm and the overlying dorsal ectoderm, as well as within the dorsal ectoderm, to induce and pattern the nervous system (Figure 1.3) (reviewed Doniach, 1992). By the end of gastrulation, a neural plate with an elaborate gene expression pattern along the A/P and D/V axes has formed.

Candidate neural inducers have been identified that are expressed in the right place and time (gastrula organizer) and directly induce neural tissue. Noggin, chordin and follistatin are all expressed in the organizer, and their mis-expression in isolated ectoderm led to formation of neural tissue by molecular and histological criteria (Hemmati-Brivanlou, et al., 1994; Lamb, et al., 1993; Sasai, et al., 1995; Sasai, et al., 1994; Smith and Harland, 1992). Furthermore, these molecules are also active in dorsalizing ventral mesoderm (Figure 1.2) (Sasai, et al., 1995; Sasai, et al., 1994; Smith, et al., 1993), another key property of the classically defined organizer.

It is now thought that neural induction results from inhibition of an endogenous ventralizing signal that normally determines epidermis in ectoderm (reviewed in Hemmati-Brivanlou, et al., 1997). This mechanism was first suggested by experiments where dissociation of isolated ectoderm

led to autonomous expression of neural markers and loss of epidermal markers (Godsave, et al., 1989; Grunz, et al., 1989). Presumably, this is due to the loss of an endogenous inhibitor(s) that maintains epidermal specification in ectoderm. The Bone Morphogenetic Protein family member, BMP-4, has been implicated as the signal that maintains ventral epidermis specification (Wilson and Hemmati-Brivanlou, 1995). Graded levels of BMP-4 activity also induce a spectrum of epidermal (high BMP-4) to anterior neural (low BMP-4) markers (Knecht, et al., 1995; Wilson, et al., 1997). A similar activity gradient was observed by varying the level of a BMP4 signaling effector, Smad1 (Wilson, et al., 1997). Overexpression of dominant negative mutant receptors that interfere with BMP-4 signaling induce ectopic neural tissue (Hawley, et al., 1995; Hemmati-Brivanlou, et al., 1994; Sasai, et al., 1995; Wilson and Hemmati-Brivanlou, 1995). Noggin, chordin, follistatin and xnr-3 have all been shown to bind BMP-4 and inhibit its activity (Hemmati-Brivanlou and Melton, 1997; Piccolo, et al., 1996; Re'em-Kalma, et al., 1995; Sasai, et al., 1995; Zimmerman, et al., 1996). The current hypothesis is that active BMP-4 signaling maintains the ventral epidermal specification, and neural induction occurs by inhibiting this signal with organizer-derived neural inducers in the dorsal ectoderm (reviewed in Hemmati-Brivanlou and Melton, 1997; Sasai, et al., 1997).

1.3C Initial A/P patterning of the neurectoderm

Many have reported that A/P specification is quite labile in the gastrula ectoderm (Saha, et al., 1992; Sharpe, et al., 1990; Sive, et al., 1989). The current data support the hypothesis that induced neurectoderm undergoes an initial, transient anterior specification before committing to its final A/P fate. This transient anterior specification of posterior ectoderm has been observed. Eyal-Giladi observed that forebrain specification in the induced neurectoderm moved anteriorly as anterior mesoderm involuted more anteriorly, and posterior ectoderm is re-specified to its final posterior fate (Eyal-Giladi, 1954). Sive and colleagues observed the similar progressive determination of induced gastrula ectoderm by assaying markers specific to the anterior ectodermal organ, cement gland (Sive, et al., 1989). This is consistent with the activity of the candidate neural inducers (noggin, chordin, follistatin), which activate primarily general and anterior neural markers (Hemmati-Brivanlou, et al., 1994; Lamb, et al., 1993; Sasai, et al., 1994).

These observations also predict that tissue fated to become posterior neurectoderm is first induced to an anterior state, and another signal(s) is required for induction of the final posterior fate.

Several models have been proposed for establishing the A/P neural axis. The first is a regional inducer model, where the A/P state of the induced neurectoderm is directly induced by the A/P specification of the underlying mesoderm: anterior mesoderm would induce posterior ectoderm to an anterior state via one inducer; then further mesoderm involution during gastrulation would place posterior mesoderm under the posterior ectoderm, and a posterior inducer would then specify posterior neural fates. What is inconsistent with this model is that several studies have shown that posterior mesoderm can induce both anterior and posterior markers by itself (Doniach and Musci, 1995; Hemmati-Brivanlou, et al., 1990; Sharpe and Gurdon, 1990). Another model proposes a neural inducer gradient, with the highest level at the posterior mesoderm that induces posterior neurectoderm, and the lowest level at the anterior mesoderm that induces anterior neural tissue (reviewed in Doniach, 1993). But this is inconsistent with the finding that regardless of the amount of posterior mesoderm present, an anterior neural marker is still induced in isolated ectoderm-mesoderm conjugates (Doniach and Musci, 1995).

The commonly accepted model involves two signals in an 'activation-transformation' model described by (Nieuwkoop, 1952b; Nieuwkoop, 1952c; Nieuwkoop, 1952a), and recently modified by Kolm, et al., 1997). It is based on Nieuwkoop's experiments, where ectoderm explants were implanted at different A/P positions along the presumptive neural plate in embryos. Neural tissue was induced in the ectodermal transplants with characteristics related to its implanted A/P position along the neural plate. The proximal part of the transplant was induced to form neural tissue with the same A/P level as that of the insertion site, and the distal parts of the transplant were induced to form neural tissue of more anterior character. Explants implanted in the most anterior position formed the most anterior neural tissue. This led to the hypothesis that there is first an 'activation' of the anterior neural state in the dorsal ectoderm by neural inducers.. Then a second signal, with an activity gradient highest in the posterior dorsal mesoderm, 'transforms' the induced tissue to more posterior fates. This model correlates with the observations that formation of posterior neurectoderm requires passage

through a labile anterior state. Posterior neurectodermal precursors may be independently specified as posterior or neural in a recently proposed modification of the Nieuwkoop model (Kolm and Sive, 1997). This takes into account the observations that lateral ectoderm fated to become posterior neural tissue is already induced to express the posterior marker, *HoxD1*, by mid-gastrula. Subsequently, convergent movement of those cells towards the dorsal midline during gastrulation allows exposure to neural inducers, and completes the induction of posterior neural tissue.

As detailed below, other signals are required for posterior neural induction and result in the final A/P pattern.

1.3D Posterior neural inducing molecules

Secondary treatment of neuralized tissues with several factors, including retinoic acid (RA), fibroblast growth factor (FGF), wnt-3A can induce expression of neural markers more posterior than the forebrain markers induced by organizer signals (Cox, et al., 1995; Kengaku, et al., 1995; Lamb, et al., 1995; McGrew, et al., 1997; McGrew, et al., 1995; Papalopulu, et al., 1996; Sive, et al., 1991; Sive, et al., 1990; Taira, et al., 1997). As predicted by Nieuwkoop for posteriorizing ('transformation') signals, these molecules alone are unable to neuralize isolated ectoderm, and can only act on already induced ('activated') neural tissue.

Much reported data support the role of retinoic acid as an endogenous posteriorizing factor. Retinoic acid eliminates formation of anterior tissue by posteriorizing both mesoderm and ectoderm (Ruiz i Altaba, et al., 1991a; Sive, et al., 1990). Several different retinoids, receptors and related signal transducing proteins are present in gastrula embryos (rev. in Kolm and Sive, 1997). Dominant negative retinoic acid receptors block proper patterning of the dorsal ectoderm (Blumberg, et al., 1997; Kolm, et al., 1997).. Retinoic acid response elements have also been identified in the promoter regions of many genes, such as the anterior patterning gene *otx2* (Simeone, et al., 1995) and posterior expressed *Hox* genes (rev. in (Marshall, et al., 1996). These findings all suggest that retinoic acid has an endogenous role in determination of posterior neural tissue.

A candidate FGF for posterior neural induction is embryonic FGF (eFGF), which is expressed in a ring around the blastopore, with timing and location consistent with an endogenous posteriorizing role (Isaacs, et al.,

1992). eFGF overexpression from the start of gastrulation results in a posteriorized phenotype (embryos with a reduced head and enlarged proctodeum), and an increase in expression level and anterior expansion in the territory of posterior neural markers (Isaacs, et al., 1994; Pownall, et al., 1996). By expressing dominant negative FGF receptors to block FGF signaling in embryos, it has also been shown that FGF signaling is required for expression of the spinal cord markers *Xcad3* and *HoxA7,* but not needed for patterning anterior neural tissue (Kroll, et al., 1996; Northrop, et al., 1994; Pownall, et al., 1996).

It was reported that overexpression of wnt-3A in noggin-induced neurectoderm led to expression of posterior neural markers (midbrain marker *en2*, hindbrain marker *krox20*, and spinal cord marker *HoxB9*) and repression of anterior ectodermal markers (cement gland marker *XAG-1,* anterior neural markers *XANF-2* and *otx2)*(McGrew, et al., 1995, 1997). Wnt3a and wnt-8 are two viable candidates for the posteriorizing activity, since they are expressed in the lateral and ventral marginal zones next to the ectoderm fated to become posterior neural tissue, and can repress anterior neural markers (Christian and Moon, 1993; Fredieu, et al., 1997; McGrew, et al., 1997; Moon, 1993). Consistent with the above hypothesis, expression of dominant negative Xwnt8 caused decreased expression of posterior neural markers and increased expression of anterior neural markers in embryos and in isolated neurectoderm (McGrew, et al., 1997). Intriguingly, these wnts overlap with eFGF and FGF3 expression domains at gastrula stages, suggesting that wnt and FGF signaling pathways interact with each other in neurectodermal patterning. Using the relevant dominant negative mutant factors, it was found that FGF signaling is required for wnt3a-mediated downregulation of anterior neural markers, and that FGF posteriorization of neurectoderm also requires active wnt signaling (McGrew, et al., 1997). These interesting studies suggest that retinoic acid, wnt and FGF signaling pathways all contribute to the 'transformation' of initially induced anterior neurectoderm to elaborate the A/P neural axis.

1.3E Initial neurectodermal patterning along the future D/V neural tube axis

Similar to A/P patterning, initial D/V neural tube patterning also occurs early during gastrula stages (reviewed in Placzek, et al., 1996).

During gastrula and early neurula stages, the presumptive neural plate stays open, and the medio-lateral axis is equivalent to the future D/V axis of the neural tube. The lateral margins will form the future neural crest and dorsal neural tube. Organizer-derived factors, such as noggin and chordin, could initiate patterning in the medio-lateral neurectodermal axis (the future D/V neural tube axis) (Sasai and DeRobertis, 1997). Such organizer-derived factors are expressed in the chordamesoderm (the notochord precursor) that underlies the neural plate midline (the future floor-plate) (Sasai, et al., 1995; Smith and Harland, 1992). As gastrulation proceeds, organizer signals diffuse from the underlying mesoderm to the ectoderm, sequestering BMPs to establish neurectodermal territory. BMP4 has been shown to specify epidermis, the major ventral ectodermal fate (Wilson and Hemmati-Brivanlou, 1995), and BMPs and the related TGF-β family member dorsalin-1, have also been shown to act later in D/V neural tube patterning by antagonizing the floor-plate signal, shh (Liem, et al., 1995).

One testable hypothesis is that a gradient of organizer signal activity exists at a high level at the midline and drops to a low level at the lateral margins of the presumptive neural plate, therefore regionalizing the future D/V neural tube axis. This was shown as a plausible model in a recent study that compared induction of the general neural marker *NCAM*, the neural crest marker *Xslu* and epidermal keratin at different doses of neural inducing activities (noggin, dominant negative BMP-4 receptor and *Xlpou2*) (Morgan and Sargent, 1997). Upregulation of the epidermal keratin occurred at low doses, activation of the neural crest marker occurred at intermediate doses, and *NCAM* was activated at high doses of neural inducing activity (Morgan and Sargent, 1997). The changes in marker expression occurred over only a two-fold range of RNA concentrations, suggesting an exquisitely sensitive patterning response apparatus. Dorsal ectoderm-specific expression of the translation initiation factor eIF4AII is correlated with this effect (Morgan and Sargent, 1997).

1.3F Later D/V patterning of the neural tube

The tissue interactions that contribute to D/V patterning in the neural tube are well studied in several vertebrate species. In chick studies, the notochord was shown to be required for inducing floor plate in the overlying neurectoderm (Holtfreter and Hamburger, 1955). Ectopic notochord grafts

induced ectopic floor-plates and ventral cell types (motor neurons) (Yamada, et al., 1993; Yamada, et al., 1991). Floor plate grafts could also mimic notochord grafts (Placzek, et al., 1991). There are several reports suggesting a requirement for neural and non-neural ectoderm-derived signals in the development of the dorsal neural tube(Dickenson, et al., 1995; Liem, et al., 1995; Mancilla, et al., 1996; Moury, et al., 1990; Selleck, et al., 1995). Neural crest are formed at the borders of neural tissue transplanted to ventral ectoderm, and neural crest markers can be induced by the ventral epidermal-derived signals (Liem, et al., 1995; Mancilla and Mayor, 1996).

The molecules underlying many of these interactions have been identified. A great deal of work in many species implicates the ventral floor-plate signal, sonic hedgehog (shh), and dorsal neural tube and epidermal factors (e.g. *dorsalin-1, BMP4,*) in patterning the D/V neural tube axis during neurula stages (discussed below). Shh is expressed both in the notochord and the floor-plate, and ectopic misexpression of shh induces floor-plate markers in mouse, zebrafish and *Xenopus* (Echelard, et al., 1993; Riddle, et al., 1993; Roelink, et al., 1994). Both Shh-expressing COS cells and the amino terminal shh autocleavage product can induce ventral neural cell types from lateral and dorsal neural tube explants in vitro (Echelard, et al., 1993; Marti, et al., 1995; Riddle, et al., 1993; Roelink, et al., 1994). The winged helix transcription factor hepatocyte nuclear factor 3β (HNF-3β) is also required for notochord and floor-plate formation (Ang, et al., 1994; Weinstein, et al., 1994), and HNF-3β misexpression in dorsal neural tube also leads to expression of floor-plate markers in mouse and *Xenopus* (Ruiz i Altaba, et al., 1993; Sasaki, et al., 1994). The current model is that ventral neural tube specification is established by the positive feedback loop of HNF-3β and shh expression, first in the notochord then in the floor-plate, leading to a ventral neural tube shh gradient.

The ventral neural tube shh gradient is antagonized by TGF-β-like factors present in the dorsal neural tube and adjacent non-neural ectoderm (epidermis). BMP4 and 7 and dorsalin-1 have been identified in chick, BMP2 in mouse, and radar (BMP-related molecule) in zebrafish (Basler, et al., 1993; Liem, et al., 1995; Rissi, et al., 1995). These factors originating from the dorsal neural tube and epidermis can induce dorsal neural tube markers (wnt-1, pax3) and cell types (neural crest, roof-plate cells and commisural neurons) (Moury and Jacobson, 1990; Selleck and Bronner-Fraser, 1995).

The interplay between ventral-derived shh and dorsal factors contribute to D/V neural tube patterning.

A large repertoire of neural tube markers have been identified that aid current studies on neural tube patterning. Many are transcription factors that serve as regional markers of the neural tube, and some have been shown to be required for differentiation of specific types of neurons. The following classes of DNA-binding proteins have been found in the neural tube: 1) winged helix (HNF-3β; (Dirksen, et al., 1992; Ruiz i Altaba, et al., 1992b), 2) pax (pax2, 3, 5, 6, 7, 8; reviewed in Gruss, et al., 1992), 3) lim (lim-1, islet-1; (Dawid, et al., 1995; Tsuchida, et al., 1994), 4) msx (msx1, 2; (Davidson, et al., 1991) and 5) nkx (nkx2.2; (Price, 1993; Saha, et al., 1993). Gene targeting studies in mouse showed that pax3 is essential for proper dorsal neural tube formation and closure, and is the gene responsible for the mouse *Splotch* mutation(Epstein, et al., 1991). Another set of mouse knockout studies show that islet-1 is required for motor neuron development, and for secondary development of adjacent interneurons (Pfaff, et al., 1996). Intensive work is continuing on neural tube patterning by studies on the functional roles of these and other genes.

1.4 What genes are expressed in the gastrula dorsal ectoderm?

During the course of this work, many genes were isolated that are expressed in the gastrula dorsal ectoderm. The following descriptions are of some general classes of genes found by ourselves and others. Identified gastrula dorsal ectodermal genes can be tested for functional involvement in early neural determination: mediating the ectodermal response to neural inducers, patterning the induced neurectoderm, or activating the genetic programs of neuronal types.

1.4A A growing number of neural competence factors

A group of diverse genes have been identified as neural competence factors, regulating the ability of the dorsal ectoderm to respond to neural induction via many different mechanisms. As described before, when expressed in ectoderm, the translation initiation factor eIF4AII (Morgan and Sargent, 1997) and the signaling kinase isozyme PKC-α (Otte, 1992a) increase sensitivity to neural inducers, and a constitutively active Xotch receptor extends ectodermal competence (Coffman, et al., 1993). The Sry-

related HMG box chromatin factor, Sox-2, was also reported to sensitize the gastrula ectoderm to FGF signals and cooperatively mediate neuralization (Mizuseki, et al., 1998). Sry-related proteins are thought to regulate gene expression by binding to and causing changes in the topology of promoter regions (Werner, et al., 1995). Chapter 3 details the ability of opl, a zinc finger transcription factor we identified, to sensitize ectoderm to the neural inducer noggin.

1.4B Genes that determine and pattern neurectoderm

During the course of our work, many other genes expressed early in the dorsal ectoderm have also been reported and were tested for possible involvement in neural determination and patterning. Examples include the anterior patterning homebox gene *otx2* (Blitz and Cho, 1994; Gammill, et al., 1997; Pannese, et al., 1995) and *distal-less* genes (Dirksen, et al., 1994; Papalopulu, et al., 1993), the posterior patterning homebox gene *HoxD1* (Kolm, et al., 1994; Kolm, et al., 1995a), the neural crest/neural plate border determining factor eIF4AII (Morgan and Sargent, 1997) and the anterior neural fold genes *Xanf-1*(Zaraisky, et al., 1992) and *Xanf-2* (Mathers, et al., 1995). Some of these genes have also been isolated in other vertebrates, and studies across species yield complementary information on their functional roles in neural patterning.

The vertebrate homologs of two types of genes involved in *Drosophila* neurogenesis, the proneural and neurogenic genes, are also extensively studied for their activity in neural determination. Proneural genes are defined in *Drosophila* as genes involved in determinining the neurectoderm, a region of ventral ectoderm in the fly embryo that gives rise to neurons. Neurogenic genes are defined in *Drosophila* as genes that select specific neurectodermal cells for differentiation into neurons. This arbitrary grouping of neural determination genes in flies is partly based on timing of expression and mechanisms of action, and has been used in the vertebrate neural determination literature loosely as well.

Identified by homology, multiple basic Helix-Loop-Helix (bHLH) proteins were shown to function as proneural genes in vertebrates. Some examples including vertebrate *achaete-scute* homologs (*Mash-1, Xash-1, Xash-3*) (Ferreiro, et al., 1992; Johnson, et al., 1990; Turner, et al., 1994; Zimmerman, et al., 1993), *atonal* homologs (*neuroD, neurogenin, math-1,*

math-3, nex-1, ATH3) (Akazawa, et al., 1995; Bartholoma, et al., 1994; Lee, et al., 1995; Ma, et al., 1996) and negative proneural regulators (e.g. *id*, *HES*) (Benezra, et al., 1990; Sasai, et al., 1992) have been identified. In addition to intriguing expression neural patterns that usually correlate with neural precursors undergoing differentiation, many are also able to convert non-neural cells to the neural fate (Lee, et al., 1995; Ma, et al., 1996; Turner and Weintraub, 1994). The proneural bHLH genes, *neurogenin* and *Xash3*, are expressed very closely after induction of neurectoderm in early gastrula (Ma, et al., 1996; Turner and Weintraub, 1994) in the regions that will subsequently express neurogenic genes and give rise to primary differentiated neurons (Ma, et al., 1996; Turner and Weintraub, 1994).

Neurogenic genes involved in the well-characterized *Drosophila* lateral inhibition signaling pathway (*notch* and *delta*) play a role in selecting cells for neural differentiation in vertebrate species. In *Drosophila* proneural cell clusters (neurectoderm), delta is the ligand expressed by the cell committed to neural differentiation to inhibit surrounding cells in the cluster via the Notch receptor. This mechanism of selecting cells for neural differentation is preserved in vertebrate species (mouse, chick, *xenopus*). *Xenopus delta* expression demarcates differentiating neurons, and ectopic expression inhibits production of neurons (Chitnis, et al., 1995). Expression of a constitutively active Xotch receptor inhibits expression of neural markers and extends neural competence by delaying differentiation (Coffman, et al., 1993). A related ligand and receptor (jagged, serrate) have analogous activities in mouse and chicks (Lindsell, et al., 1995; Myat, et al., 1996).

1.4C Vertebrate "odd-paired-like" genes

Odd-paired-like (opl) is a zinc finger transcription factor gene we identified that reaches maximal expression in the gastrula ectoderm and has multiple roles in early neural development (Chapter 3, Kuo et al., 1998). Opl and its zebrafish, mouse and human homologs have zinc finger domains that are highly similar to those encoded by the *Drosophila* pair-rule gene, *odd-paired* (Aruga et al., 1996; Kuo et al., 1998; Grinblat et al., 1998). *Drosophila odd-paired (opa)* and related vertebrate genes also share the same exon-intron boundary (Aruga et al., 1996; Grinblat et al., 1998), further suggesting that they evolved from a common ancestral gene.

In *Drosophila*, *opa* is involved in early ectodermal segmentation and also required later for proper midgut morphogenesis (Benedyk et al., 1994; Cimbora and Sakonju, 1995). Genetic analysis defined *opa* as an essential activator for *wingless* (*wg*) activation in odd parasegments, and important for the timely activation of both *wg* and *engrailed* (*en*) in all parasegments (Benedyk et al., 1994). Unlike the segmental expression of other pair-rule genes, *opa* is ubiquitously expressed in all regions of the blastoderm segment primordium. Therefore, Benedyk and colleagues proposed that *opa* mediates different functions by cooperating with other, spatially restricted pair-rule factors, in the proper segmentation of *Drosophila* blastoderm (Benedyk et al., 1994).

Another group identified *opa* as a gene required in the later development of the visceral mesoderm and proper formation of midgut constrictions (Cimbora and Sakonju, 1995). Their data suggest that *opa* regulation of the homeobox gene *bagpipe* (*bap*) is important for visceral mesoderm determination, and that *opa* is later regulated by homeotic genes (*antennapedia, abdominal-A, ultrabithorax*) and secreted *decapentaplegic* in determining the midgut constrictions. Furthermore, proper expression of the endodermal POU-domain gene, *pdm-1*, depends on *opa* function in the mesoderm. These *Drosophila* studies demonstrate that *opa* is a gene with many roles in different embryonic processes.

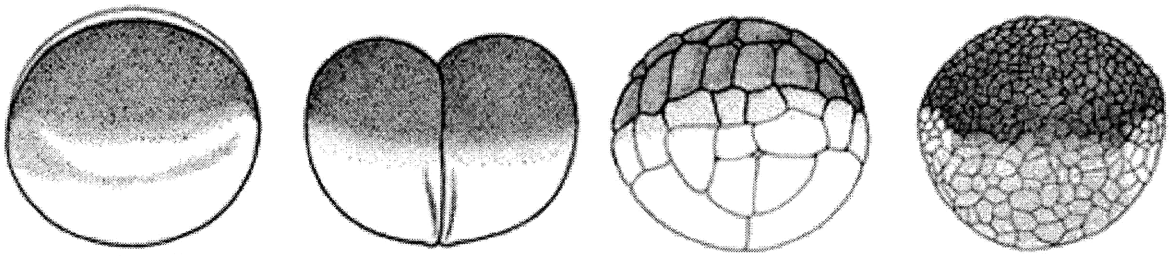
Based on our analysis of *Xenopus odd-paired-like* (*opl*), we propose a model (Figure 3.10) for *opl*'s multiple roles in early neural determination. It may mediate different functions by acting alone or by interacting with other spatially restricted factors. Similar to *opa*, *opl* and its vertebrate homologs may have roles in multiple distinct developmental processes. The mouse homologs were originally identified as zinc finger genes (*zic*) in the cerebellum, and *zic-1* was recently shown to be required for proper cerebellar development (Aruga et al., 1994, 1998). It is likely that in vertebrates, as in *Drosophila*, these evolutionarily related factors have multiple functions and interact with distinct regulatory hierarchies of genes during embryogenesis.

With the twin goals of obtaining a repertoire of molecular markers for the induced gastrula ectoderm, and elucidating the molecular mechanism of neural fate determination, we set out to identify genes expressed in gastrula dorsal ectoderm and test them for possible roles in early neural

determination. Chapter 2 of this thesis will describe the successful use of PCR-based subtractive cloning to identify more than 40 genes expressed in the gastrula ectoderm, and the expression profiles of five candidates for neural patterning genes. Chapter 3 presents the expression and analysis of one of the cloned genes, *opl*, and its multiple roles in early neural development: regulating neural competence, posteriorizing induced neurectoderm, and activating dorsal neural tube and neural crest fates (Kuo et al, in press). Chapter 4 will discuss future directions for using these genes to understand neural determination. Appendix I details the Materials and Methods. Appendix II lists the references. Appendix III presents a study on translational inhibition by 5' polycytidine tracts in *Xenopus* embryos and in vitro.

Figure 1.1 Early stages of Xenopus development

Early stages of *Xenopus* development, with the stage number and the hours post-fertilization at room temperature below each drawing (d: day). The top row are egg to mid-blastula stages shown in lateral view. The initial gastrula (stage 10) is shown with a vegetal view of the initial dorsal blastopore. Mid-gastrula (stage 11.5) to late neurula (stage 19) are shown in progressively dorsal views. Lateral views of embryos are shown from early tailbud (stage 22) on. All embryos up to stage 30 are drawn to scale. Major morphological features are labeled on the tailbud and hatching stage embryos: CG (cement gland), FB (forebrain), MB (midbrain), HB (hindbrain), E (eye), OV (otic vesicle), TB (tailbud), H (heart). Stage 1 through stage 8 are shown animal pole up, with the view indicated. Stage 10 through stage 11.5 are shown dorsal side up. Stage 12.5 through stage 19 are shown anterior side up. Stage 22 through 25 are shown anterior up and dorsal to the right. Stage 30 is shown anterior to the left and dorsal up. Taken from Nieuwkoop (1994), *Normal Tables of Xenopus laevis (Daudin)*.

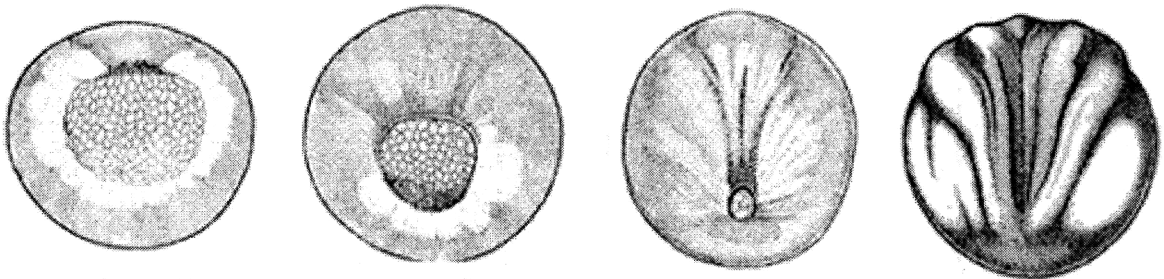


stage1
one-cell
0 hours

stage 2
two-cell
1.5 hours

stage 6.5
48-cell
3.5 hours

stage 8.5
mid-blastula
5 hours

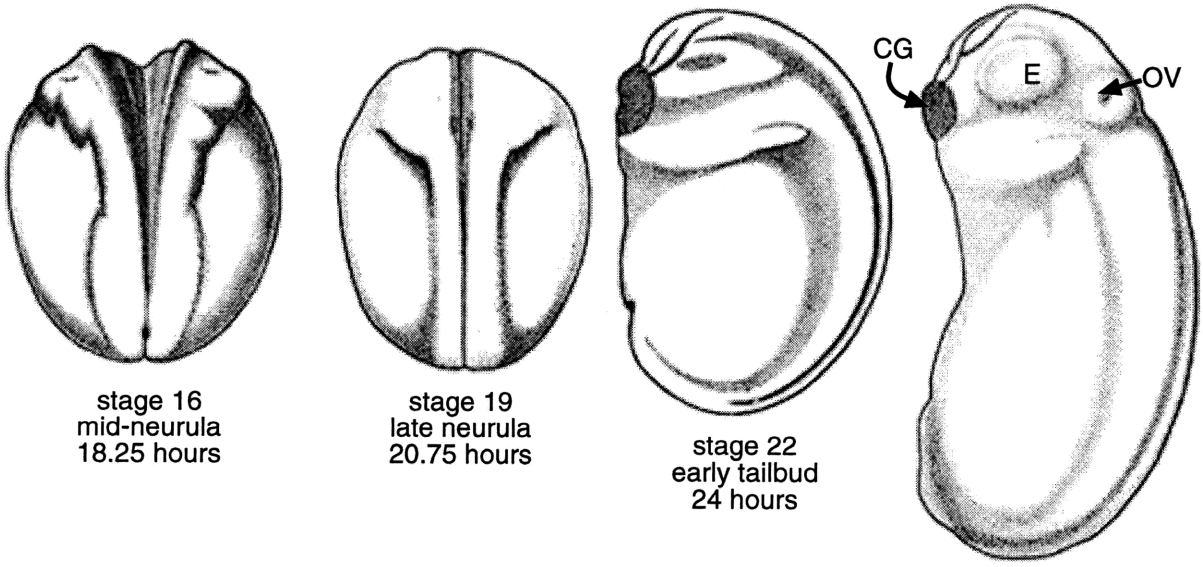


stage 10
initial gastrula
9 hours

stage 11.5
mid-gastrula
12.5 hours

stage 12.5
late gastrula
14.25 hours

stage 14
early neurula
16.25 hours

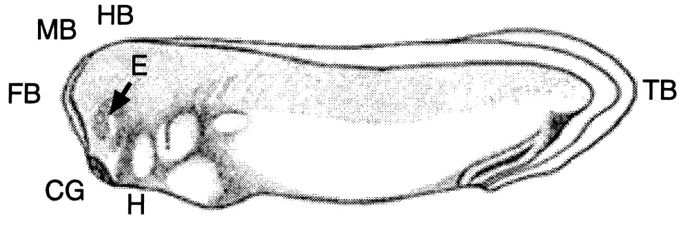


stage 16
mid-neurula
18.25 hours

stage 19
late neurula
20.75 hours

stage 22
early tailbud
24 hours

stage 25
tailbud
1d. 5.5 hours



stage30
hatching
1d. 11 hours

Figure 1.2 Establishing the D/V axis during early development
The *Xenopus* egg begins with radial symmetry and has clear animal pole-vegetal asymmetry. Dorso-ventral asymmetry is introduced by the entry of the sperm in the animal hemisphere (light grey shading) at fertilization. Fertilization causes a 30° cortical rotation, and localizes a dorsal determinant (β-catenin) in the dorsal side of the embryo (hatching). In the vegetal half of the embryo the dorsalized region becomes the Nieuwkoop signaling center (bold hatching); in the dorsal animal hemisphere there is now a 'pre-pattern', observed as enhanced competence to neural inducers. During cleavage stages the Nieuwkoop center induces the formation of the organizer (vertical closed arrow), while a general mesodermalizing signal induces ventral-like mesoderm throughout the remainder of the marginal zone (vertical open arrows). Mesoderm patterning is further refined during blastula and early gastrula stages when dorsalizing signals (e.g. noggin, chordine, follistatin) from the organizer (horizontal closed arrow) and a ventralizing signal (e.g. BMP4, Xwnt8) from the ventral mesoderm (horizontal open arrow) interact to induce intermediate mesoderm types. In the animal hemisphere, unknown animal pole determinant(s) combine with β-catenin to activate early ectodermal genes in a rudimentary pattern. By gastrula stage, organizer signals induce neural tissue in the ectoderm by blockade of ventralizing signals. D: dorsal; V: ventral; O: organizer; DL: dorsolateral mesoderm; VL: ventrolateral mesoderm; V: ventral mesoderm. Based on (Sive, 1993).

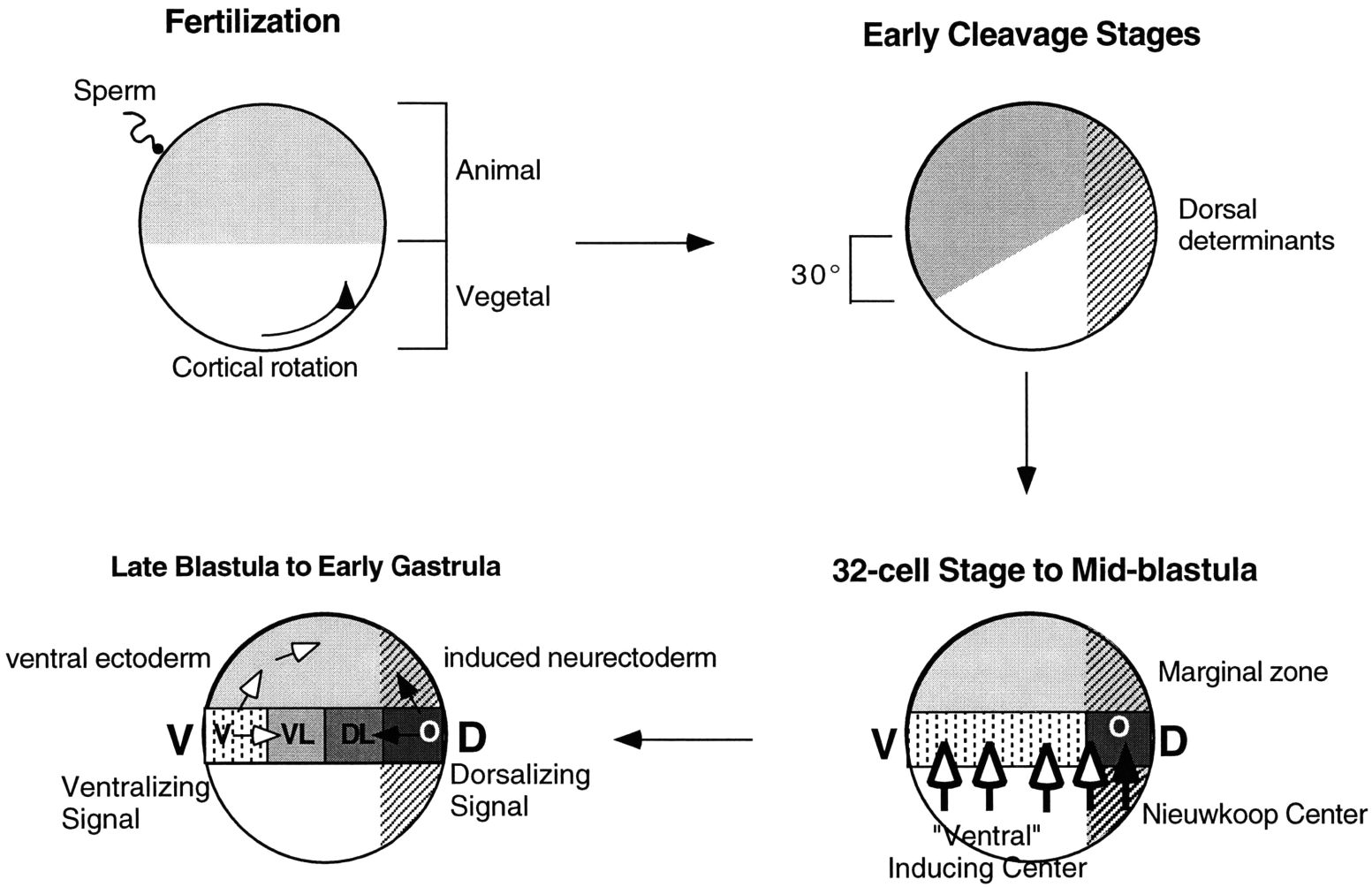
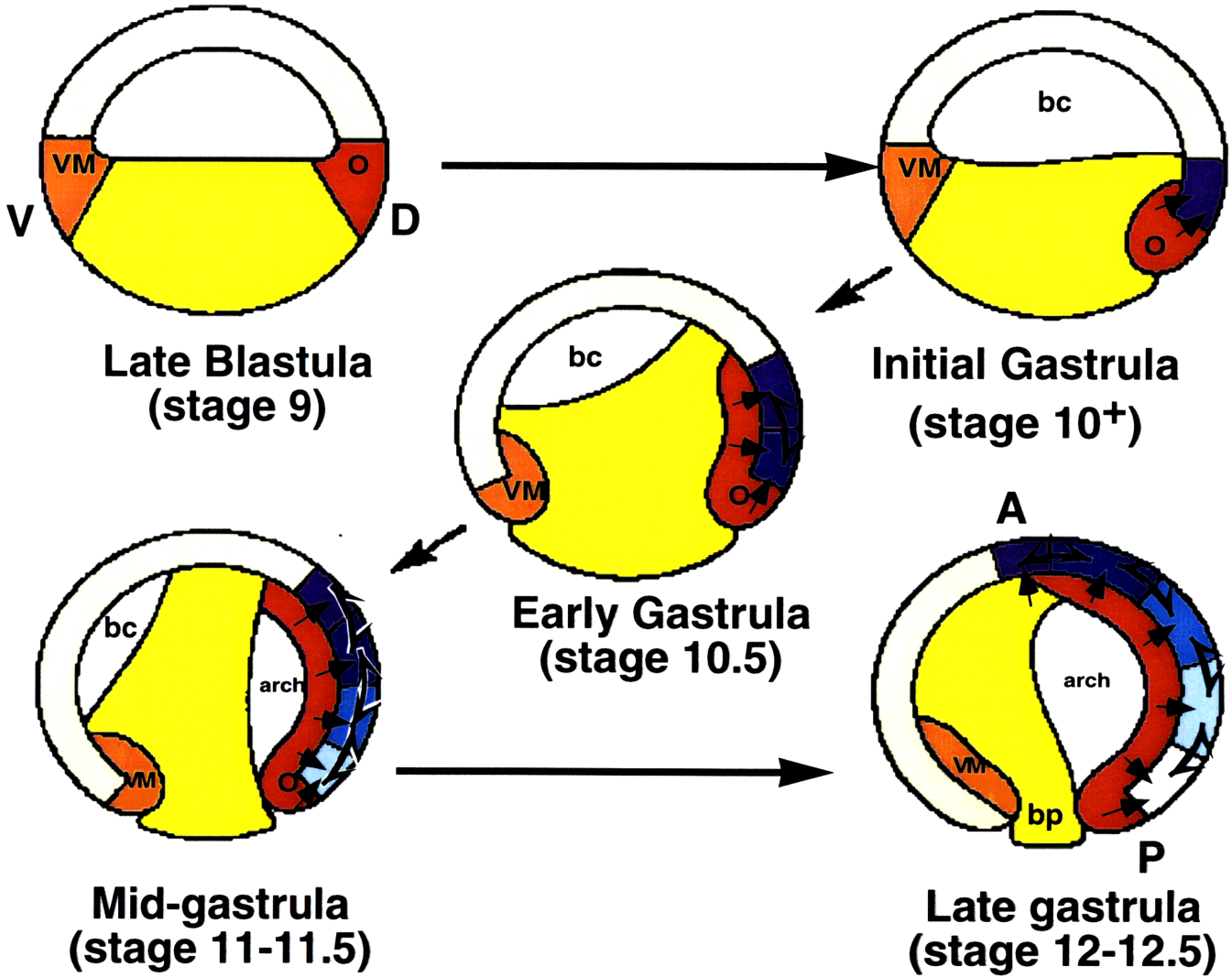

Fertilization
Sperm
Animal
Vegetal
Cortical rotation
Early Cleavage Stages
30°
Dorsal
determinants
32-cell Stage to Mid-blastula
Marginal zone
V
O
D
Nieuwkoop Center
"Ventral"
Inducing Center
Late Blastula to Early Gastrula
ventral ectoderm
induced neurectoderm
V
VL
DL
O
D
Ventralizing
Signal
Dorsalizing
Signal
Animal Hemisphere (Ectoderm)
Vegetal Hemisphere (Endoderm)
Dorsal
Nieuwkoop Center
Dorsal Mesoderm (organizer, presumptive notochord)
Dorsolateral Mesoderm (Presumptive muscle and some notochord)
Ventrolateral Mesoderm (presumptive lateral plate and some muscle)
Ventral Mesoderm (presumptive blood)

Figure 1.3 Neural induction and early patterning occurs during gastrulation

Diagrams of sagittal sections of Xenopus embryos during gastrulation. Dorsal is to the right. Before the onset of gastrulation, the embryo is divided into three germ layers; endoderm (yellow) at the vegetal pole, ectoderm (green) at the animal pole, and mesoderm (red, orange) at the marginal zone. At this stage the ectoderm is specified to form epidermis. At initial gastrula (stage 10^+), the mesoderm invaginates on the dorsovegetal side of the embryo. As the dorsal mesoderm (red) passes beneath the ectoderm, there is a series of inductive interactions between the mesoderm and the overlying and adjacent ectoderm (↗), as well as in the plane of the ectoderm itself (↕). The dorsal ectoderm is initially specified as anterior (purple), then is respecified to more posterior fates (dark blue, light blue). By the end of gastrulation the dorsal ectoderm has been regionalized along the entire anteroposterior axis. Note that as gastrulation proceeds, the population of cells that make up the dorsal lip (organizer) becomes progressively more posterior. D: dorsal, V: ventral, VM: ventral marginal zone, DM: dorsal marginal zone, O: organizer; bp: blastopore, bc: blastocoel, arch: archenteron. Diagram is adapted from Slack and Tannahill (1992) and taken from Kolm (1997).


VM
O
V
D
Late Blastula
(stage 9)
bc
VM
O
Initial Gastrula
(stage 10+)
bc
VM
O
Early Gastrula
(stage 10.5)
A
bc
arch
VM
O
Mid-gastrula
(stage 11-11.5)
arch
VM
bp
P
Late gastrula
(stage 12-12.5)

Chapter 2. Identification of early neural-specific genes in *Xenopus* by subtractive cloning

2.1 Preface

The subtractions were performed by M. Patel, and the screening of clones was carried out jointly by J.S. Kuo and M. Patel with substantial help from J. Gamse and David Willison.

2.2 Summary

The molecular basis of early neural determination and patterning in the *Xenopus* gastrula dorsal ectoderm is largely unknown. Previous efforts to identify genes involved in neural development relied on homology with known genes, therefore potentially missing many novel neural-specific genes. We used a PCR-based subtraction strategy to identify neural-specific genes that are differentially expressed in *Xenopus* gastrula dorsal ectoderm, by subtracting an uninduced ectoderm cDNA library from a mid-gastrula dorsal ectoderm cDNA library. Subtractive enrichment was monitored by general removal of radioactive tracer cDNA, removal of ubiquitous translation factor *EF-1α* and enrichment for known dorsal ectoderm genes. 75 different clones were identified by differential blot analysis and DNA sequencing from 800 cDNAs randomly chosen from the subtracted dorsal ectoderm library. In situ analysis showed that 44 different clones were specifically expressed in gastrula ectoderm, with 26 of 44 clones specific to dorsal regions, and 30 other clones (undetectable in gastrula) later expressed in restricted domains in the developing nervous system. The complex molecular pattern in the gastrula dorsal ectoderm revealed by these clones show that gastrula stage neurectoderm is determined and initially regionalized. Some of the isolated clones were novel sequences, while others contain DNA-binding motifs suggesting that they encode transcriptional regulatory proteins. Five isolated clones (including the known homeobox gene *otx2*) are candidate genes for involvement in early neural determination and patterning because they are abundantly and differentially expressed in presumptive neurectoderm at gastrula stage, and later persist in various parts of the nervous system.

2.3 Introduction

The molecular basis of the early neural determination and patterning is starting to be revealed by identifying the genes involved. Classical embryologists showed that the vertebrate nervous system is induced by the dorsal organizer from embryonic ectoderm during gastrula stages. During gastrulation, the dorsal mesoderm involutes to underlie the dorsal ectoderm and organizer-secreted signals mediate neural induction (reviewed in Harland and Gerhart, 1997; Sasai and DeRobertis, 1997).

Experiments with transplants, or with peptide-treated explants or conjugates have shown that the maximal competence of the *Xenopus* ectoderm to respond to neural induction is at early gastrula (Jones and Woodland, 1987; Kintner and Dodd, 1991; Nieuwkoop, 1985; Servetnick, et al., 1991; Sive, et al., 1989). By mid-gastrula stage, anterior neural tissue has been specified in the dorsal ectoderm (Sharpe and Gurdon, 1990; Sive, et al., 1989) by organizer-derived neural inducers (such as noggin, chordin, follistatin) antagonizing BMP4, the signal specifying epidermis (Knecht and Harland, 1997; Lamb, et al., 1993). It is now accepted that posterior ectoderm is transiently specified as anterior neural tissue, before being re-specified to its final posterior neural fate (Nieuwkoop, 1952a;; reviewed by Kolm and Sive, 1997). Retinoids, FGF and wnt signaling are all implicated in posteriorizing initially induced anterior neurectoderm (Blumberg, et al., 1997; Isaacs, et al., 1992; Kolm, et al., 1997; McGrew, et al., 1997; McGrew, et al., 1995).

Dorsoventral (D/V) neural tube patterning also starts during gastrula. One possible mechanism for D/V neural tube patterning is a gradient of organizer-secreted neural inducer that acts across the medio-lateral extent of the open neural plate (Morgan and Sargent, 1997). The notochord/ floor-plate sonic hedgehog signal and TGF-β related factors from the non-neural ectoderm are also involved in determining D/V neural tube fates (Bang, et al., 1997; Liem, et al., 1995). Many transcription factors have been identified in restricted neural tube domains, and may determine specific neuronal cell types. Examples include the pax (Bang, et al., 1997); lim, islet-1 (Pfaff, et al., 1996; Tsuchida, et al., 1994), nkx (Price, 1993; Saha, et al., 1993), msx (Davidson and RE, 1991), and HNF-3β (Dirksen, et al., 1994; Ruiz i Altaba and Jessell, 1992b) gene families. Furthermore, bHLH genes involved in neurogenesis (i.e. *neurogenin, Xash3, neuroD*) have also been isolated.

However, few molecular markers expressed in the gastrula dorsal ectoderm have been isolated (i.e. *eIF4AII* (Morgan and Sargent, 1997); *otx2* (Blitz and Cho, 1994; Pannese, et al., 1995); *HoxD1* (Kolm and Sive, 1995a). So there is still little known about the molecular basis of the ectodermal response to neural induction, and the initial steps in neural patterning. We used subtractive cloning to identify genes specifically expressed in the mid-gastrula dorsal ectoderm after neural induction, but before neural differentiation. Our goal was to generate a repertoire of early neural-specific genes that may mediate early neural determination. We identified five candidate neural patterning genes on the basis of their abundant expression in gastrula dorsal ectoderm and continued expression later in neural tissue. Four encode putative DNA-binding proteins (DB9, fkh5, opl, otx2), suggesting that they regulate early neural development at a transcriptional level.

2.4 Results

2.4A Subtractive cloning strategy

Details of the embryological tissue used and subtraction strategy are shown in Figures 2.1 and 2.2. Please see Methods for details on the protocol.

In summary, 800 explants of dorsal ectoderm were dissected from mid-gastrula (st. 11.5) embryos, and mRNA harvested for the Dorsal ectoderm (D) cDNA library. A similar number of uninduced ectodermal explants (animal caps) were dissected from late blastula (st. 9) embyros, and aged until control embryos reached mid-gastrula stage before mRNA was harvested for the Uninduced ectoderm (U) cDNA library. After cDNA synthesis and restriction digestion to generate uniformly sized partial cDNAs, different sets of PCR primer-linkers were ligated onto each library. Two parallel sets of subtractions were performed: one to enrich for D-specific sequences, and the other to enrich for U-specific sequences. The U-specific subtractions also serve to generate subtracted drivers for subsequent rounds of subtractions. The subsequent screen was carried out on the subtracted D-specific library to search for neural-specific markers.

2.4A Monitoring subtractive enrichment

The success of subtractive enrichment was monitored in two ways: 1) an expected decrease in the amount of tracer cDNA after each subtraction, and 2) the relative enrichment for known tracer-specific genes (*Xotx2*, *XANF-1*), and the removal of a known common gene (translation factor *EF-1α*).

The amount of tracer cDNA should decrease after each successful round of subtraction, and is expected to plateau as subtraction reaches completion. This is determined by measuring the removal of radioactively labeled tracer. In the D-specific subtraction, 20% of tracer remained after two rounds, 12% remained after four rounds, 4% remained after six rounds, 1.8% remained after eight rounds, and 0.9% after ten rounds (Figure 2.3). In the U-specific subtraction, 12% of tracer remained after two rounds, 4% remained after four rounds, 2% remained after six rounds, 1% remained after eight rounds, and 0.9% remained after ten rounds (Figure 2.3). The logarithmic removal of tracer after each round suggested successful subtractions, and these data suggested that greater than 99% of starting cDNAs were removed after ten rounds for both subtractions. Still, the failure to reach a stable plateau in tracer removal after ten rounds of subtraction

suggested that more subtractions were needed for optimal enrichment. Monitoring tracer removal is a useful indication of successful bulk subtraction of starting tracer material, but does not indicate what genes are represented in the enriched population.

To confirm that D-specific subtractions were enriching for D-specific genes, the tracer population was evaluated for increased representation of known D-specific genes (*Xotx2*, *XANF-1*), and decreased representation of a known common gene (translation factor *EF-1α*). *Xotx2* and *XANF-1* are known mid-gastrula dorsal ectodermal markers (Blitz and Cho, 1994; Pannese, et al., 1995; Zaraisky, et al., 1992). Shown in Figure 2.4, *Xotx2* and *XANF-1* increased in abundance during successive subtractions, and reached 40-fold and 15-fold maximal enrichment after ten rounds, respectively. The ubiquitously expressed translation factor gene *EF-1α* was enriched in the first two rounds of subtraction, then removed in successive rounds, and decreased 10-fold in representation in the tracer after ten subtractions. Mesodermal contamination was assessed by analysis of *brachyury* RNA, which was undetectable in the D tracer cDNA (data not shown).

In summary, the progress of subtractions was monitored by measuring general removal of tracer cDNA and successful enrichment for known D-specific genes. Removal of tracer cDNA did not reach a stable plateau after ten rounds of subtraction, which indicated that more subtractions were needed to achieve maximal enrichment. In this subtraction, removal of greater than 99% of the original complex tracer cDNA population was achieved. Analyses of the D_{10} tracer population for *Xotx2* and *XANF-1* showed 15 to 40 fold enrichment for dorsal-specific genes, and efficient removal of 90% of the common, ubiquitously expressed gene *EF-1α* after ten rounds of subtraction.

2.4B Screening the subtracted gastrula dorsal ectoderm library

The objective of the subtraction screen was to identify neural-specific genes expressed by gastrula stage in the dorsal ectoderm. To identify clones that are differentially expressed in the gastrula dorsal ectoderm within the D_{10} tracer population, dot blot analysis was first used as a preliminary screen. A subgroup of clones that hybridized more strongly with subtracted dorsal-specific probe was further analyzed by DNA sequencing. Then, whole-mount in situ hybridization analysis was used to define the expression

domains of putative dorsal ectodermal genes in gastrula, neurula and tailbud embryos. A summary of the screen is shown in Table 2.1.

2.4B1 Characterizing the subtracted dorsal ectoderm library

The D_{10} tracer cDNA population with average size of 300 bp (range 150 to 1000 bp) was subcloned into CS2+ vector (Rupp, et al., 1994). 800 clones were randomly selected and screened by dot blot analysis with subtracted probes (prepared from D_{10} and U_{10} cDNAs) to identify cDNAs expressed more strongly in the dorsal pool. The resulting 194 putative dorsal-specific clones were sequenced and grouped into 75 different sequences. Seven were identified as partial sequences of known genes, including the ubiquitous translation factor *EF-1α*, the structural genes *centrosominA* and *cytochrome P450*, and genes encoding putative DNA-binding proteins (*NGF-IB, Xotx2, krox24, SP120*). Some of remaining novel clones also contained sequences with high similarity to known DNA binding motifs, suggesting that they may encode transcriptional regulatory proteins. Among the 75 different DNA sequences, 41 (55%) were unique clones (represented only once), suggesting that we successfully enriched for rare sequences in the starting tracer population. Some of these unique clones may also represent rare common sequences that were not sufficiently removed in this subtraction series. All of the common structural and housekeeping genes identified fell into the group of 34 multiply-represented clones, as expected from their abundance in the starting tracer population. The translation factor *EF-1α* was found 15 times, *centrosominA* 8 times, and *cytochrome P450* twice. The other 31 multiply-represented clones includes genes containing DNA-binding motifs such as *NGF-IB, Xotx2, krox24, SP120*, and is probably an enriched population of unknown, rare dorsal ectodermal transcripts. 14 sequences (41% of all multiply-represented clones) are found only twice, 6 sequences(18%) are found 3 times, and 11 sequences (32%) are found more than 4 times.

2.4B2 In situ analysis of dorsal-specific clones

In situ analysis revealed that many of the isolated dorsal ectodermal cDNAs were primarily expressed in neural-specific precursors or neural-derived tissues. Despite the small size of in situ probes generated from the subtracted tracer cDNA (average size 300 bp), signal could be detected reliably in embryos after whole mount in situ hybridization (faint signals were better visualized after clearing the embryos). The expression domains

visualized with short cDNA probes were later confirmed in several cases with larger cDNA probes made from isolated full-length cDNAs.

By in situ hybridization, 45 clones were detected in the gastrula ectoderm, and 44 of these were detected in gastrula dorsal ectoderm: 26 of 44 were strongly expressed in dorsal ectoderm and either undetectable or very weak in ventral ectoderm, 18 of 44 were expressed equally in both dorsal and ventral ectoderm, 1 of 44 was not expressed in gastrula ectoderm. Of the known gastrula dorsal ectodermal genes *Xotx2* was identified, but *XANF-1* was not found. *XANF-1* was found after more D-specific subtractions were carried out (Gamse et al., unpublished), and probably would have been isolated in this subtraction series if more than 800 clones were initially chosen for analysis. Of the 30 sequences not detectable in gastrula stage embryos, 19 were expressed in dorsal ectoderm-derived tissues of neurula or tailbud stage embryos. Expression patterns of novel dorsal clones at specific embryonic stages are described in Tables 2.2. Figure 2.2 presents the significant neural-specific expression patterns of 34 novel dorsal ectodermal clones in tailbud stage embryos. In addition to neural expression, nine cDNAs are also expressed in dorsal mesoderm-derived tissues like somites and notochord (DA68, DA99, DB24, DB178, DB187, DC44, DC108, DC190, DD167), with one cDNA (DD92) expressed only in ventral ectoderm-derived tissues.

In summary, many novel cDNA clones that are expressed in neural precursor or neural tissue were identified in the enriched D_{10} tracer cDNA population. 75 different sequences were found with 44 different clones detectable in gastrula dorsal ectoderm. Of the 44 gastrula dorsal ectoderm clones, 26 were dorsal ectoderm-specific transcripts, and the remaining 18 were expressed throughout both dorsal and ventral ectoderm. 30 other clones were undetectable in gastrula ectoderm, but 19 of these 30 clones were later expressed in neurula or tailbud nervous systems. Many novel cDNA clones identified contain known DNA-binding motifs, suggesting that they encode transcriptional regulators.

2.4C Interesting clones

Our criteria for interesting clones were as follows: strong expression in the gastrula ectoderm (better if restricted to dorsal ectoderm), and persistent expression in presumptive neural tissue. Those genes that match these

criteria were thought to be more likely to mediate ectodermal response to neural induction. The five genes shown in Figure 2.6 are clones that match the above criteria. Each is abundantly expressed in gastrula dorsal ectoderm, persists in neural folds, and continues to be expressed in the tailbud nervous system.

2.4C1 DD135

At the gastrula stage, DD135 is strongly expressed throughout the dorsal ectoderm, with faint signal in ventral ectoderm (panel a). It is strongly expressed in an anterior neural fold domain in neurula (panel b), and then continues to be strongly expressed in forebrain, midbrain, hindbrain and spinal cord, the eyes and branchial arches (panel c). The high expression level of this clone is reflected in the fact that its sequence was identified 17 times in the screen. BLAST searches with the partial cDNA showed no homology with known genes in the database. Work in progress to isolate a full-length cDNA to continue further study on this abundantly expressed novel, neural-specific clone.

2.4C2 DB9

Clone DB9 is also strongly expressed in the gastrula dorsal ectoderm (panel d), and restricted to a smaller anterior neural fold domain by neurula stage (panel e), then later expressed strongly in the forebrain, forebrain/midbrain junction, midbrain, hindbrain and spinal cord, eye and weakly in otic vesicle and branchial arches (panel f). Northern analysis showed that DB9 corresponds to a 3.2 kb mRNA in embryos (data not shown), and comparison of a partial cDNA with the known database indicated high similarity with the gene for XLSSB2, a *Xenopus* mitochondrial single-stranded DNA-binding protein (Champagne, et al., 1997). XLSSB2 encodes subunit polypeptides of a DNA-binding protein complex involved in mitochondrial DNA replication. Given its early and persistent expression in a restricted ectodermal domain, DB9 may encode an XLSSB2-like DNA-binding protein involved in early neural development.

2.343 fkh5

Shown in the next row is the in situ analysis of a novel clone which was named *forkhead-5* (*fkh5*); its full length cDNA sequence encodes a protein with an N-terminal forkhead DNA binding domain, and a high degree of similarity to the mouse winged helix transcription factor FKH5 (Kaestner, et al., 1996; Knöchel, et al., 1992). It is expressed in two bilateral dorsal

ectoderm regions by mid-gastrula (panel g), and restricted to domains in the anterior and posterior neural folds by neurula (panel h), then expressed in midbrain and hindbrain domains by tailbud stage (panel i). A highly similar *Xenopus forkhead* gene has been identified by Dirksen and colleagues, but its expression is limited to a subset of epidermal cells and floor plate of neural tube (Dirksen, et al., 1995) . Detailed analysis of *fkh5* is in progress by J. Gamse in our lab.

2.4C4 opl

Next shown is *opl*, a gene very abundantly expressed in dorsal ectoderm at the gastrula stage (panel j), then localized to the lateral margins of the anterior neural folds by neurula stage (panel k), and later expressed in a complex pattern throughout the dorsal brain and spinal cord at tailbud stage (panel l). *opl* encodes a zinc-finger transcription factor highly related to the *Drosophila* pair-rule gene, *odd-paired*, and most similar to the mouse *zic-1* gene. Its detailed analysis and possible roles in neural patterning are reported in Chapter 3 (Kuo, et al., 1998).

2.4C5 otx2

Also isolated is *otx2*, the *Xenopus* homologue of *Drosophila orthodenticle,* a homeobox gene implicated in *Xenopus* cement gland induction and anterior neurectoderm patterning (Blitz and Cho, 1994; Gammill and Sive, 1997; Pannese, et al., 1995). We successfully used *otx2* to check for subtractive enrichment of dorsal ectoderm genes (panels m, n o). Its detailed analysis in cement gland induction and neural patterning in *Xenopus* has been reported elsewhere (Blitz and Cho, 1994; Gammill and Sive, 1997; Pannese, et al., 1995).

In summary, these five genes identified in our subtraction screen are candidate neural patterning genes because they are expressed in the right place and at the right time to play a regulatory role in early neural determination or patterning. All (except DD135 so far) are identified as genes that encode DNA-binding proteins. Persistent expression in restricted parts of the nervous system suggest they may also be involved in later steps of nervous system development.

2.5 Discussion

2.5A Identification of candidate early neural patterning genes

We successfully used PCR-based subtractive cloning to identify many novel cDNAs that are specifically expressed in the gastrula dorsal ectoderm. Since neural determination occurs in dorsal ectoderm starting at gastrula stage, such genes are present in the right place and time to mediate ectodermal response to neural inducers. Many identified sequences contain DNA-binding motifs, and may encode transcriptional regulators. We identified five cDNAs strongly expressed in gastrula dorsal ectoderm that are candidates for early neural patterning genes. *Xotx2*, a *Drosophila orthodenticle* homologue, was already shown by others to be involved in cement gland induction and anterior neural patterning in *Xenopus* (Blitz and Cho, 1994; Gammill and Sive, 1997; Pannese, et al., 1995). Chapter 3 of this thesis describes the analysis of *opl*, which encodes a zinc-finger transcriptional activator with multiple roles in neurogenesis (Kuo, et al., 1998). Further analysis of other candidate genes (DD135, DB9) is in progress (Gamse et al., in preparation).

2.5B Advantages of subtractive cloning

Others have chosen to identify candidate genes involved in neural development by cloning homologues of known genes encoding DNA-binding proteins (Dawid, et al., 1995; Lee, et al., 1995; Ma, et al., 1996; Pannese, et al., 1995; Ruiz i Altaba and Jessell, 1992b; Tsuchida, et al., 1994), or by searching for genes encoding components of growth factor signaling pathways or those with oncogenic potential (McMahon, 1992; Sirad, et al., 1998). Although successful, these strategies may miss possible candidate genes due to the starting bias for a subset of known genes. PCR-based subtractive cloning was used to identify a non-biased repertoire of gastrula dorsal ectodermal genes that are expressed early in the presumptive neurectoderm. We used subtraction to enrich for rare, dorsal ectoderm-specific transcripts, followed by random sampling and differential hybridization of the subtracted dorsal ectoderm library (Patel, et al., 1996).

Subtraction is a powerful method to identify genes differentially expressed in one tissue versus another tissue population. Due to the high complexity of the starting tracer cDNA population, reiterative rounds of subtraction were needed both to enrich for rare differential transcripts and to

remove abundant common transcripts. PCR amplification between subtraction rounds was used to regenerate tracer, and made subtractive cloning feasible with the limited amount of source tissue: dissected dorsal ectoderm. Of note, PCR introduced a bias for amplifying short products in the 300 bp range, so the initial tracer cDNA population was first digested with AluI and RsaI in order to maintain proportionate representation of long and short transcripts. Enzymatic digestion into cDNA fragments gave rise to two potential problems: loss of directionality and loss of sensitivity for in situ analysis. The first problem was resolved by testing in situ probes in both directions, but the second issue was not a problem: short in situ probes (average size 300 bp) were successfully used in whole-mount in situ analysis. Using partial cDNAs for subtraction necessitated an extra step of isolating full length cDNAs from another library in order to fully test desired genes.

Ultimately, ten rounds of subtraction were not sufficient to reach saturation for this screen, as seen in the lack of a plateau in removal of *EF-1α* cDNAs (Figure 2.4). It was still a very successful subtraction screen in terms of identifying many previously unknown transcripts expressed in neural-specific tissues. A random sampling of 800 clones from the subtracted tracer population yielded 75 different sequences, with 44 already detectable in gastrula ectoderm (Table 2.1). 5 genes were chosen as candidate genes for early neural determination due to their high level of gastrula dorsal ectodermal expression and persistence in specific neural structures later in development.

For a comprehensive review of the theory and application of subtractive cloning, see Sagerström, et al., (1997). Differential display is another powerful technique for identifying differences in gene expression between tissues, but is less likely to yield a complete repertoire of differential genes (Liang, et al., 1995; Patel and Sive, 1996). Positive selection was not used because rare differential transcripts in complex starting cDNA populations are usually lost in the such schemes (Sagerström, et al., 1997).

2.5C Improvements on the current subtraction

The use of PCR to regenerate tracer cDNA between subtractions is a key advantage when working with limited amounts of starting tissue, but its use introduces a bias for small cDNA products (average size 300 bp). Instead of restriction digestion of the starting cDNA, another method for generating

uniformly sized small tracer cDNAs is to use high concentrations of random hexamers to limit the size of first-strand cDNA synthesis. Theoretically, the representation of long versus short transcripts of equal abundance should be maintained by the different primer annealing rates on long versus short transcripts. There may also be increased overlap between clones that represent fragments of the same transcript, thereby increasing the likelihood that slot blot analysis and DNA sequencing would more efficiently group clones for in situ analysis. This modification was used successfully in our lab in another later subtraction cloning project (Sagerström et al., in preparation).

Directionality can be introduced in the initial cDNA synthesis by using a specific 3' random hexamer-linker. This will enable directionality to be maintained throughout the reiterative subtractions and later subcloning of the subtracted tracer cDNA. Knowledge of the orientation will allow correct synthesis of probes for in situ analysis, and speed isolation of the full length cDNA.

2.5D Analysis of interesting clones

2.5D1 Identified clones are early markers of prospective neurectoderm

This subtraction screen generated a panel of molecular markers that are specifically expressed in neural progenitors. When detected in the gastrula dorsal ectoderm, these markers are expressed in regions determined to become neurectoderm. The very early expression of many markers (*opl*, *otx2*, *fkh5*, DD135) indicates that neural determination may have already started by early gastrula stage. The different, restricted expression domains of these markers (i.e. *opl, otx2, fkh5*) suggest that a complex molecular pattern in the mid-gastrula dorsal ectoderm, and may reflect early regionalization of the presumptive neurectoderm. Characterization of the expression domains of multiple markers via whole-mount in situ analysis will yield detailed information about the molecular pattern in induced neurectoderm.

2.5D2 Early neural-specific genes may regulate early neural determination

This subtraction yielded candidate genes that are expressed in the gastrula dorsal ectoderm that may regulate the ectodermal response to neural induction and early neural patterning. At least five clones are expressed at high level at the right place and time for involvement in early

neural determination functions (Figure 2.5). What are possible roles for mid-gastrula dorsal ectodermal genes?

Since they are expressed during the time of neural induction, one possible role is regulation of neural competence. Others have shown that protein kinase C-α isozyme activity (Otte, 1992a; Otte, et al., 1991) , Xotch receptor signaling (Coffman, et al., 1993) and overexpression of RNA helicase translation initiation factor eIF4AII increase ectodermal competence to respond to neural inducers (Morgan and Sargent, 1997).

Another process that occurs in the gastrula ectoderm is specification of A/P or mediolateral neurectodermal pattern. Not only are isolated genes useful as regional dorsal ectodermal markers in gastrula ectoderm, they may also be involved in specifying cell fates along either the A/P or mediolateral axes.

Misexpression in *Xenopus* embryos and isolated ectoderm is a useful technique to uncover possible functional roles. Functional ablation studies will reveal the functional requirements for such genes in neural development. Such a genetic 'knock-out' experiment is now theoretically feasible in *Xenopus* embryos via the introduction of antisense transgenes (Kroll and Amaya, 1996). Comparative and complementary studies with isolated homologues in other vertebrate species (zebrafish, mouse) will be important to define common molecular mechanisms in neurogenesis. Chapter 3 will describe the detailed analysis of one very interesting clone identified in this screen, *opl*.

Future challenges include understanding the upstream regulation of these early neurectodermal genes, defining their roles in early neural determination, and elucidating their downstream targets in order to formulate a molecular mechanism for early neural development.

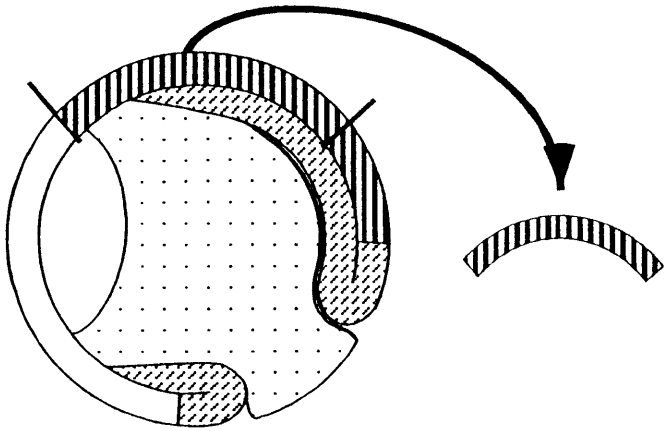
2.6 Acknowledgments

We thank Joshua Gamse for substantial help with the screen, David Willison for help with sequencing the dorsal-specific clones, Cathy Nocente-McGrath for making the mid-gastrula λZap phage library, Melissa Rones for helping isolate the full length *opl* cDNA. We greatly appreciate the helpful comments and discussions with members of the Sive lab during the long course of this work. This work was supported by grants from the NSF and NIH to H.L.S. We appreciate support from Genetics Institute, Cambridge MA. J.S.K. was the recipient of a Medical Scientist Training Program Fellowship (PHS grant 2-T32-GM07753) from the Harvard MD-PhD Program. M.P. was the recipient of a fellowship from the Human Frontiers Science Program. H.L.S. was the recipient of a NSF Young Investigators Award and the Latham Family Development Professorship at MIT.

Figure 2.1. Tissues used in subtraction.

Induced dorsal ectoderm were dissected from mid-gastrula (st. 11.5) stage embryos as shown and harvested immediately for dorsal ectoderm (D) mRNA. Uninduced ectoderm were dissected as animal caps from late blastula (st. 9) stage embryos and aged (with sibling control embryos) to mid-gastrula stage (st. 11.5) equivalent before being harvested for uninduced ectoderm (U) mRNA.

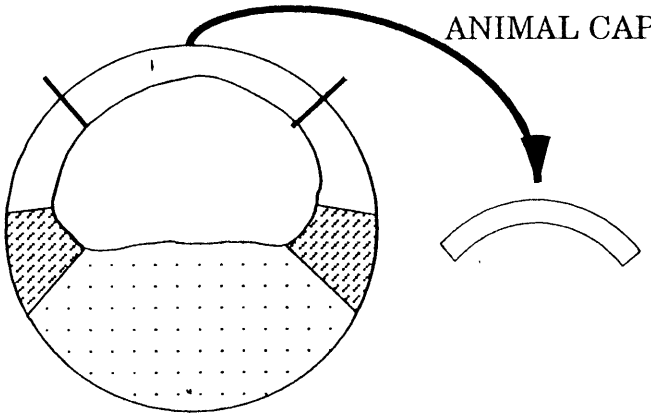
DORSAL ECTODERM (D)



HARVEST IMMEDIATELY → **mRNA (D)**

ST. 11.5 (MID-GASTRULA)

UNINDUCED ECTODERM (U)



AGE TO ST. 11.5 EQUIV. → **mRNA (U)**

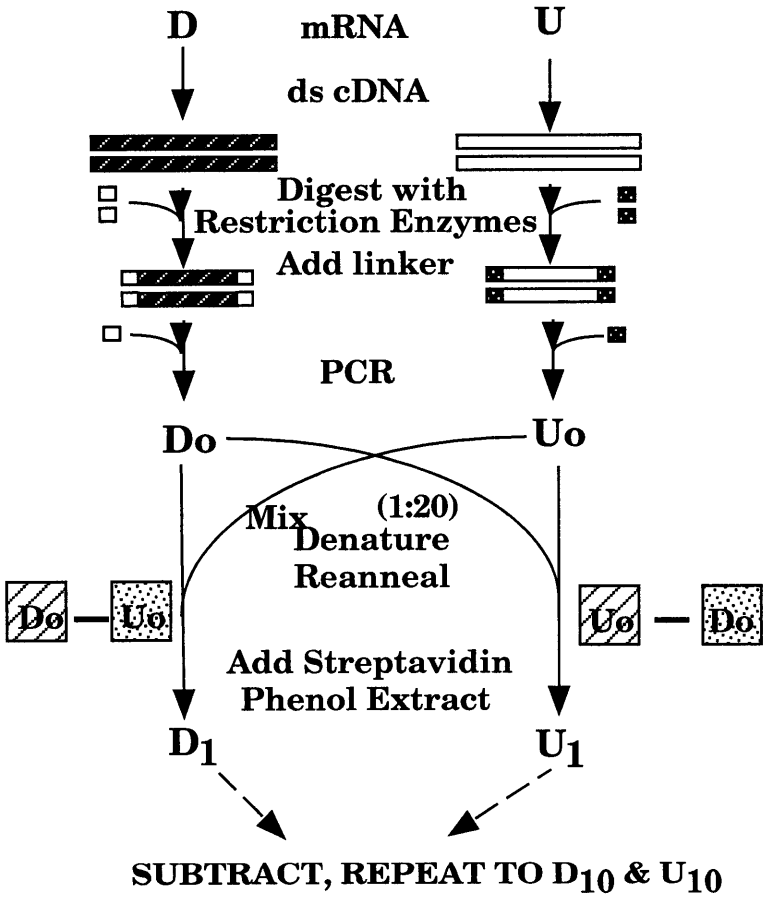
ST. 9 (BLASTULA)

UNINDUCED ECTODERM
INDUCED DORSAL ECTODERM
MESODERM
ENDODERM

Figure 2.2. Subtraction scheme.

The detailed methodology is described in Sive and Patel (1997 REF). Briefly, mRNAs prepared from dorsal ectoderm (D) and uninduced ectoderm (U) were converted to cDNA (Pharmacia Timesaver cDNA synthesis kit). The cDNAs were digested with AluI and RsaI, then ligated with different sets of linkers (D-specific PCR primer + EcoRI site/ U-specific PCR primer + EcoRV site). Before each round of subtraction, tracer cDNA (from the D population) was PCR-amplified in the presence of ^{32}-P-dCTP, and driver cDNA (from the U population) was PCR-amplified in the presence of biotinylated-11-dUTP (Enzo Diagnostics). Subtractive hybridization was carried out at 68°C in a Perkin-Elmer thermocycler by mixing the tracer to driver at 1:20 proportion, with long hybridizations (40 hours) alternating with short hybridizations (2 hours) to remove rare common sequences and abundant common sequences, respectively. Biotinylated duplexes were removed by treatment with streptavidin and phenol extraction (Sive and St. John., 1988). The enriched tracer and driver cDNAs were amplified for the next round of subtraction. Ten rounds of subtraction were performed to produce D_{10} and U_{10} cDNAs. A similar procedure was performed in parallel (with the U cDNA serving as tracer, and D cDNA serving as driver) to generate enriched U drivers for subsequent D-specific subtractions. This figure was taken from (Patel and Sive, 1996)

SUBTRACTION PROTOCOL.



Tracer is labelled with trace α ^{32}*P-dCTP.*

Driver is labelled with bio-11-dUTP.

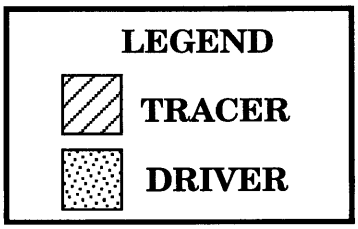


Figure 2.3. General removal of tracer by subtraction

Removal of tracer was monitored by assaying for the remaining fraction of ^{32}-P-labeled tracer after each round of subtraction. On this graph, the round of subtraction is shown on the x-axis, and % DNA remaining after each subtraction is shown on the y-axis. The dorsal-specific subtraction (D-U) is plotted with squares, and the uninduced-specific subtraction (U-D) is plotted with open circles. The amount of tracer before the first round of subtraction is set at 100%. Both subtractions show 0.9% of starting tracer remaining after 10 rounds of subtraction, with similar removal kinetics.

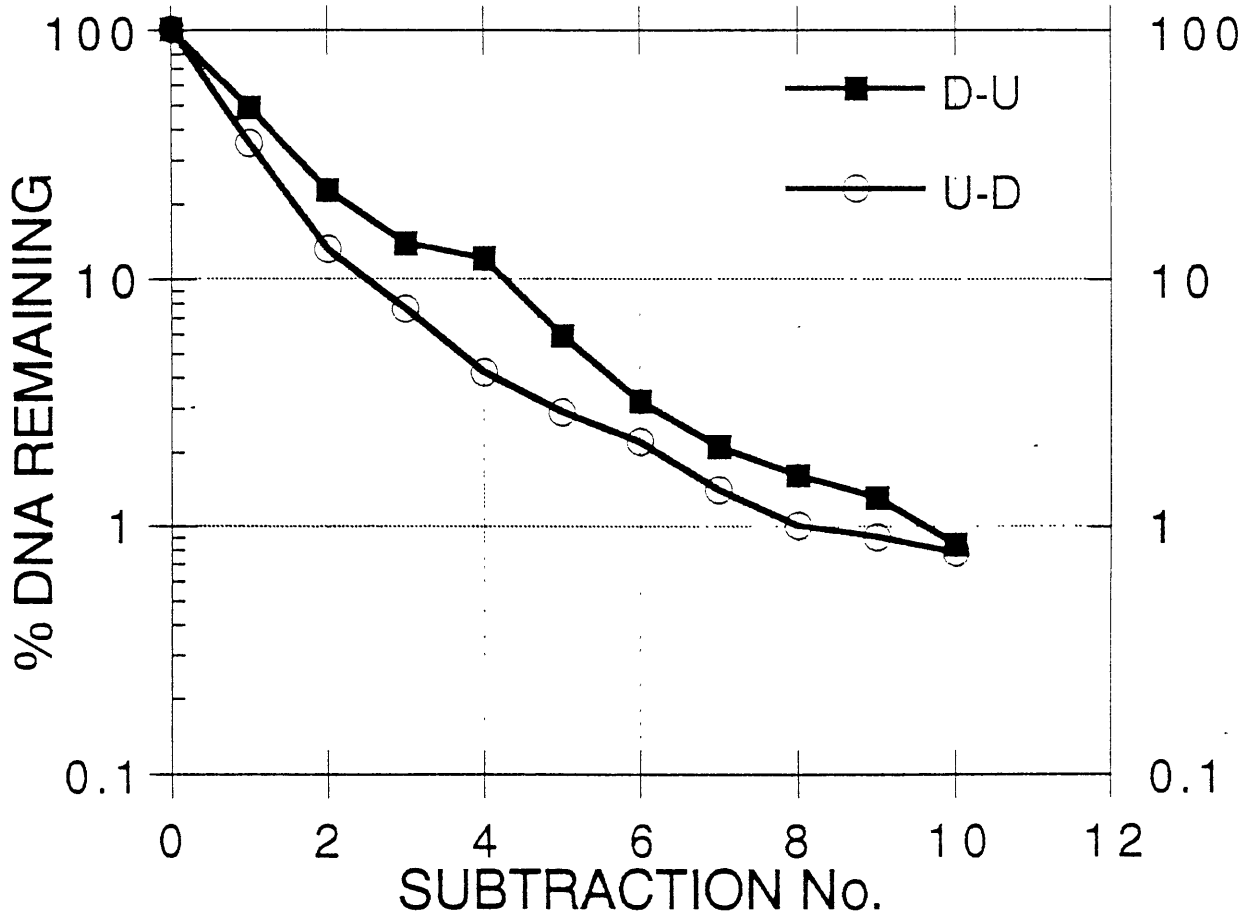

100
10
1
0.1
% DNA REMAINING
D-U
U-D
0
2
4
6
8
10
12
SUBTRACTION No.

Figure 2.4. Specific enrichment and removal of genes by subtraction.

The abundance of specific genes were monitored by dot blot analysis during the subtractions: dorsal ectoderm specific genes (*otx2*, *XANF-1*) and the ubiquitous translation factor gene *EF-1α* The round of subtraction is shown on the x-axis, and the % of maximal enrichment is shown on the y-axis. The values for *XANF-1* are plotted with squares, for *otx2* with open circles, and for *EF-1α* with triangles. The dorsal-specific genes are progressively enriched, with *otx2* enriched maximally at 40X and *XANF-1* enriched to 15X. *EF-1α* is enriched in the first two rounds, then progressively removed until on 0.1X remain after ten rounds of subtractions.

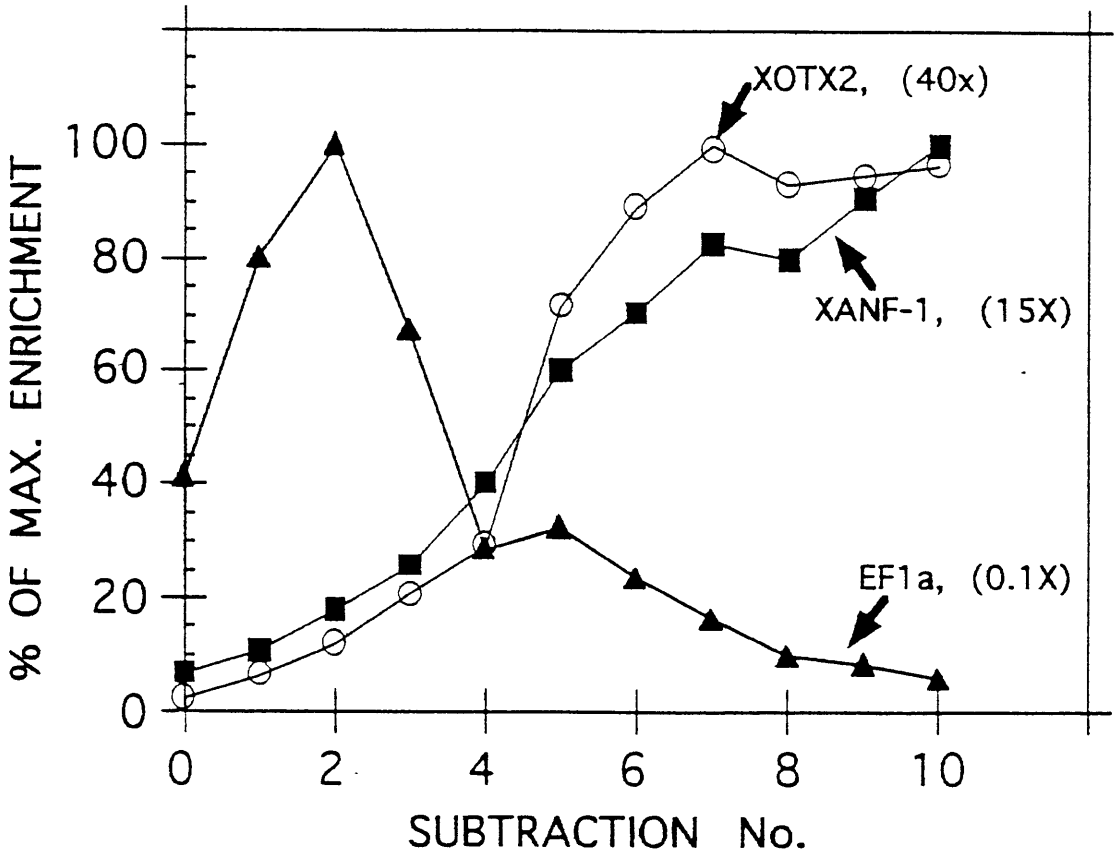
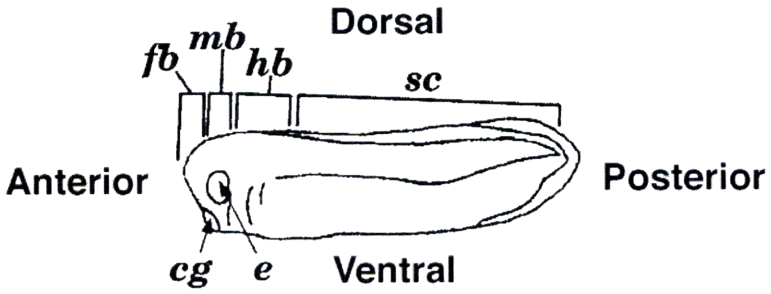
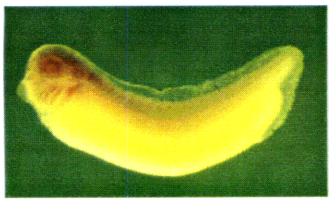

XOTX2, (40x)
XANF-1, (15X)
EF1a, (0.1X)
% OF MAX. ENRICHMENT
100
80
60
40
20
0
0
2
4
6
8
10
SUBTRACTION No.

Figure 2.5. Isolated dorsal-specific clones are expressed in the tailbud nervous system.

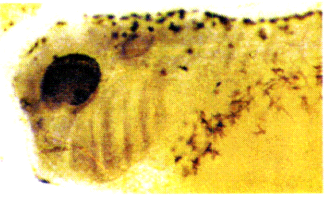
Whole mount in situ analyses of 34 of the isolated gastrula dorsal ectodermal clones are shown here. Antisense controls for all probes showed background staining (data not shown). Shown at the top is a schematic of a tailbud embryo with anterior to the left, posterior to the right, dorsal to the top and ventral to the bottom. cg: cement gland, e: eye, fb: forebrain, mb: midbrain, hb: hindbrain, sc: spinal cord. In all panels, the same general orientation (anterior to the left, dorsal to the top) is used. The probe used is indicated under each panel. In some panels (i.e. DA142, DB8), later stage tailbuds were used in in situs, and show background melanophore pigment-containing cells along the dorsal and lateral lines which are not part of the detected expression domains. For details on the expression domains, please refer to Table 2.2



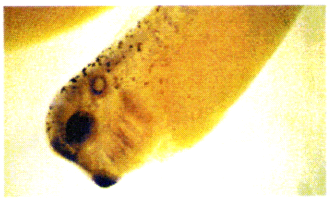

DA23

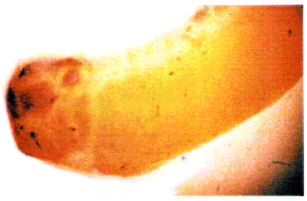

DA68

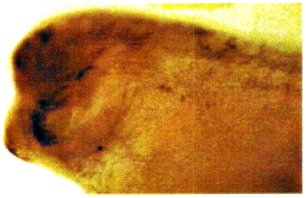

DA99

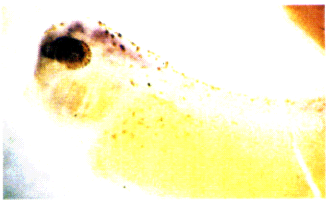

DA142


DA149

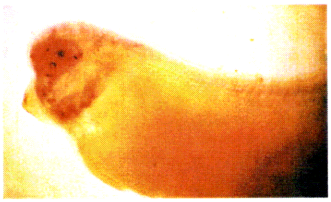

DA169

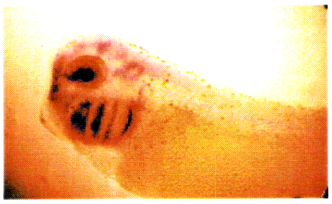

DA170


DA176


DB8

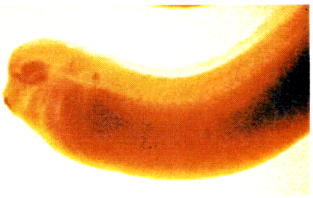

DB18


DB22

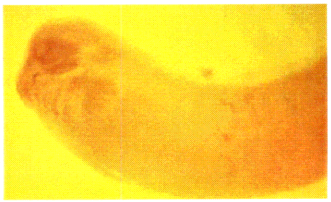

DB24


DB50


DB119


DB178

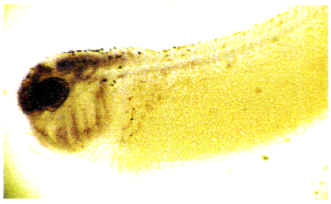

DB187


DB191

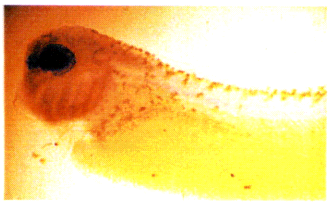

DB197


DC44


DC57



DC108

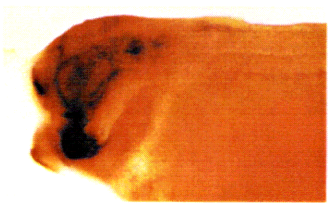


DC143

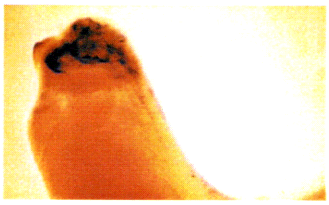
DC190

DC191

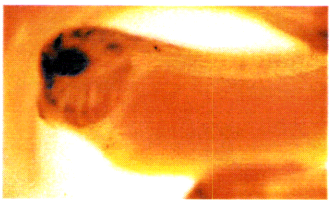
DD3



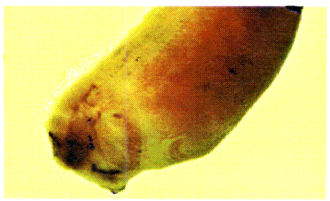
DD50



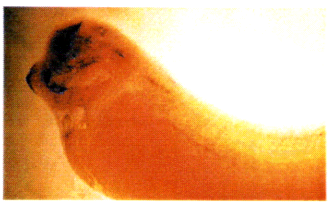
DD64



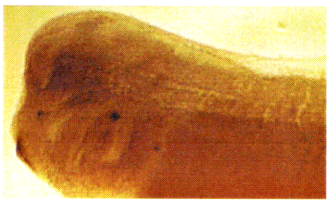
DD65



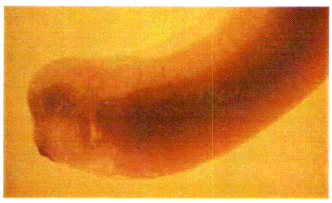
DD96



DD109

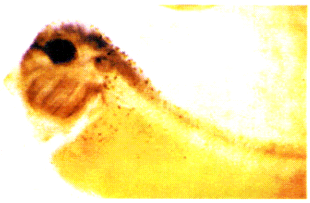


DD110



DD113

DD167



DD196

Figure 2.6. Five interesting clones identified in the subtraction screen.

These were deemed candidate neural patterning genes because they were expressed early at high level in gastrula dorsal ectoderm, and persists in neural domains through neurula and tailbud stages. Whole mount in situ analysis of gastrula, neurula and hatching stage embryos are shown here for each clone: DD135, panels a, b, c; DB9, panels d, e, f; *fkh5*, panels g, h, i; *opl*, panels j, k, l; *otx2*, panels m, n, o. In all the gastrula panels (a, d, g, j, m), dorsal views are shown with D=dorsal, V=ventral, and the black arrow points to the dorsal blastopore lip. In neurula panels (b, e, h, and n) lateral views are shown; in panel k, a dorsoanterior view is presented. In all the neurula panels, A=anterior, P=posterior, and some background staining in the archenterons are seen. In the hatching tailbud stage panels (c, f, i, l, o), anterior is to the left, and dorsal to the top, with f=forebrain, fm=forebrain/ midbrain boundary, m=midbrain, h=hindbrain and sc=spinal cord.

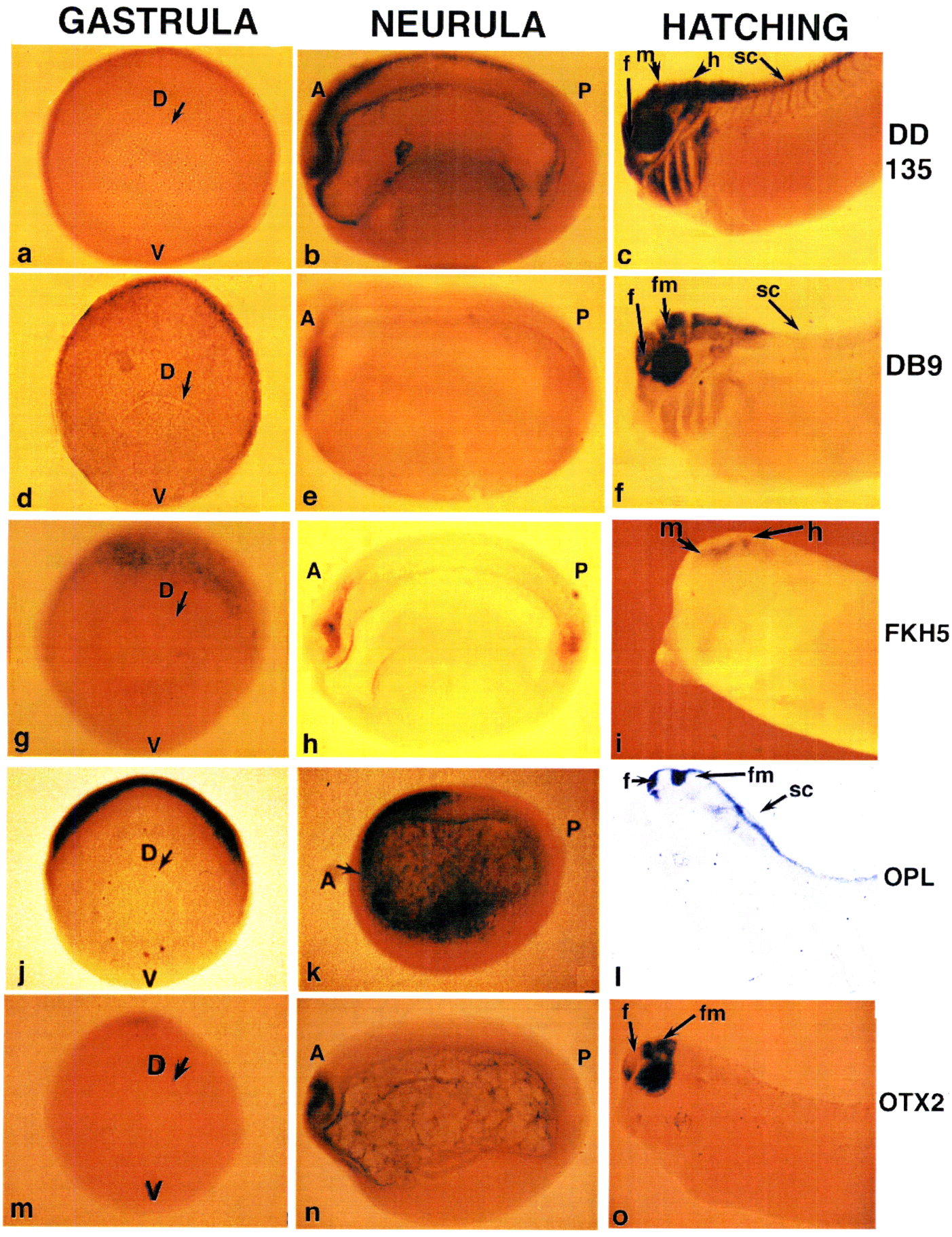

GASTRULA
NEURULA
HATCHING
D
V
A
P
f
m
h
sc
fm
DD
135
DB9
FKH5
OPL
OTX2
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o

Table 2.1. Summary of screen

# of clones screened by differential hybridization	800
# of dorsal-specific clones[a]	194
# analyzed by DNA sequencing	194
# of different sequences[b]	75
# of previously identified sequences[c]	7
# of clones represented more than once	34
# of unique clones[d]	41
# of dorsal-specific signal by Northern analysis on gastrula	7
clones detected in gastrula by in situ analysis[e]	
# giving DE > VE signal	26
# giving DE = VE signal	18
# giving non-ectodermal signal	1
total # of gastrula-specific transcripts	45
clones not detected in gastrula by in situ analysis[e]	
# with late expression in DE-derived tissues[f]	19
# undetectable	11
total # of clones not detectable in gastrula	30

[a] average insert size: 300 bp; range: 150-1000 bp.

[b] sequences were compared to each other with Seqman (DNA Star) software.

[c] sequences were compared to Genbank using BLAST program.

[d] some clones may represent non-overlapping fragments of the same gene.

[e] clones were chosen from the initial 194 putative DE-specific pool either randomly, or after sequence identification.

[f] these clones may be present as rare transcripts in gastrulas.

Table 2.2. Expression pattern of isolated gastrula dorsal ectodermal cDNAs by in situ hybridization analysis

		Stage-specific expression		
Clone[a]	In situ probe[b]	Gastrula[c]	Neurula[d]	Tailbud[e]
DA23	T7	D>V	NF	CNS, eye, OV, BA
DA68	T3	D>V	NF	FB, MB, HB, eye, OV, BA, somites
DA99	T7	faint D>V	faint anterior NF	FB, MB, HB, eye, OV, BA, somites
DA142	T3	faint D>V	none detected	faint FB, MB, HB, OV, BA
DA149	T7	D>V	NF	CNS, eye, OV, CN V, BA
DA169	T7	D>V	inner and posterior NF	faint CNS, OV, BA
DA170	T7	faint D=V	faint anterior inner NF	faint CNS, eye
DA176	T3	faint D>V	anterior NF	FB, MB, HB, anterior SC, eye, OV
DB8	T3	none detected	faint NF	faint FB, MB, HB, eye
DB9	T3	D>V	NF, placodes	CNS, eye, CN V
DB12	T3 + T7	none detected	none detected	none detected
DB18	T7	faint D>V	NF	CNS, eye, OV, BA
DB22	T7	none detected	faint NF	FB, MB, eye, BA
DB24	T3	not done	not done	CNS, eye, OV, BA, CN V, notochord

DB50	T7	faint D>V	NF	FB, MB, eye, OV, BA
DB101(fkh5)	T7	D>V	NF	FB, MB, SC
DB107	T7	faint D=V	NF	CNS
DB119	T7	none detected	NF	MB, HB, anterior SC, OV
DB178	T7	none detected	NF	CNS, somites
DB187	T7	faint D>V	NF	CNS, somites
DB191	T7	faint D>V	NF	faint FB, MB, HB
DB197	T3	faint D=V	NF	CN V
DC44	T3	faint D>V	NF	CNS, eye, somites
DC57	T3	none detected	NF	FB, MB, HB, anterior SC, eye
DC78	T7	none detected	NF	none detected
DC108	T3	not done	not done	CNS, eye, OV, BA, notochord
DC143	T3	not done	not done	faint FB, MB, HB, eye, OV, BA
DC167	T3 + T7	none detected	none detected	none detected
DC190	T7	D>V	NF	CNS, eye, OV, CN, somites
DC191	T3	faint D>V	NF	CNS, eye, OV, BA
DD3	T3	faint D>V	faint NF	faint CNS, OV
DD6	T7	D=V	NF	CNS, eye, OV, BA, somites
DD43 (opl)	T3	D>V	NF	CNS

DD50	T3	faint D>V	faint NF	faint FB, MB, HB, anterior SC, eye, OV
DD64	T3	faint D>V	faint inner NF	faint FB
DD65	T3	D>V	NF	FB, MB, eye, OV
DD77	T7	none detected	none detected	faint eye
DD92	T3	punctate D=V	punctate lateral etoderm	punctate lateral ectoderm
DD96	T7	faint D>V	faint posterior NF	faint FB, MB, HB
DD109	T3	faint D>V	faint NF	faint CNS
DD110	T3	faint D=V	faint NF	none detected
DD113	T7	none detected	NF	faint FB, MB, HB, eye, OV
DD135	T7	D>V	NF	CNS, eye, OV, BA
DD167	T7	faint D>V	faint NF	faint somites
DD185	T3	none detected	none detected	none detected
DD186	T3	none detected	faint inner NF	none detected
DD196	T3	none detected	NF	CNS, eye, OV, BA

[a] independent cDNA clones identified in subtraction screen.

[b] phage polymerase used to generate in situ probe.

[c] In situ analysis at gastrula stages (st. 10-12). D=dorsal ectoderm, V=ventral ectoderm, D>V indicates higher or expression only detectable in dorsal ectoderm. D=V indicates equal expression throughout ectoderm. Faint expression required clearing of embryos before visualization of signal. (See Methods for details).

[d] In situ analysis at neurula stages (st. 16-18). NF=neural folds. General staining of neural folds was observed except where restricted domains are indicated. Placodes indicate the placodal precursor region. Faint expression

required clearing of embryos before visualization of signal. (see Methods for details).

[e] In situ analysis at tailbud stages (st. 24-28). CNS=central nervous system, including all regions of brain and spinal cord. FB=forebrain, MB=midbrain, HB=hindbrain, SC=spinal cord, eye, OV=otic vesicle, BA=branchial arches, CN=cranial nerve, somites. Specific regions of expression are indicated when observed. Faint expression required clearing of embryos before visualization of signal. (see Methods for details).

Chapter 3. Analysis of *opl*: a gene with multiple roles in early neural development

3.1 Preface

This chapter was accepted for publication in *Development* as

Kuo, J.S., Patel, M., Gamse, J., Merzdorf, C., Liu, X., Apekin, V., and Sive, H. (1998) "opl: a zinc finger protein that regulates neural determination and patterning in *Xenopus*." *Development*, in press.

3.2 Summary

In order to study the mechanism of neural patterning in *Xenopus*, we used subtractive cloning to isolate genes activated early during this process. One gene isolated was *opl*, (*odd-paired*-like) that resembles the *Drosophila* pair-rule gene *odd-paired* and encodes a zinc finger protein that is a member of the *Zic* gene family. At the onset of gastrulation, *opl* is expressed throughout the presumptive neural plate, indicating that neural determination has begun at this stage, while by neurula, *opl* expression is restricted to the dorsal neural tube and neural crest. *opl* encodes a transcriptional activator, with a carboxy terminal regulatory domain, that when removed increases *opl* activity. *opl* both sensitizes animal cap ectoderm to the neural inducer *noggin* and alters the spectrum of genes induced by *noggin*, allowing activation of the midbrain marker *engrailed*. Consistent with the later dorsal neural expression of *opl*, the activated form of *opl* is able to induce neural crest and dorsal neural tube markers both in animal caps and whole embryos. In ventral ectoderm, *opl* induces formation of loose cell aggregates that may indicate neural crest precursor cells. Aggregates do not express an epidermal marker, indicating that *opl* suppresses ventral fates. Together, these data suggest that *opl* may mediate neural competence, and be involved in activation of midbrain, dorsal neural and neural crest fates.

3.3 Introduction

One of the first decisions made during neural determination in *Xenopus* is a general increase in the competence of the ectoderm to respond to subsequent neuralizing signals. Both transplantation experiments and exposure of explants to pure factors or to natural inducing tissue have shown that neural competence in *Xenopus* is maximal at early gastrula; by the end of gastrulation, ectoderm displays little competence to form neural tissue in response to mesoderm-derived signals (Nieuwkoop et al., 1985; Jones and Woodland, 1987; Sive et al., 1989; Servetnick and Grainger, 1991; Kintner and Dodd, 1991; Lamb et al., 1993; Knecht and Harland, 1997).

Superimposed on an increase in neural competence of the entire ectoderm is an enhanced competence of the dorsal ectoderm (the presumptive neurectoderm) for dorsal fates. The dorsal ectoderm is already biased towards dorsal fates by early blastula stages, since isolated dorsal ectoderm treated with activin goes on to form more dorsal mesoderm than does ventral ectoderm (Sokol, et al., 1991) . By early gastrula, isolated dorsal ectoderm fails to activate expression of the epidermal antigen, Epi-1, whereas this antigen is strongly specified in the ventral ectoderm, which gives rise to epidermis (Savage, et al., 1989) . Consistently, the dorsal ectoderm is more responsive than ventral ectoderm to neural inducing signals arising from the dorsal mesendoderm (Sharpe, et al., 1987).

By mid-gastrula, specification assays indicate that neural determination has occurred (Sharpe and Gurdon, 1990; Sive, et al., 1989). Initial neural induction is thought to be anterior in character, and appears to be mediated by antagonists of Bone Morphogenetic Proteins (BMPs) such as the secreted factors *noggin* and *chordin* (Eyal-Giladi, 1954; Sive, et al., 1989) , rev. in (Sasai and DeRobertis, 1997). Later, the posterior part of the neural plate is converted into posterior neural tissue. Retinoids, fibroblast growth factor and wnt proteins may all be involved in this process, perhaps acting at different anteroposterior levels (rev. in Kolm, et al., 1997).

Initiation of dorsoventral neural tube patterning also occurs during gastrulation (rev. in (Placzek and Furley, 1996). In *Xenopus* ventral parts of the neural tube have been specified by mid-gastrula (Saha, et al., 1997) presumably under direction of ventral midline-derived signals, particularly sonic hedgehog (Roelink, et al., 1994). Similarly, at this time, expression of *pax3* (a dorsal neural tube marker) is beginning to be restricted to the future

dorsal neural plate showing that dorsal neural fates have started to be determined (Bang, et al., 1997). Final dorsal neural tube fates require input from adjacent non-neural ectoderm (Bang, et al., 1997; Liem, et al., 1995) including members of the TGFβ gene family. The neural crest is specified later than dorsal neural tube, by the end of gastrulation, with specification increasing as the neural tube closes (Mayor, et al., 1995). Neural crest induction requires signals both from the neural plate and from the juxtaposed non-neural ectoderm, and perhaps also signals from underlying paraxial mesoderm (rev. in Bronner-Fraser, 1995).

Neural differentiation is regulated by genes such as *neurogenin*, *Xash3* and other members of the bHLH class (Turner and Weintraub, 1994; Zimmerman et al., 1993; Ma et al., 1996). Interfacing with neural differentiation is the correct patterning of the nervous system that must be controlled within the neurectoderm by a distinct set of genes. However, few genes that control early patterning decisions within the neurectoderm have been defined (for examples (Blitz, et al., 1995; Kolm and Sive, 1995a; Pannese, et al., 1995) . In order to address this deficit, we have used subtractive cloning (Sagerström, et al., 1997) to identify genes expressed specifically in the dorsal ectoderm during gastrula stages, after neural determination has occurred, but before neural differentiation has begun. To date, we have isolated more than 40 genes expressed preferentially in the dorsal ectoderm by mid-gastrula (Patel and Sive, 1996; Gamse et al, unpublished). One of the earliest markers of the neurectoderm we identified is a zinc finger gene that is similar to *Drosophila odd-paired* (*opa*) (Benedyk, et al., 1994) that we called *opl* for *odd-paired*-like. *opl* is a member of the *Zic* gene family, that comprise at least four members (Aruga, et al., 1996).

Here we analyse the expression and activity of the *opl* gene. We show that *opl* expression demarcates the presumptive neural plate very early, by the onset of gastrulation, and later defines the dorsal neural tube and neural crest. *opl* can sensitize the neurectoderm for induction, suggesting that *opl* may regulate neural competence. *opl* is also able to potentiate expression of a midbrain marker, as well as dorsal neural and neural crest marker genes, delineating a role in neural tube patterning for this gene.

3.4 Results

3.4A opl encodes a zinc finger protein with similarity to the Zic family

In order to isolate genes activated soon after neural determination has begun in *Xenopus*, we subtracted cDNAs from microdissected mid-gastrula dorsal ectoderm with those from uninduced ectoderm aged until mid-gastrula (Patel and Sive, 1996) and see Methods). One of the genes we isolated encodes a 443 amino acid protein with five zinc fingers of the C2H2 class (Genbank accession no. AF028805). By comparison with invertebrate zinc finger genes, the zinc fingers of this gene were most similar (85%) to those of the *Drosophila odd-paired* gene (*opa*), a pair-rule gene (Benedyk, et al., 1994), and we therefore named this gene *opl* for *odd-paired*-like. Among the vertebrate zinc finger genes, the zinc fingers of *opl* were similar to those of the *Gli* family genes (53% identity to the zinc fingers of human *Gli3* (Ruppert, et al., 1988)), but even more similar to the family of mouse *Zic* genes. The *Zic* genes share intron/exon boundaries with Drosophila *odd-paired* (Aruga, et al., 1996) indicating that these are true homologs of *opa* (Fig.3.1A). Over the entire coding region, opl was very similar to mouse Zic1, with 91% overall amino acid identity (Fig.3.1B, (Aruga, et al., 1994)). It was much less similar to the proteins encoded by other members of the *Zic* gene family, with 63% identity to mouse Zic2, 60% identity to Zic3 and 54% identity to Zic4 (Aruga, et al., 1996). Recent reports describing isolation of *Xenopus* Zic members include Zic3 (Nakata, et al., 1997) which has 60% identity to *opl*, while the percent identity of *opl* and Zic-r1 (Mizuseki, et al., 1998) is not clear. On the basis of these data, *opl* may be the *Xenopus* homolog of *Zic1*. However, since we cannot conclude this unequivocally, and in order to reflect the similarity of this gene to Drosophila *opa*, we chose the name *opl*.

3.4B Temporal and spatial characterization of opl expression

We next examined the timecourse of *opl* expression by Northern analysis (Fig.3.2A). *opl* RNA is activated zygotically by late blastula (st.9.5, lane 3). Expression was maximal by mid-gastrula (lanes 4-7), with lower expression persisting into tailbud stages (lanes 8-11).

By whole mount in situ hybridization, *opl* expression was biphasic. By early gastrula, (Fig.3.2B), *opl* was expressed strongly in the dorsal ectoderm (a, b), with much lower levels in the ventral ectoderm, and trace levels of expression in the marginal zone. Strong expression throughout the

presumptive neural plate was observed at mid-gastrula, with increased *opl* expression at the lateral edges of the neural plate (c, d). By early neurula, *opl* expression had entered a second phase, with restriction to the lateral edges of the neural plate, that is the presumptive dorsal neural tube and neural crest (e, f). As the neural tube closed, expression was predominant in the anterior dorsal neural tube and the pre-migratory presumptive cranial neural crest (g, h). The precise region of presumptive neural crest expressing *opl* changes during neurulation. At early neurula (stage 15), *opl* expression was in the most anterior mandibular crest (not shown), while by stage 18 (g, h), *opl* was expressed predominantly in the more posterior hyoid and branchial neural crest (Sadaghiani, et al., 1987). By tailbud, expression was high in two stripes located in the telencephalon and diencephalon of the forebrain, and more posteriorly along the entire length of the neural tube (i, j).

In order to analyse *opl* expression in more detail, embryos stained for *opl* RNA were sectioned sagittally. At st. 10+ (the onset of gastrulation), *opl* expression was observed in both the inner and outer layers of the dorsal ectoderm (Fig.3.2C; a, b) in a region largely without underlying mesendoderm (bracket). By mid-gastrula (st.11) (c, d) and late gastrula (e), *opl* expression was excluded from the extreme posterior neurectoderm. Sequential transverse sections of a tailbud embryo showed that within the spinal cord, *opl* expression was restricted to the roof plate of the neural tube (f, black arrow) and the migratory neural crest (f, white arrow). In the hindbrain, *opl* expression extended into the more ventral alar plate region of the neural tube (g, h). At the midbrain level, *opl* expression extended through the dorsal half of the neural tube (i), while more anteriorly, in the forebrain, *opl* expression was lost from roof plate and retained in the alar plate (j).

In order to delineate the expression of *opl* relative to other positions in the neural tube, we performed comparative in situ hybridization with other dorsal ectodermal markers (Fig.3.2D). At mid-neurula, expression of the forebrain and cement gland marker *otx2* (Blitz and Cho, 1995; Pannese, et al., 1995) overlapped with that of *opl* anteriorly, extending to the anterior boundary of the neural plate, but *opl* is excluded from the dorsal medial region and the future cement gland (bracket) (a). Consistently, *opl* was expressed more posteriorly than *XCG*, a marker of the cement gland (Sive, et al., 1989 that lies anterior to the neural plate (b). Expression of *slug* (Mayor, et al., 1995) in the pre-migratory cranial neural crest overlapped that of *opl*,

confirming that *opl* expression extended beyond the edges of the neural plate (c). Relative to *engrailed 2* (*en2*) at the midbrain/hindbrain junction (Hemmati-Brivanlou, et al., 1989) and *krox20*, demarcating future rhombomeres 3 and 5 of the hindbrain (Bradley, et al., 1993), *opl* expression at early neurula extends posterior to the rhombomere 5 stripe of *krox20* (d).

In summary, *opl* expression is an early marker of the presumptive neurectoderm with later expression marking the dorsal neural tube and neural crest. Recent analyses of the *Xenopus Zic3* (Nakata, et al., 1997) and a *Zic1*-related gene (Zic-r1) (Mizuseki, et al., 1998) indicate that the expression patterns of these genes and *opl* are similar.

3.4C opl is a nuclear protein whose carboxy terminal encodes a regulatory domain

We next characterized the activity and localization of the opl protein. Since mouse Zic1 is a nuclear protein that can bind the DNA target site of the related zinc finger protein Gli3 (Fig.3.1A; (Aruga, et al., 1994), we assumed that opl was a transcription factor and asked whether it had activator or repressor functions. The carboxy terminus of opl is highly serine-rich, suggesting it may be a phosphorylation domain (see Fig.3.1B). We removed this region (to create oplΔC) and asked whether it played a regulatory role in opl activity, using a reporter assay. The various constructs used are diagrammed in Fig.3.3 and described in Methods. The reporter consisted of *luciferase* linked to three copies of the Gli3 consensus DNA binding site (Fig.3.4A; (Vortkamp, et al., 1995). The activity of intact opl or oplΔC was assayed in transient transfection assays (see Methods). The averaged results of three experiments are shown in Fig.3.4A. While opl was able to activate reporter gene expression 2.8 fold above background (a), oplΔC was able to activate reporter gene expression 14.6 fold above background, indicating that this mutant allele of *opl* has an enhanced transcriptional activation capacity, and designating the carboxy terminus of opl as a domain that is able to suppress opl activity. This transactivation requires Gli3 binding sites, since a reporter lacking these sites (containing an SV40 promoter) was not activated above background levels by either opl or oplΔC (not shown).

We also asked whether the opl protein was localized to the nucleus, and whether C-terminal removal altered its nucleocytoplasmic distribution using myc-epitope tagged versions of both opl and oplΔC , MT-opl and MT-

oplΔC (see Methods). One cell of a two cell embryo was injected with RNA encoding either of these constructs and examined at mid-gastrula for distribution of the epitope-tagged opl using whole mount immunocytochemistry. As shown in Fig.3.4B, both the intact (a) and carboxy terminal truncated (b) MT-opl proteins were nuclear, with no obvious differences in the distribution of either construct. We were also unable to detect any differences in the amount of opl protein produced in embryos from either MT-opl or MT-oplΔC (not shown).

In summary, this analysis suggested that opl is a transcriptional activator, and defined a putative regulatory domain in the carboxy region of the opl protein. This region repressed the ability of opl to act as an activator but had no effect on the nucleocytoplasmic distribution of opl.

3.4D oplΔC activates neural crest and dorsal neural tube markers

The neurectodermal restriction of *opl* expression suggested that it may play a role in neural determination. We tested this possibility in an animal cap assay. RNA encoding opl, oplΔC or CAT (as control) was injected into the animal pole region of two cell embryos. At late blastula (st.9), animal caps were removed, cultured until tailbud (st.22) and marker gene expression was analysed using a reverse transcriptase-polymerase chain reaction (RT-PCR) assay (Fig.3.5A and Methods).

One of three representative experiments is shown in Fig.3.5B. After injection of *CAT* RNA we observed only background levels of the neural-specific markers *Xash3* (Zimmerman, et al., 1993) , *neurogenin* (Ma, et al., 1996), *NCAM* (Kintner, et al., 1987) , the neural crest marker *slug* (Mayor, et al., 1995), the dorsal neural tube marker *pax3* (Espeseth, et al., 1995) and the ventral neural tube marker Sonic hedgehog (*shh*) (Ekker, et al., 1995) (Fig.3.5B, lane 2). In contrast, high levels of the ventral ectodermal markers *XK81* (Jonas, et al., 1985) and *GATA2* (Walmsley, et al., 1994) were observed after this treatment (lane 2). Injection of *opl* or *oplΔC* RNAs did not induce expression of *NCAM*, *Xash3* or *neurogenin* (lanes 3 and 4), however *oplΔC* (lane 3), but not *opl* (lane 4) was able to activate expression of *slug* (4-fold over *CAT*-injected levels, averaged over three experiments) and *pax3* (3.5-fold over *CAT*-injected levels). *shh* was not activated by injection of either construct. We also asked whether *opl* or *oplΔC* could suppress non-neural ectodermal differentiation, by assaying the expression of *XK81* and *GATA2* as

ventral markers. Both *opl* and *oplΔC* were able to suppress expression of *XK81* by 2-fold relative to *CAT* injected samples. Additionally, while *oplΔC* was unable to suppress *GATA2* expression, *opl* consistently suppressed expression of *GATA2* by 2-fold.

In summary, these data showed that neither *opl* nor its activated form, *oplΔC,* could activate expression of neural-specific genes in uninduced ectoderm. However, consistent with the later expression pattern of *opl* in the dorsal neural tube, *oplΔC* efficiently induced expression of neural crest and dorsal neural tube markers. These data contrast with those obtained after injection of *Zic3* and *Zic-r1* RNAs, where multiple neural-specific markers as well as *slug* were activated (Mizuseki, et al., 1998; Nakata, et al., 1997). It is not clear whether this represents a difference in the experimental conditions used, or a difference in the activities of *opl* and these related genes.

3.4E oplΔC sensitizes the ectoderm to induction by noggin

The early expression domain of *opl* in the dorsal ectoderm correlated with the region of the gastrula ectoderm that is most competent to respond to neural induction. We therefore asked whether *opl* could act as a modulator of neural competence and sensitize ectoderm to the neural inducing activity of noggin, a factor that acts by antagonizing BMP signaling (Lamb, et al., 1993), using the animal cap assay diagrammed in Fig.3.5A. Embryos were injected with varying amounts of *noggin* RNA alone or together with *oplΔC* RNA or with *globin* RNA as a control and caps later analysed by RT-PCR for expression of two neural-specific genes, *NCAM* and *Xash3* .

A representative experiment (of three) is shown in Fig.3.6. Neither *globin* RNA (lane 1) nor *oplΔC* alone (lane 2) induced *NCAM* or *Xash3* RNA expression, consistent with results shown in Fig.3.5. 0.1 pg *noggin* RNA did not induce either *NCAM* or *Xash3* (lane 3) and 1 pg *noggin* RNA did not induce *NCAM* and activated *Xash3* only weakly (lane 4). 10 pg *noggin* RNA strongly activated both *NCAM* and *Xash3* expression (lane 5). At 0.1 pg *noggin* RNA plus *oplΔC* RNA, no *NCAM* and very weak *Xash3* expression was observed (lane 6), however at 1 pg *noggin* RNA plus *oplΔC,* strong expression of both *NCAM* and *Xash3* was observed (lane 7), to levels that were almost as strong as seen with 10 pg of *noggin* alone. At higher *noggin* concentration (10 pg) plus *oplΔC*, no further potentiation of *NCAM* and *Xash3* was observed (lane 8) and in the experiment shown, *Xash3* RNA accumulation declined

relative to 1 pg *noggin* plus *oplΔC*, perhaps indicating that saturation of the induction machinery had occurred.

In summary, these data showed that *oplΔC* could sensitize isolated ectoderm to the neural inducing effects of *noggin*, indicating that *opl* can regulate neural induction, and consistent with a role for *opl* in mediating neural competence.

3.4F In conjunction with noggin, opl activates posterior and dorsoventral neural markers

Having shown that *oplΔC* could sensitize the ectoderm for responsiveness to noggin, we next asked whether *opl* or *oplΔC* could alter the spectrum of neural markers induced by high levels of noggin that were characteristic of particular anteroposterior or dorsoventral axial positions, using the animal cap assay diagrammed in Fig.3.5A with *CAT* RNA as control. Expression levels of the general neural marker *NCAM*, and marker genes differentially expressed along the anteroposterior (A/P) or dorsoventral (D/V) axes of the neural tube were assayed by RT-PCR.

One of four representative experiments is shown in Fig.3.7. Uninjected caps (or caps injected with *CAT* RNAs (not shown) failed to express all dorsal ectodermal and mesodermal markers examined (lane 2). Injection of *noggin* plus *CAT* RNAs (lane 3) led to activation of *NCAM*, the cement gland marker *XCG* (Sive, et al., 1989) and the forebrain plus cement gland marker *otx2* (Blitz and Cho, 1995; Pannese, et al., 1995), but activated only low levels of markers that were more posterior. Injection of *oplΔC* (lane 3) or intact *opl* (not shown) RNAs alone led to weak activation of the hindbrain markers *krox20* (Bradley, et al., 1993), *HoxD1* (Kolm and Sive, 1995a) and less reproducibly, the spinal cord marker *HoxB9* (Wright, et al., 1990), but failed to activate either *NCAM* or the anterior markers *XCG*, *otx2* and *en2* (Hemmati-Brivanlou and Harland, 1989). However, expression of *oplΔC* plus *noggin* (lane 5) or *opl* plus *noggin* (lane 6), reproducibly led to very strong activation of *en2*, expressed at the midbrain/hindbrain junction (25-fold for *oplΔC*, and 7-fold for *opl*, averaged over four experiments). *oplΔC* (but not *opl*) was also able to activate expression of *krox20* and *HoxD1*, (lane 5), with the extent of activation varying between experiments, while *HoxB9* expression was not reproducibly activated.

We also examined synergy of *opl* or *oplΔC* with *noggin* with respect to markers expressed along the D/V aspect of the neural tube. Injection of *noggin* RNA together with control *CAT* RNA (lane 3) induced low levels of the dorsal marker *pax3* (Espeseth, et al., 1995) , the ventrolateral marker *NK2* (Saha, et al., 1993) and the ventral marker *shh*. Consistent with the data shown in Fig.3.5, *oplΔC* alone (lane 4) was very effective at activating expression of the neural crest markers *slug, twist* (Hopwood, et al., 1989) (not shown) and *pax3*. In conjunction with *noggin*, *oplΔC* (lane 5) also activated expression of genes normally restricted to the ventrolateral and ventral neural tube including *NK2*, *shh* and *F-spondin* (Ruiz i Altaba, et al., 1995) (not shown). Intact *opl* protein in conjunction with *noggin* activated high levels of *pax3*, *NK2* and *shh*, but did not efficiently activate *slug* or *twist* (not shown) expression (lane 6). Expression of mesodermal genes, including muscle actin, was not activated by any of these injections.

Overall, this assay demonstrated that *opl* and *oplΔC* were able to alter the A/P and D/V spectrum of genes activated by noggin, suggesting that opl may act as a modulator of positional information along the A/P and D/V neural axes. As in the assay for transcriptional activation, oplΔC was more active than native opl protein.

3.4G opl induces cellular aggregates in the absence of DNA synthesis

Since *opl* and *oplΔC* were able to alter neural marker gene expression in animal cap assays, we wanted to determine whether ectopic *opl* expression could alter neurogenesis in the intact embryo. To do this, we compared the effects of native opl protein, oplΔC, and a hormone inducible version of opl, oplGR, on the morphology of whole embryos. oplGR comprises the opl coding region linked in frame to the ligand binding domain of the glucocorticoid receptor (GR). We have previously shown that such constructs are post-translationally hormone inducible in *Xenopus* embryos, and can be activated at the time when the endogenous gene is maximally expressed (Kolm, et al., 1995b). Embryos were injected with RNA encoding either oplΔC or *oplGR*, along with *lacZ* RNA as a lineage tracer (Fig.3.8A). oplGR constructs were activated by dexamethasone (dex) at early gastrula, and embryos later assayed morphologically.

The results of this analysis are shown in Fig. 8B and collated in Table 3.1. Without dex treatment embryos appeared normal (panel a), while after

activation of the oplGR protein at early gastrula, large pigmented aggregates were seen in the ventral and lateral ectoderm (b, c) in 50% of all embryos. These aggregates were also observed after ectopic *opl* misexpression in 15% of embryos, and after ectopic *oplΔC* expression in 64% of embryos, consistent with the greater activity of *oplΔC* (Table 3.1). Aggregates were observed in the ectoderm and adjacent to the neural plate, but not within the neural plate itself. The increased activity of oplGR relative to opl in this assay is similar to that of oplΔC and is likely to be due to the presence of a weak activation domain in the GR, that can overcome the inhibitory domain of intact opl protein (Mattioni, et al., 1994).

We next asked whether the aggregates observed after *opl* misexpression were a result of increased proliferation or cell aggregation. Embryos injected with *opl*GR were pre-treated with hydroxyurea and aphidicolin (HUA) to inhibit DNA synthesis and cell division (Harris, et al., 1991), before dexamethasone treatment(Fig.3.8A). After this treatment, cells remained large consistent with the absence of cell division (not shown). As shown in Fig.3.8B, panel d, cell aggregates were observed even in the absence of DNA synthesis in 58% of embryos, similar to the percentage observed without HUA treatment (66%; Table 3.1). Similar results were obtained after injection of *oplΔC* in the presence of HUA (Table 3.1).

A section of an embryo that was injected with *oplGR* RNA, treated with dex at st.10, that formed a ventral aggregate is shown in Fig.3.8B, panel e. In close up, the uninjected side shows the three germ layers, including a tightly packed ectodermal epithelium (bracket, f). In contrast, on the injected side of the embryos the ectoderm was organized as a loosely packed mass of cells, that included pigmented cells not arranged in the characteristic epithelium seen in control embryos (g). The mesodermal and endodermal layers appeared morphologically normal.

Additionally, we asked when the embryo was competent to form aggregates. After injection of *oplGR* RNA followed by dex treatment at various times after injection (Fig.3.8A), embryos were later scored for appearance of maximally sized aggregates. The results are collated in Table 3.2. After dex induction at mid-blastula (st.8), embryos took 12.5 hours to form aggregates of maximal size, whereas by early gastrula (st.10) only 8 hours elapsed before aggregate formation. By mid-gastrula (st.11.5), only 4 hours elapsed after dex addition before maximal aggregate formation

occurred, and this was the shortest lag time observed. Older embryos (late gastrula (st.12), early neurula (st.15) and late neurula (st.18) all formed aggregates, but at 8 to 9 hours after dex addition. No aggregates were observed after dex addition at tailbud (st.24), but it was not clear whether this reflected a lack of competence to form aggregates or degradation of *opl*GR RNA.

In summary, opl, oplΔC and oplGR proteins were able to activate formation of large pigmented aggregates in the ventral and lateral ectoderm. These appeared to be loose associations of cells and were not caused by an increase in cell proliferation. Embryos were competent to form aggregates from mid-blastula through late neurula, with the time of maximal competence at mid-gastrula.

3.4H opl activates expression of neural crest and dorsal neural tube markers and represses epidermal gene expression in whole embryos

We extended the analysis in whole embryos to ask whether *opl* constructs can activate ectopic expression of neural crest and dorsal neural tube markers and suppress ventral ectodermal (epidermal) fates. Embryos were injected with either *opl*GR or *opl*ΔC RNA, along with *lacZ* RNA as lineage tracer. For *opl*GR injections, dexamethasone (dex) was added at stage 11 when response to *opl*GR was maximal as indicated in the aggregate formation assay (Fig.3.9A, and not shown). Neural crest formation was assayed by expression of *slug*, dorsal neural tube fates were assayed by expression of *pax3,* and epidermal fate was assayed by expression of *XK81.*

Representative data is shown in Fig.3.9B and the results collated in Table 3.3. Without dex, uniform *slug* expression was observed on both sides of the embryo (panel a). After dex treatment, concentration of *lacZ* lineage tracer was observed reflecting the cell aggregates previously seen in pigmented embryos (see Fig.3.8). In dex-treated embryos, *slug* expression expanded anteriorly on the injected side of the embryo (b) in 42% of injected embryos. Similarly, a large expansion of the *pax3* expression domain was observed after dex addition in 75% of injected embryos. In a few cases (4% of injected embryos) patches of *pax3* expression were not connected to the neural plate.

We also asked whether *opl* altered expression of the ventral epidermal marker, *XK81*. Since epidermis is specified early, we used the constitutively

active construct *opl*Δ*C*. Embryos injected with *globin* RNA as a control (Fig.3.9C, panel a) along with *lacZ* showed *XK81* expression over the entire ventral ectoderm, obscuring the βGal staining. After injection of *opl*Δ*C* (b), however, 51% of embryos showed large patches of *lacZ* expression since *XK81* is not expressed in these regions (Table 3.4). Regions of ventral ectoderm that did not express *XK81* always coincided with regions of *lacZ* expression.

In summary, in whole embryos, *opl* constructs were able to expand the expression domains of a neural crest and a dorsal neural tube marker, and was able to inhibit expression of an epidermal cytokeratin gene, that is a marker of the ventral ectoderm. The *slug* expression domain also expands in *Zic3* and *Zic-r1* injected embryos, consistent with these results.

3.5 Discussion

We report isolation of *opl*, a gene similar to Drosophila *odd-paired* and a new member of the *Zic* gene family in *Xenopus*. *opl* expression indicates that neural determination begins by the onset of gastrulation, and the opl protein appears to have three activities. First, *opl* can sensitize the presumptive neurectoderm for induction, suggesting that it may be a neural competence factor. Second, consistent with its later expression in the dorsal neural tube and neural crest, an activated form of *opl*, *opl*ΔC, can activate neural crest and dorsal neural tube markers. Third, *opl* can synergize with noggin to induce expression of engrailed. These data indicate that *opl* may be a key regulator of *Xenopus* neurogenesis.

3.5A opl expression defines an early neurectodermal domain

opl is most similar to the mouse *Zic1* gene that is expressed in the embryonic mesoderm and presumptive neurectoderm of mice (Nagai, et al., 1997) , with later expression persisting in the dorsal neural tube and the dorsomedial region of the somites. *Xenopus opl* is not expressed at significant levels in the mesoderm. However, the early expression of *Xenopus opl*, mouse *Zic1* and zebrafish *opl* (Grinblat, et al., 1998) during neurogenesis, and their later restriction of expression to the dorsal neural tube suggests that their function may be conserved during vertebrate neural development.

At early gastrula, when *opl* is first expressed, neural tissue is not yet specified, as judged by isolation and culture of dorsal ectoderm and later analysis for markers of neural differentiation (Sive, et al., 1989). However, restricted *opl* expression in dorsal ectoderm clearly shows that the neurectoderm has begun to be set aside by the onset of gastrulation. A large part of the early expression domain of *opl* is in dorsal ectoderm not yet underlain by the involuting mesendoderm. This dorsal restriction of *opl* RNA is dependent on dorsal signals since it is abolished in UV-treated embryos (Gamse and Sive, unpublished). These data indicate that *opl* RNA accumulation may be activated by planar signals from the organizer, or by patterning signals present in the dorsal ectoderm prior to the onset of gastrulation (Sokol and Melton, 1991; Wylie, et al., 1996). Recent reports concerning other *Xenopus* Zic family members, *Zic3* and *Zic-r1* indicates that their expression requires removal of BMP signaling (Mizuseki, et al., 1998; Nakata, et al., 1997). Consistent with this, *opl* is induced by *noggin* (Kuo et

al., unpublished), however, it is not clear whether such signaling initiates *opl* expression, or is required for a later maintenance step.

3.5B opl can modulate neural competence

The early *opl* expression domain suggested that it may be a competence factor, and indeed *opl* could increase the responsiveness of isolated ectoderm to *noggin* by ten-fold. Our data indicate that expression of *opl* may account for some of the enhanced sensitivity of the dorsal ectoderm to neural induction as noted by other investigators (Otte, et al., 1991; Sharpe, et al., 1987). The translation initiation factor *EIF4AII* has recently been shown to synergize with *noggin* to activate expression of some neural crest markers as well as markers of the neurectoderm proper (Morgan and Sargent, 1997). *EIF4AII* is expressed considerably later than *opl*, by mid-gastrula, and may be a target of *opl* activation. Protein kinase C (PKC) activity has also been implicated in conferring neural competence on the dorsal ectoderm (Otte, et al., 1991), while the *Notch* signaling pathway may also be involved in directing neural competence (Coffman, et al., 1993). It is unknown whether *opl* can modulate or be modulated by either of these pathways.

3.5C opl can activate engrailed

In conjunction with *noggin*, *opl* and *opl*ΔC strongly potentiated expression of the posterior neural marker *engrailed*, expressed in the midbrain/hindbrain region. Although *opl* is expressed in presumptive posterior neurectoderm, we did not expect this result, since *opl* RNA is present throughout the anteroposterior extent of the future brain at early stages. It is possible, however that *opl* protein activity is modulated along the A/P neural axis. One intriguing connection between the ability of *opl* to potentiate expression of *engrailed* and *Drosophila opa* is that *opa* is necessary for the correct temporal activation of *Drosophila wingless* (Benedyk, et al., 1994), while in *Xenopus*, the wingless homologs *wnt3A* and *wnt8* can posteriorize neurectoderm in combination with *noggin* (Fredieu, et al., 1997; McGrew, et al., 1997; McGrew, et al., 1995) This suggests that *opl* may activate *wnt* genes or act downstream of wnt proteins. Indeed the expression patterns of *opl* and *wnt3A* are very similar from late gastrula (Wolda, et al., 1993). However, unlike *wnt3A*, *opl* expression did down-regulate anterior

neurectodermal markers, suggesting that the activities of these proteins are not entirely equivalent.

3.5D opl as an activator of dorsal neural tube and neural crest fates

The later expression of *opl* in the dorsal neural tube and neural crest correlated with the ability of a truncated form of *opl*, *oplΔC*, to activate genes characteristic of these fates. Like *opl*, the dorsal neural tube marker, *pax3*, is transiently expressed initially across the entire dorsoventral extent of both the *Xenopus* and chick neural plates and is later expressed solely in the future dorsal neural tube (Bang, et al., 1997; Liem, et al., 1995). However, *opl* is expressed earlier than *pax3*, consistent with a role for *opl* in activating *pax3*. As *pax3* expression increases in the future dorsal neural tube, *opl* expression also increases and later becomes dorsally restricted. The expression of *opl* in presumptive neural crest from late gastrula again precedes the expression of neural crest-specific markers in this region (Mayor, et al., 1995), consistent with a role for *opl* in regulating neural crest formation. *opl* expression across both presumptive dorsal neural tube and neural crest regions at late gastrula suggests that these two neural domains are initially defined as a unit that later becomes subdivided into dorsal neural tube and neural crest subdomains. Since recent studies have shown that wnt proteins can also activate neural crest markers in neuralized ectoderm (Chang, et al., 1998; Saint-Jeannet, et al., 1997), another connection between opl and wnt expression is possible.

3.5E opl activity is modulated by synergizing factors

Several lines of evidence indicate that opl protein requires synergizing factors. First, oplΔC, but not full length opl protein could activate dorsal neural tube and neural crest markers in animal caps. In other respects, opl appears to have similar, though weaker activity to oplΔC, as both constructs could repress ventral ectodermal markers, synergize with noggin and catalyse formation of pigmented aggregates. This suggests that oplΔC is an activated form of opl, although we cannot rule out that oplΔC has a novel activity. Second, we showed that in animal caps, intact opl in conjunction with noggin was able to activate a dorsal neural tube marker, but not neural crest markers. This suggests that factors induced by noggin modulate opl activity, however since opl could not activate neural crest markers even in

conjunction with noggin, some other factor may allow opl to activate neural crest-specific gene expression. Such a factor may act by de-repressing a carboxy terminus inhibitory function in the opl protein, perhaps by altering opl phosphorylation. Third, in whole embryos, activated opl constructs could expand the domain of *slug* and *pax3* expression but only adjacent to the normal expression domains of these genes, and never in the extreme anterior neural plate. This further suggests that *opl* activity requires cofactors that are present in the neural plate, neural crest and in early gastrula animal caps, but that are absent from or counteracted by inhibitors in the ventral ectoderm of older embryos.

These considerations raise the question of whether the activation of dorsal neural tube fates, the *engrailed*-inducing capacity and the competence-promoting activity of *opl* are distinct functions, perhaps requiring different domains of *opl* or different cofactors. In animal caps, *opl* in conjunction with *noggin* was able to promote expression of both dorsal and ventral neural tube markers, indicating that expression of ventral neural tube markers is not incompatible with *opl* activity. Thus *opl* may act by a similar mechanism throughout the neural plate, and may play a role in early gastrula to activate both dorsal and ventral neural tube markers. Since by the end of gastrulation, *opl* is not expressed ventrally, ventral *opl* activity is only transient.

Unlike *opl*, two related genes, *Zic3* and *Zic-r1* are able to activate both general neuronal markers and a neural crest marker in animal caps without requiring *noggin* addition (Mizuseki, et al., 1998; Nakata, et al., 1997). The differences in our results may reflect a difference in assay conditions that were employed, or may indicate different activities for these related genes. Since the neuronal markers that were examined in these studies are expressed in restricted areas of the neural plate, whereas expression of *Zic3* and *Zic-r1* is much broader, activity of these genes may also be modulated by cofactors. Similarly, *Zic3* and *Zic-r1* increased *slug* expression only adjacent to the normal *slug* expression domain and could not induce it in ventral ectoderm.

3.5F opl modulates cell adhesion

One of the most striking phenotypes consistently produced by *opl* and also produced by *Zic3* (Nakata, et al., 1997), was the appearance of cellular

aggregates in the ventrolateral ectoderm, but not within the neural plate itself. Aggregates did not express neural or neural crest markers unless they directly contacted the neural plate. In both whole embryos and in animal caps injected with *opl* RNA, aggregates comprised cells that were loosely adherent (not shown). In both the whole embryo and the animal cap, the cells appeared healthy, surviving and dividing after disaggreagation in the culture dish. One intriguing hypothesis is that *opl* suppresses an adhesion pathway responsible for ectodermal-epithelial integrity that is normally down-regulated as neural crest fates are determined (Bronner-Fraser, 1995). Since *opl* did not induce ectopic slug in the ventral ectoderm, but did induce aggregates there, the aggregates are clearly not committed neural crest cells, but may represent some early step in neural crest commitment. Consistent with this hypothesis, *opl* is expressed sequentially in the cranial neural crest segments just before the cells mound up, lose adhesivity and begin migrating (Sadaghiani and Thiebaud, 1987).

3.5G opl and inhibition of epidermal fates

In *Xenopus*, formation of ventral fates including epidermis appears to be dependent on Bone Morphogenetic Protein (BMP) activity (Wilson, et al., 1995) . Suppression of this activity by *noggin* and *chordin* proteins during gastrulation results in neural determination (Piccolo, et al., 1996; Zimmerman, et al., 1996). In whole embryos *opl* can inhibit expression of the epidermal cytokeratin gene *XK81*, while in animal caps, *opl* also suppresses expression of *GATA2*, a ventral ectodermal marker that is regulated by BMP4 signaling. Since this suppression is not correlated with an ability of *opl* or *opl*ΔC alone to activate general neural markers, *opl* is able to inhibit only part of the dorsoventral ectodermal patterning pathway. In accord with this, *opl* expression overlaps that of BMP4, until mid-gastrula when BMP4 RNA begins to be cleared from the presumptive neural plate (Gamse and Sive, unpublished).

3.5H A model for opl function

Our data suggests a model for the role of *opl* in early neural determination (Fig.3.10). Early during gastrulation, *opl* gene expression is induced, either by planar signals from the organizer or by pre-existing signals within the dorsal ectoderm. The early expression domain of *opl* throughout

the neural plate sensitizes the neurectoderm for subsequent neuralizing signals, such as *noggin* and *chordin*, derived from the organizer. This sensitization both modulates general neural readout and is also involved in activating relatively posterior neural markers, such as *engrailed*. By the end of gastrulation, *opl* expression in the dorsal neural plate and presumptive neural crest directs fates characteristic of these regions. The activity of *opl* in the presumptive neural crest is modulated by a regulatory domain in the carboxy terminus, suggesting that a laterally, but not anteriorly, localized factor(s) may control *opl* activity. The ability of *opl* to suppress cell adhesion may be part of the commitment program for neural crest fates.

Future challenges are to analyse the consequence of *opl* ablation, to isolate the downstream direct targets of *opl*, and to further understand how *opl* fits into the hierarchy of neural regulatory genes.

3.6 Acknowledgments

We thank Igor Dawid, Richard Harland, Paul Krieg, Nancy Papalopulu, Michael Sargent, Dave Turner and Andrea Vortkamp for kind gifts of plasmids. We thank W. David Willison for help with the subtractive screen, Cathy Nocente-McGrath for the mid-gastrula λZap phage library, Melissa Rones for helping isolate the full length *opl* cDNA, Harvey Lodish for tissue culture reagents and facilities, Liuda Ziaugra for expert DNA sequencing, and Jeanne Reis for histological expertise. We greatly appreciate the helpful comments and discussions with members of the Sive lab during the interminably long course of this work, and thank Andrew Lassar for criticism of the manuscript. We also thank Jun Aruga for interesting discussion concerning *Xenopus Zic3* experiments. This work was supported by grants from the NSF and NIH to HLS. We appreciate support from Genetics Institute, Cambridge MA. J.S.K. was the recipient of a Medical Scientist Training Program Fellowship (PHS grant 2-T32-GM07753) from the Harvard MD-PhD Program. M.P. was the recipient of a fellowship from the Human Frontiers Science Program. X.L. was the recipient of an NIH postdoctoral fellowship. H.L.S. was the recipient of a NSF Young Investigators Award and the Latham Family Development Professorship at MIT.

Figure 3.1. opl protein sequence alignments.

Amino acid residues in proteins compared that are identical to *Xenopus* opl are indicated by periods. Sequence gaps introduced for optimal protein alignment are indicated by dashes. Numbers at right indicate amino acid position.

(A) Alignment of zinc fingers II-V of opl, mouse Zic proteins (Aruga, et al., 1996) : mZic1, mZic2, mZic3, mZic4, *Drosophila* Opa (Benedyk, et al., 1994) and human Gli3 (Ruppert, et al., 1988). The first zinc finger is omitted since it is incomplete. This region of the *Xenopus* opl protein (aa 265-381) is 98% identical to the equivalent regions in mZic1, 97% identical to mZic2, 90% identical to mZic3, 85% identical to Opa and 53% identical to Gli3.

(B) Alignment of conceptually translated *Xenopus* opl, mouse Zic1 and human Zic proteins. Bar above sequence indicates predicted zinc fingers. Block indicates start of polypeptide deleted in *opl*ΔC constructs. *Xenopus* opl is 92% similar to both mouse Zic1 and human Zic proteins (Yokota, et al., 1996). opl is 60% overall identical to both mouse and *Xenopus* Zic3 (Nakata, et al., 1997)

.

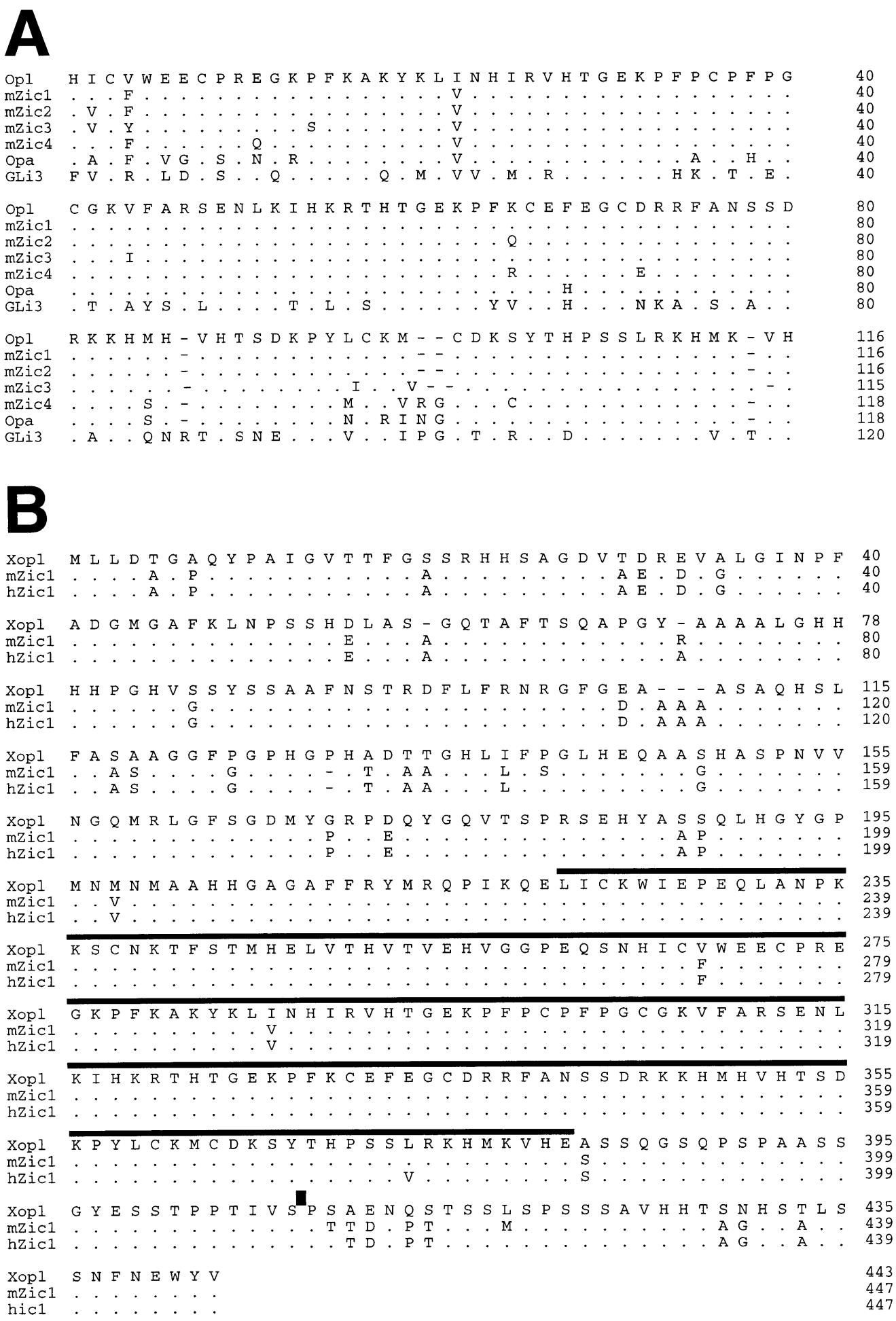

A
Opl H I C V W E E C P R E G K P F K A K Y K L I N H I R V H T G E K P F P C P F P G 40
mZic1 . . . F . . . . V 40
mZic2 . V . F . . . . V 40
mZic3 . V . Y . . . . S . . . . V 40
mZic4 . . . F . . . . . . Q . . . . V 40
Opa . A . F . V G . S . N . R . . . . V . . . . A . . H . . 40
GLi3 F V . R . L D . S . . Q . . . . . Q . M . V V . M . R . . . . . H K . T . E . 40
Opl C G K V F A R S E N L K I H K R T H T G E K P F K C E F E G C D R R F A N S S D 80
mZic1 80
mZic2 . . . . . Q 80
mZic3 . . . I 80
mZic4 . . . . . R . . . . . E 80
Opa . . . . . H 80
GLi3 . T . A Y S . L . . . . T . L . S . . . . . Y V . . H . . . N K A . S . A . . 80
Opl R K K H M H - V H T S D K P Y L C K M - - C D K S Y T H P S S L R K H M K - V H 116
mZic1 . . . . . - . . . . . - - . . . . . - . . 116
mZic2 . . . . . - . . . . . - - . . . . . - . . 116
mZic3 . . . . . - . . . . . I . . V - - . . . . . - . . 115
mZic4 . . . . S . - . . . . . M . . V R G . . . C . . . . . - . . 118
Opa . . . . S . - . . . . . N . R I N G . . . . . - . . 118
GLi3 . A . . Q N R T . S N E . . . V . . I P G . T . R . . D . . . . . V . T . . 120
B
Xopl M L L D T G A Q Y P A I G V T T F G S S R H H S A G D V T D R E V A L G I N P F 40
mZic1 . . . . A . P . . . . . A . . . . . A E . D . G 40
hZic1 . . . . A . P . . . . . A . . . . . A E . D . G 40
Xopl A D G M G A F K L N P S S H D L A S - G Q T A F T S Q A P G Y - A A A A L G H H 78
mZic1 . . . . . E . . . A . . . . . R 80
hZic1 . . . . . E . . . A . . . . . A 80
Xopl H H P G H V S S Y S S A A F N S T R D F L F R N R G F G E A - - - A S A Q H S L 115
mZic1 . . . . . G . . . . . D . A A A 120
hZic1 . . . . . G . . . . . D . A A A 120
Xopl F A S A A G G F P G P H G P H A D T T G H L I F P G L H E Q A A S H A S P N V V 155
mZic1 . . A S . . . . G . . . . - . T . A A . . . L . S . . . . . G 159
hZic1 . . A S . . . . G . . . . - . T . A A . . . L . . . . . G 159
Xopl N G Q M R L G F S G D M Y G R P D Q Y G Q V T S P R S E H Y A S S Q L H G Y G P 195
mZic1 . . . . . P . . E . . . . . A P 199
hZic1 . . . . . P . . E . . . . . A P 199
Xopl M N M N M A A H H G A G A F F R Y M R Q P I K Q E L I C K W I E P E Q L A N P K 235
mZic1 . . V 239
hZic1 . . V 239
Xopl K S C N K T F S T M H E L V T H V T V E H V G G P E Q S N H I C V W E E C P R E 275
mZic1 . . . . . F 279
hZic1 . . . . . F 279
Xopl G K P F K A K Y K L I N H I R V H T G E K P F P C P F P G C G K V F A R S E N L 315
mZic1 . . . . . V 319
hZic1 . . . . . V 319
Xopl K I H K R T H T G E K P F K C E F E G C D R R F A N S S D R K K H M H V H T S D 355
mZic1 359
hZic1 359
Xopl K P Y L C K M C D K S Y T H P S S L R K H M K V H E A S S Q G S Q P S P A A S S 395
mZic1 . . . . . S 399
hZic1 . . . . . V . . . . . S 399
Xopl G Y E S S T P P T I V S P S A E N Q S T S S L S P S S S A V H H T S N H S T L S 435
mZic1 . . . . . T T D . P T . . . M . . . . . A G . . A . . 439
hZic1 . . . . . T D . P T . . . . . A G . . A . . 439
Xopl S N F N E W Y V 443
mZic1 447
hic1 447

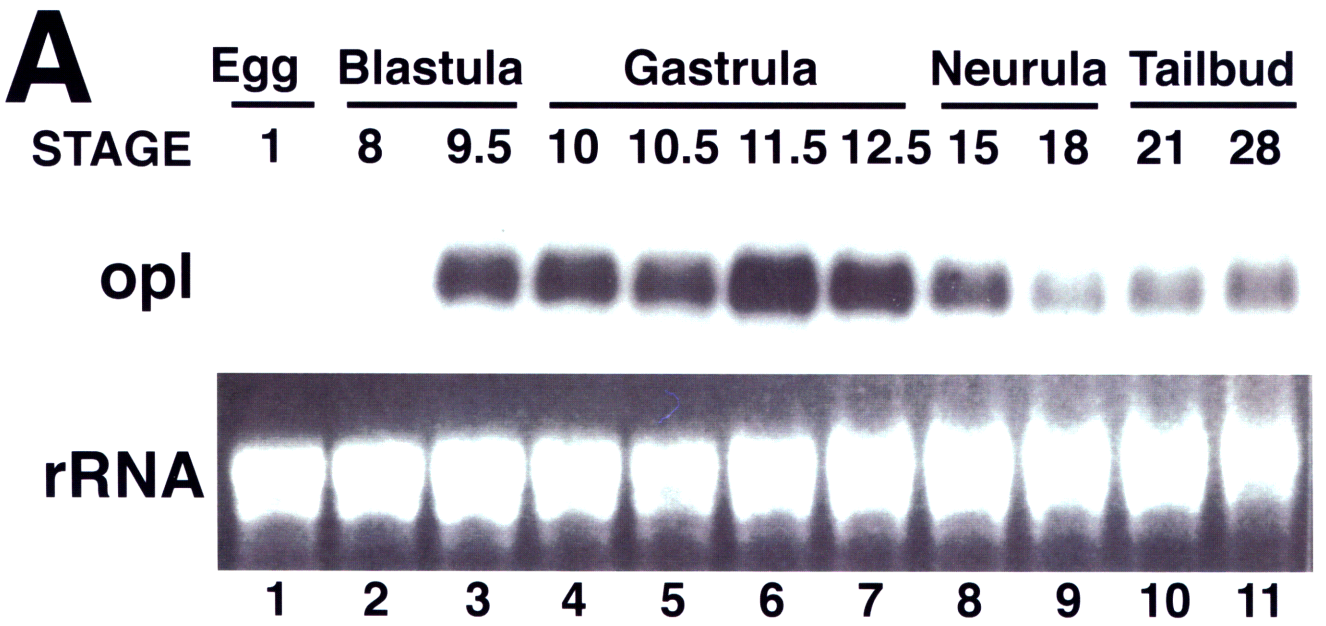
Figure 3.2. Temporal and spatial characteristics of opl expression.
(A) Northern analysis of *Xenopus* embryos. One embryo equivalent per lane was analyzed for *opl* RNA (top row) at various embryonic stages shown. See Methods. Ethidium-stained 28S rRNA is a loading control (bottom row). Lane 1: egg; lane 2: st.8; lane 3: st.9.5; lane 4: st.10; lane 5: st.10.25; lane 6: st.11.5; lane 7: st.12.5; lane 8: st.15; lane 9: st.18; lane 10: st.21; lane 11: st.28.

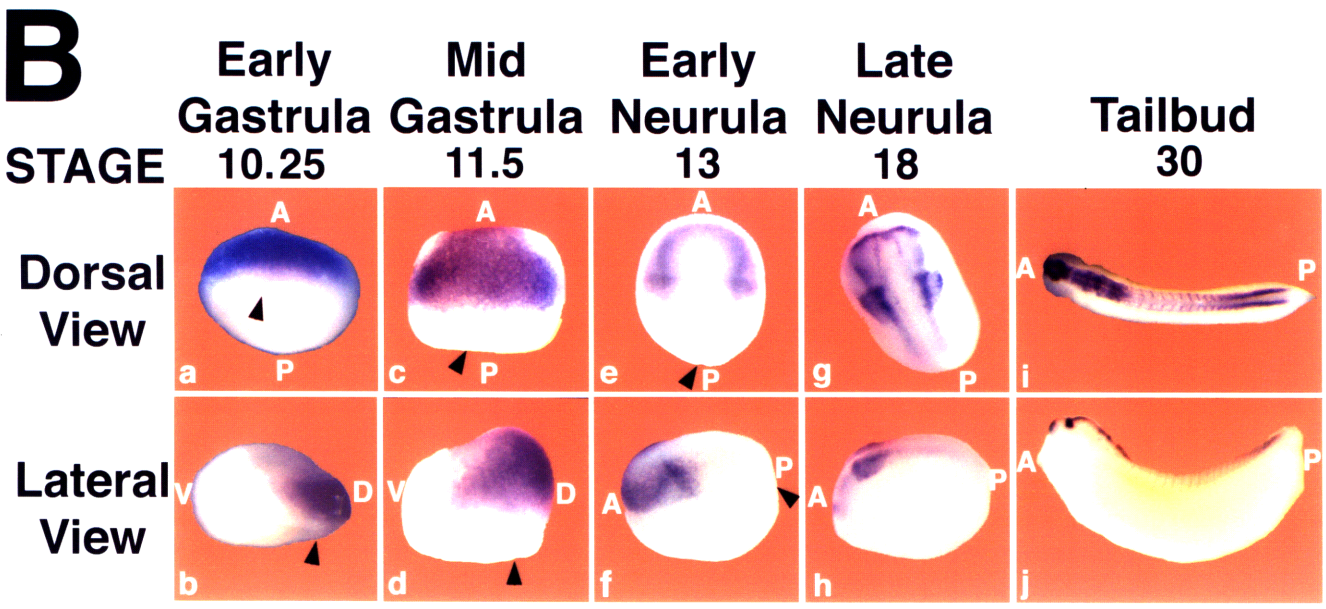
(B) Whole-mount in situ analysis of *opl* expression. Embryos were analyzed by in situ hybridization as described in Methods. The top row (panels a, c, e, g, i) shows dorsal views of embryos. The bottom row (panels b, d, f, h, j) shows lateral views of embryos. Embryo orientations are indicated by anterior (A), posterior (P), dorsal (D), and ventral (V) labels. Panels a, b: st.10.25; c, d: st.11.5; e, f: st.13; g, h: st.16; i, j: st.30. A: anterior, P: posterior, arrowhead: dorsal lip of blastopore.

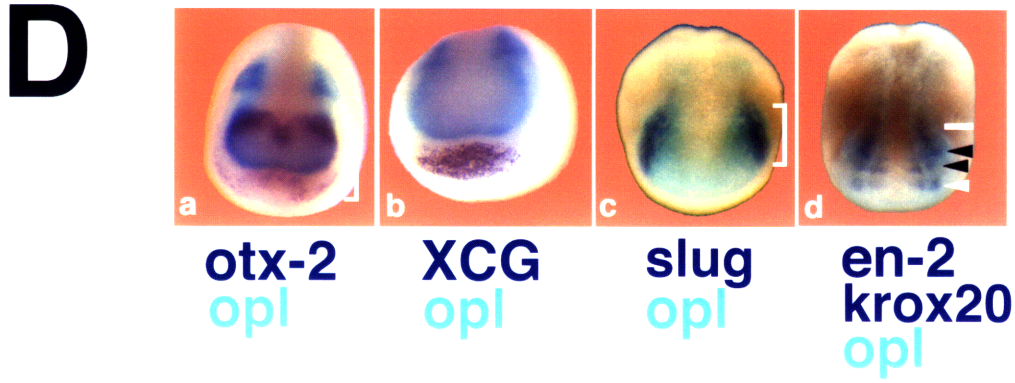
(C) Sections of embryos analyzed by whole-mount in situ hybridization for *opl* expression. Embryos were fixed, embedded and sectioned as described in Methods. Sectioning planes are shown in schematic diagrams above panels. Panels a, b, c, d, e were sagittal sections through gastrula-stage embryos as indicated. Panels, f, g, h, i, j were transverse sections of a tailbud stage (st.26-28) embryo. Panel a: st.10+, bracket indicates regions of *opl* expression not underlain by mesendoderm; b: higher magnification of embryo in a; c: st.11; d: higher magnification of embryo in c; e: st.12; f: section at trunk level; g: section at hindbrain-spinal cord level; h: section at midbrain level; panel i: section at midbrain level; panel j: section at forebrain level. Black arrowheads indicate position of dorsal blastopore lip. Black arrows indicate *opl* expression in roofplate. White arrows indicate spinal crest expression. Brackets indicate alar plate expression in fore/midbrain regions. D: dorsal, V: ventral. Scale bar=25 mm.

(D) Double whole-mount in situ analysis of *opl* . In situ analysis was as described in Methods with *opl* stained light blue and all other markers (*otx*-2, *XCG*, *slug*, *en*-2, *krox20*) are stained purple. Panel a: *opl* plus *otx*-2, a marker of forebrain and cement gland (Blitz and Cho, 1995; Pannese, et al., 1995), bracket shows *otx*-2 expression in cement gland primordium; b: *opl* plus *XCG*, a cement gland marker (Sive, et al., 1989), bracket shows *XCG* expression in

cement gland; c: *opl* plus *slug*, a neural crest marker (Mayor, et al., 1995), bracket shows *slug* domain contained within *opl* domain; d: *opl* plus *en*-2, a marker of the midbrain/hindbrain regions (Hemmati-Brivanlou and Harland, 1989) and *krox20*, a marker of presumptive rhombomeres 3 and 5 in the hindbrain (Bradley, et al., 1993). White arrowhead indicates *en*-2 expression, black arrowheads indicate *krox20* expression, white bar is posterior limit of early *opl* expression.


A
Egg
Blastula
Gastrula
Neurula
Tailbud
STAGE
1
8
9.5
10
10.5
11.5
12.5
15
18
21
28
opl
rRNA
1
2
3
4
5
6
7
8
9
10
11


B
Early Gastrula
Mid Gastrula
Early Neurula
Late Neurula
Tailbud
STAGE
10.25
11.5
13
18
30
Dorsal View
Lateral View
A
P
V
D
a
b
c
d
e
f
g
h
i
j


D
a
b
c
d
otx-2
opl
XCG
opl
slug
opl
en-2
krox20
opl

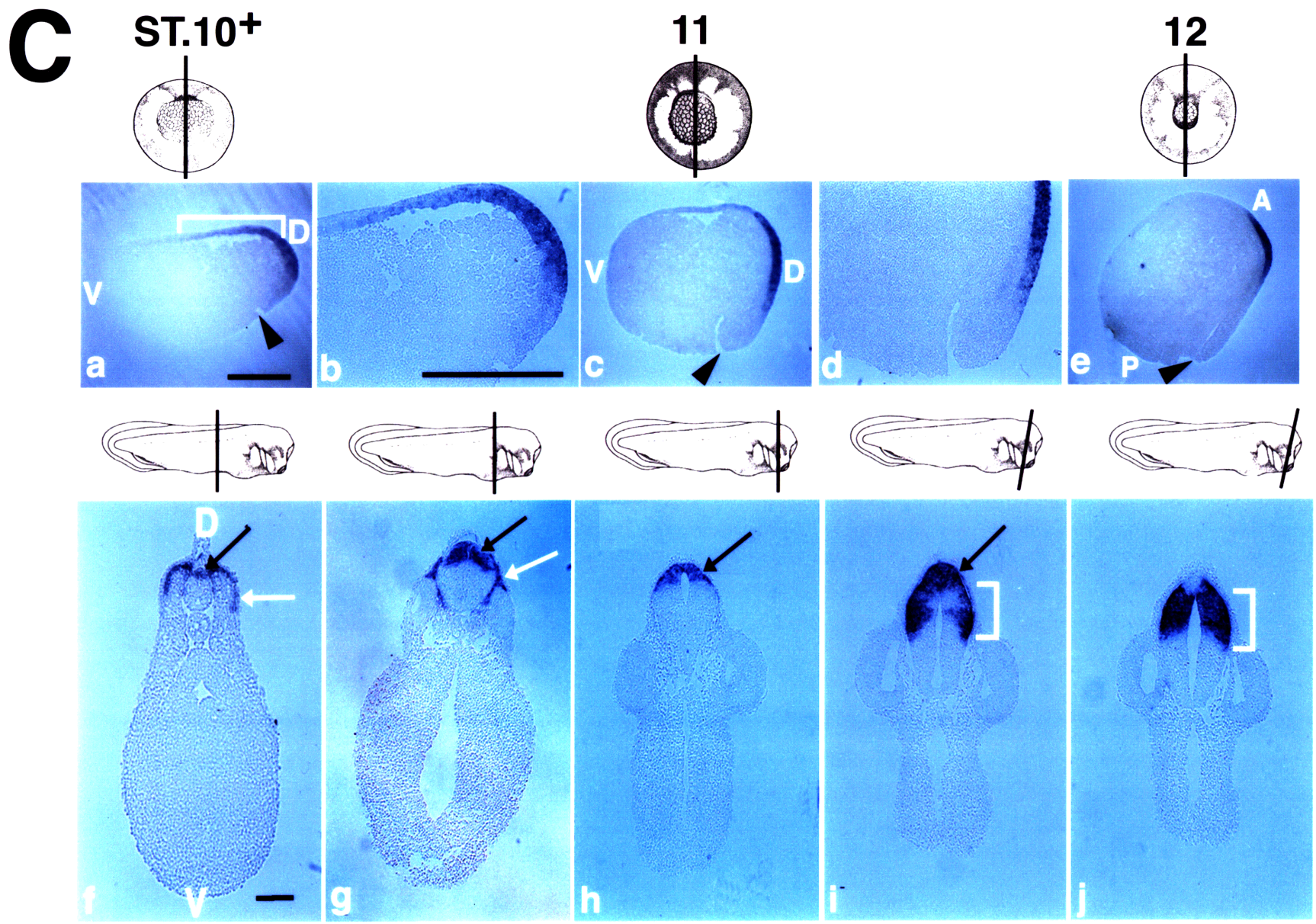

C
ST.10+
11
12
V
D
a
b
V
D
c
d
A
P
e
D
V
f
g
h
i
j

Figure 3.3. opl constructs

The *opl* construct comprises the coding region of opl; oplΔC deletes a serine-rich carboxy terminal domain (S), oplGR comprises an in-frame fusion of the opl coding sequence with the hormone-binding domain of the glucocorticoid receptor (Kolm and Sive, 1995b) . MT-opl is an in-frame fusion of the opl coding region with a multimerized myc epitope (MT, black circle) at the amino end of *opl* (Rupp, et al., 1994) , and MT-oplΔC is a similar fusion of the multimerized myc epitope with the carboxy deleted opl. See Methods for details.

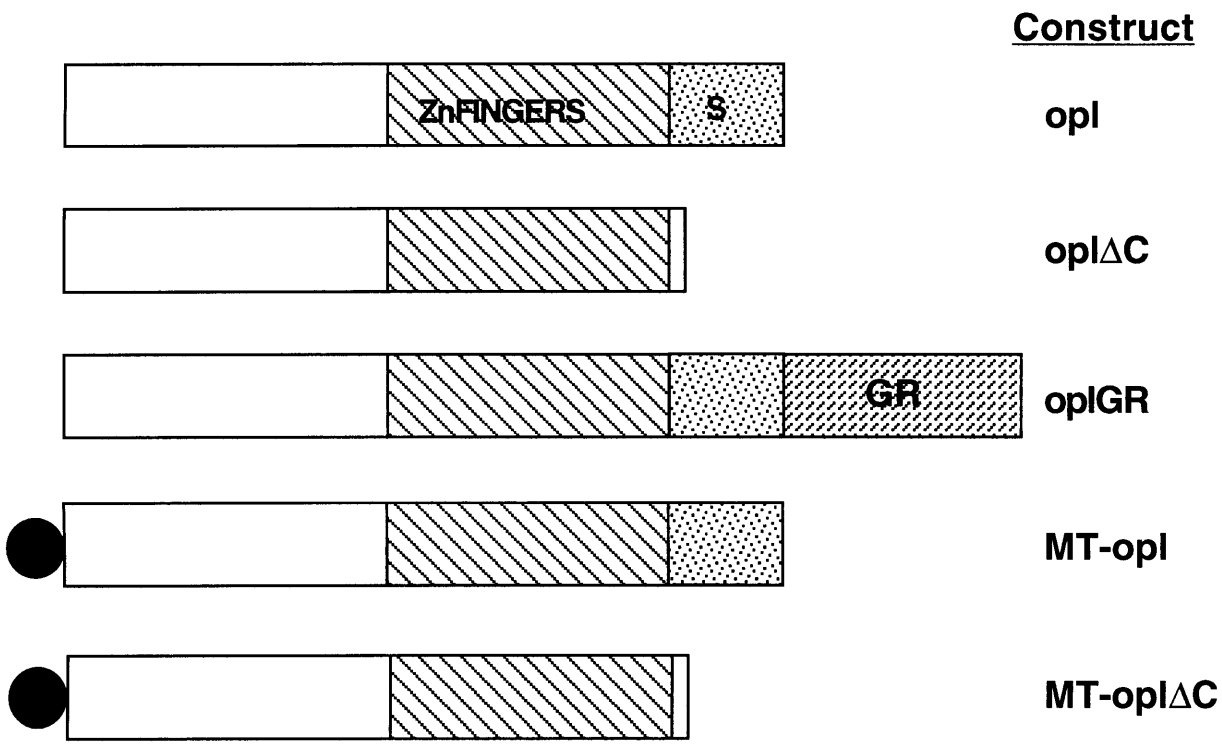

Construct
ZnFINGERS
S
opI
opIΔC
GR
opIGR
MT-opI
MT-opIΔC

Figure 3.4. opl is a nuclear protein with a regulatory domain in the COOH-terminal.

(A) opl protein transactivates reporter constructs in tissue culture. Transient transfection reporter assays were performed as described in Methods, using the human pancreatic tumor BXPC cell line. The reporter construct is shown schematically: three Gli3 DNA binding sites are located 5' to a basal promoter (TATA box) and *luciferase* reporter gene (Vortkamp, et al., 1995) Data shown is the average of three independent experiments with corresponding error bars. In the absence of the Gli3 binding sites, neither opl nor oplΔC activated a reporter construct (not shown). DNA mixes for transient transfections are as follows, Left panel: reporter plus CS2+ vector; middle panel: reporter plus CS2+*opl* ORF right panel: reporter plus pCS2-*opl*ΔC.

(B) Immunocytochemistry of injected opl and oplΔC proteins. Albino embryos were injected with 500 pg of synthetic MT*opl* or MT*opl*ΔC RNA at two- to four-cell stages and analyzed at st.11 by immunostaining with anti-myc 9E10 antibody as described in Methods. Views of the animal hemispheres are shown, left panel: MT*opl* injected embryo, right panel: MT*opl*ΔC injected embryo.

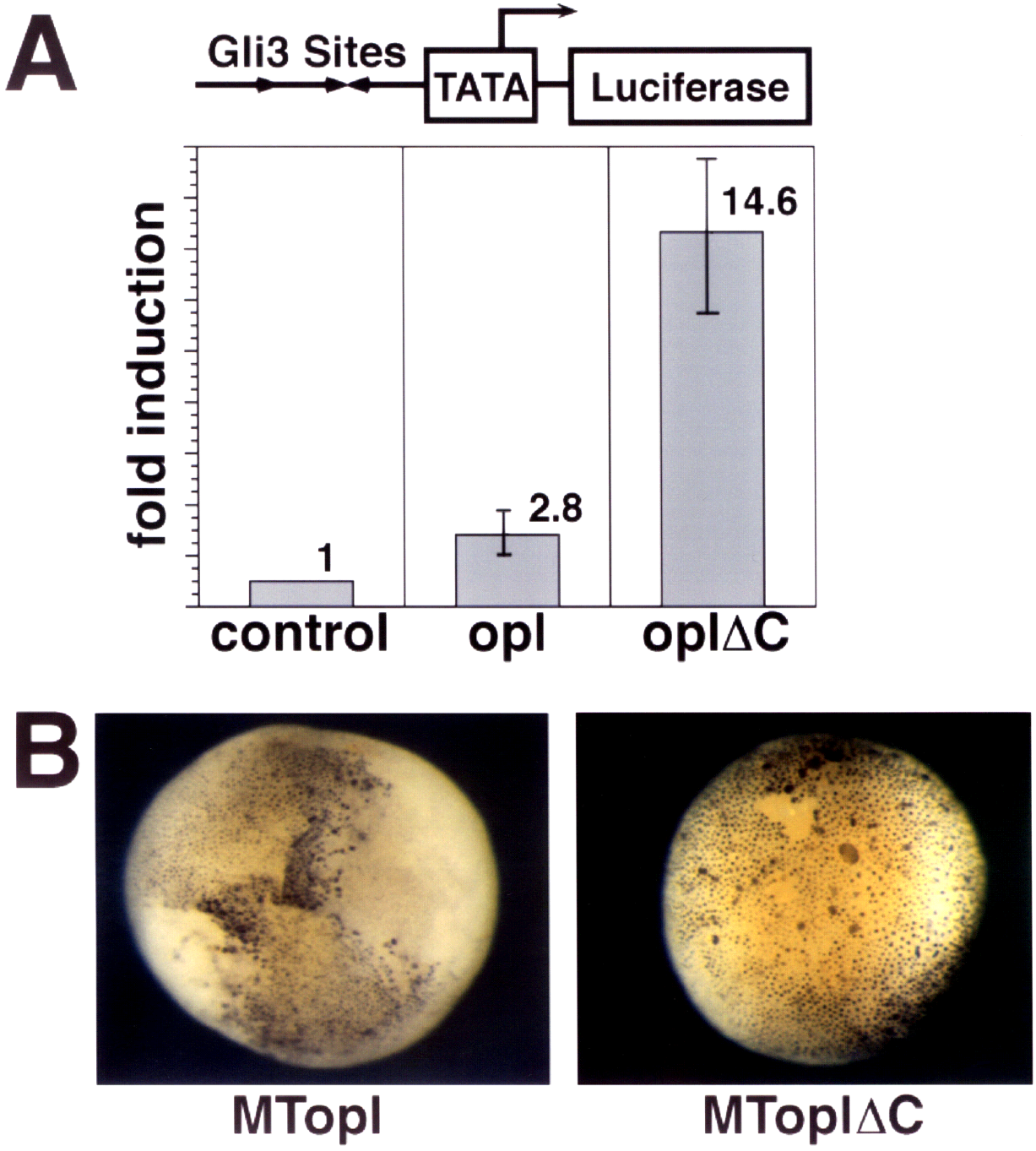

A
Gli3 Sites
TATA
Luciferase
fold induction
1
2.8
14.6
control
opl
oplΔC
B
MTopl
MToplΔC

Figure 3.5. oplΔC can activate neural crest and dorsal neural tube marker genes in animal caps.

(A) Experimental scheme. Wild-type embryos were injected in both blastomeres with 200 pg of indicated RNAs at the two-cell stage. Injection of *CAT* RNA served as negative control by equalizing the injected RNA dose. Pools of 15-20 animal caps were isolated from injected embryos at late blastula (st.9) and incubated in saline until harvest at tailbud (st.22) for RT-PCR analysis. See Methods for details.

(B) Expression of marker genes in animal caps. Uninjected embryos serve as control for baseline expression level. *Xash3* (Zimmerman, et al., 1993) , *neurogenin* (Ma, et al., 1996) and *NCAM* (Kintner and Melton, 1987) are neural-specific markers; *slug* (Mayor, et al., 1995) is a neural crest marker; *pax3* (Espeseth, et al., 1995) marks the dorsal neural tube, *shh* marks the floorplate (Ekker, et al., 1995) ; *XK81* (Jonas, et al., 1985) and *GATA2* (Walmsley, et al., 1994) are markers of the ventral ectoderm; *EF1a* (Krieg, et al., 1989) served as loading control. Samples processed without RT did not show *EF1a* signal after PCR (not shown). Data from one representative experiment is shown, similar results were obtained from three experiments. Lane 1: st.22 embryo; lane 2: *CAT* injected animal caps; lane 3: *oplΔC* injected caps; lane 4: *opl* injected caps.

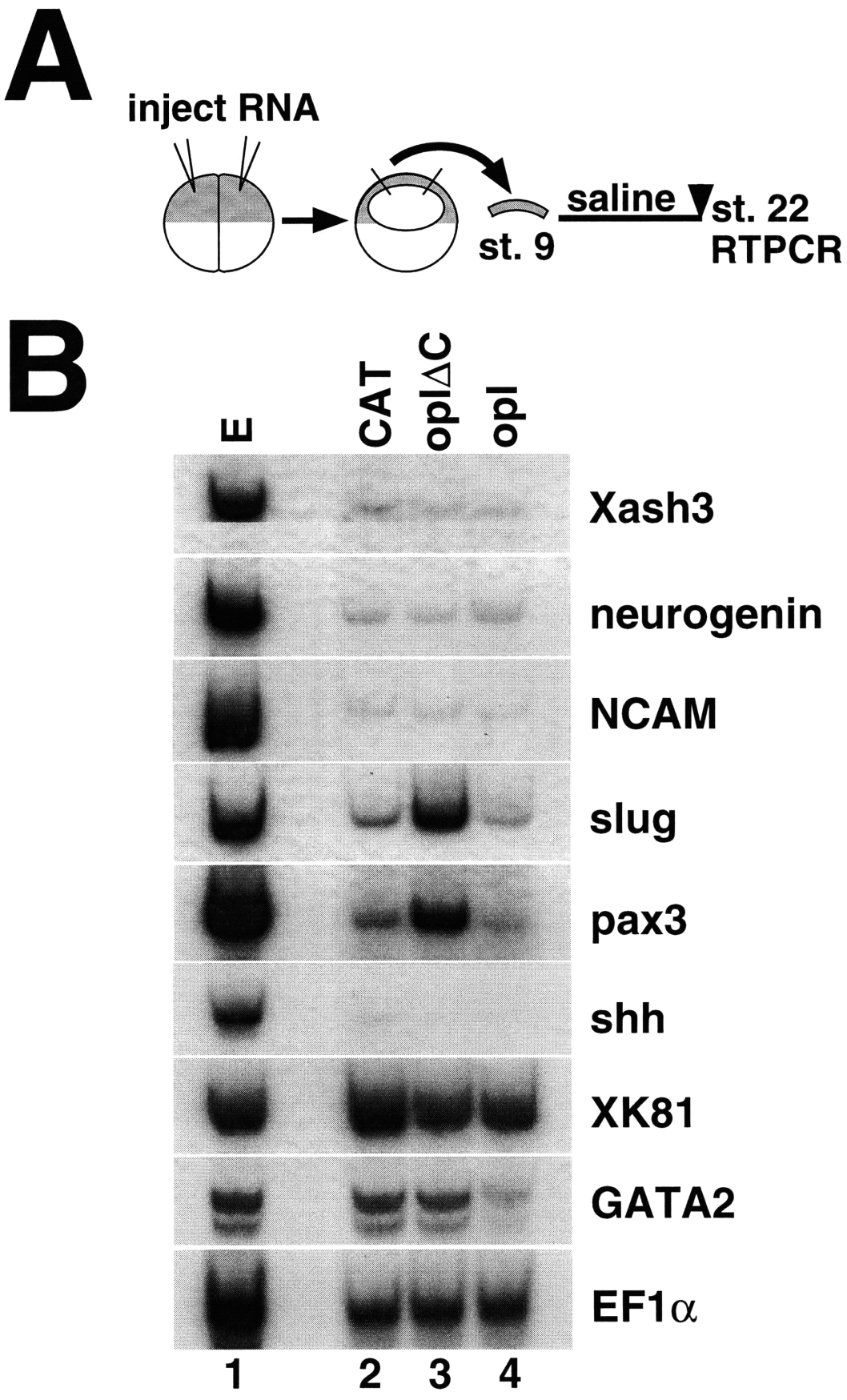

A
inject RNA
st. 9
saline
st. 22
RTPCR
B
E
CAT
oplΔC
opl
Xash3
neurogenin
NCAM
slug
pax3
shh
XK81
GATA2
EF1α
1
2
3
4

Figure 3.6. oplΔC sensitizes the ectoderm to induction by noggin.

Wild-type embryos were injected in both blastomeres with indicated RNAs at the two-cell stage, using 200 pg *globin* or *opl*ΔC, and 0.1 pg. 1 pg or 10 pg *noggin* (Smith and Harland, 1992) RNAs, as diagrammed in Fig.3.5A, except injection of *globin* RNA served as negative control by equalizing the injected RNA dose in all lanes. Pools of 15-20 animal caps were isolated from injected embryos at late blastula (st.9) and incubated in saline until harvest at tailbud (st.22) for RT-PCR analysis. See Methods for details. Expression of neural markers *NCAM* (Kintner and Melton, 1987) and *Xash3* (Zimmerman, et al., 1993) was analysed. *EF1a* served as loading control (Krieg, et al., 1989) . Samples processed without RT did not show *EF1a* signal after PCR (not shown). Data from one representative experiment is shown, similar results were obtained from three experiments. Lane 1: caps injected with 200 pg *globin* RNA; lane 2: caps injected with 200 pg *opl*ΔC RNA; lane 3: caps injected with 0.1 pg *noggin* and 200 pg *globin* RNAs; lane 4: caps injected with 1 pg *noggin* and 200 pg *globin* RNAs; lane 5: caps injected with 10 pg *noggin* and 200 pg *globin* RNAs; lane 6 caps injected with 0.1 pg *noggin* RNA plus 200 pg *opl*ΔC RNA; lane 7: caps injected with 1 pg *noggin* RNA plus 200 pg *opl*Δ*C* RNA; lane 8 caps injected with 10 pg *noggin* RNA plus 200 pg *opl*ΔC RNA.

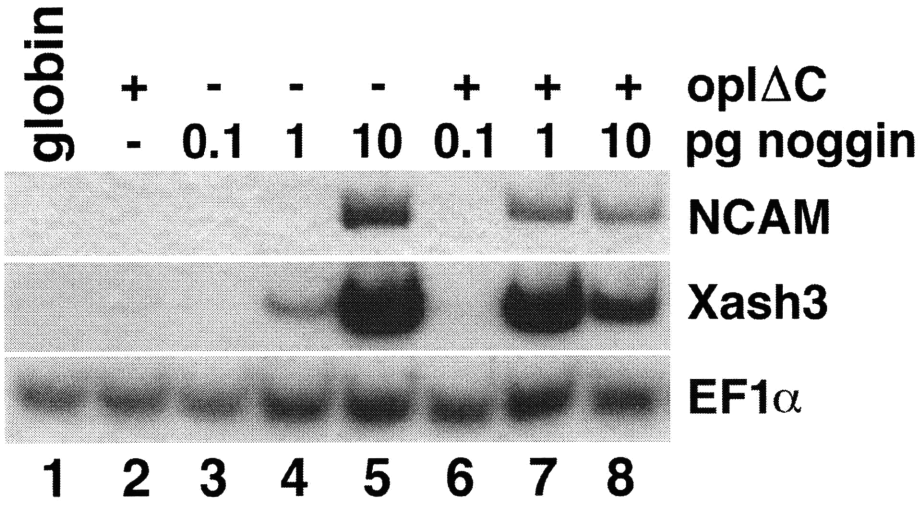

globin
+ - - - + + + opIΔC
- 0.1 1 10 0.1 1 10 pg noggin
NCAM
Xash3
EF1α
1 2 3 4 5 6 7 8

Figure 3.7. opl synergizes with noggin to activate more posterior neural markers in animal caps.

Wild-type embryos were injected in both blastomeres with indicated RNAs at the two-cell stage, using 200 pg *CAT*, *opl* or *oplΔC*, and 10 pg *noggin* (Smith and Harland, 1992) RNAs, as diagrammed in Fig.3.5A. Injection of *CAT* RNA served as negative control by equalizing the injected RNA dose. Pools of 15-20 animal caps were isolated from injected embryos at late blastula (st.9) and incubated in saline until harvest at tailbud (st.22) for RT-PCR analysis. See Methods for details. Expression of a set of neural markers expressed along the anteroposterior (A/P) or dorsoventral (D/V) neural axes was analysed. Uninjected embryos serve as control for baseline expression level. *NCAM* is a general neural marker (Kintner and Melton, 1987). Expressed in the indicated anterior (A) to posterior (P) series: *XCG* is a cement gland marker, the most anterior ectodermal tissue (Sive, et al., 1989); *otx*-2 is a forebrain and cement gland marker (Blitz and Cho, 1995; Pannese, et al., 1995) ; *en2* is a marker of mid/hindbrain boundary (Hemmati-Brivanlou and Harland, 1989); *krox20* marks rhombomeres 3 and 5 in the presumptive hindbrain (Bradley, et al., 1993); *HoxD1* is a hindbrain (posterior to rhombomere 4) and spinal cord marker (Kolm and Sive, 1995a), and *HoxB9* is a spinal cord marker (Wright, et al., 1990). Expressed in the indicated dorsal (D) to ventral (V) series of markers, *slug* (marking the neural crest, (Mayor, et al., 1995), *pax3* (marking the dorsal neural tube, (Espeseth, et al., 1995)), *NK2* (marking the ventrolateral neural tube, (Saha, et al., 1993)), and *shh* (marking the floorplate, (Ekker, et al., 1995). *M-actin* (Mohun, et al., 1984) is a mesodermal marker; *EF1a* (Krieg, et al., 1989) served as loading control. Samples processed without RT did not show *EF1a* signal after PCR (not shown). Data from one experiment is shown, comparable results were obtained in four independent experiments. Lane 1: st.22 embryo; lane 2: uninjected animal caps; lane 3: *noggin* plus *CAT* injected; lane 4: *oplΔC*-injected; lane 5: *noggin* plus *oplΔC* injected; lane 6: *noggin* plus *opl* injected.

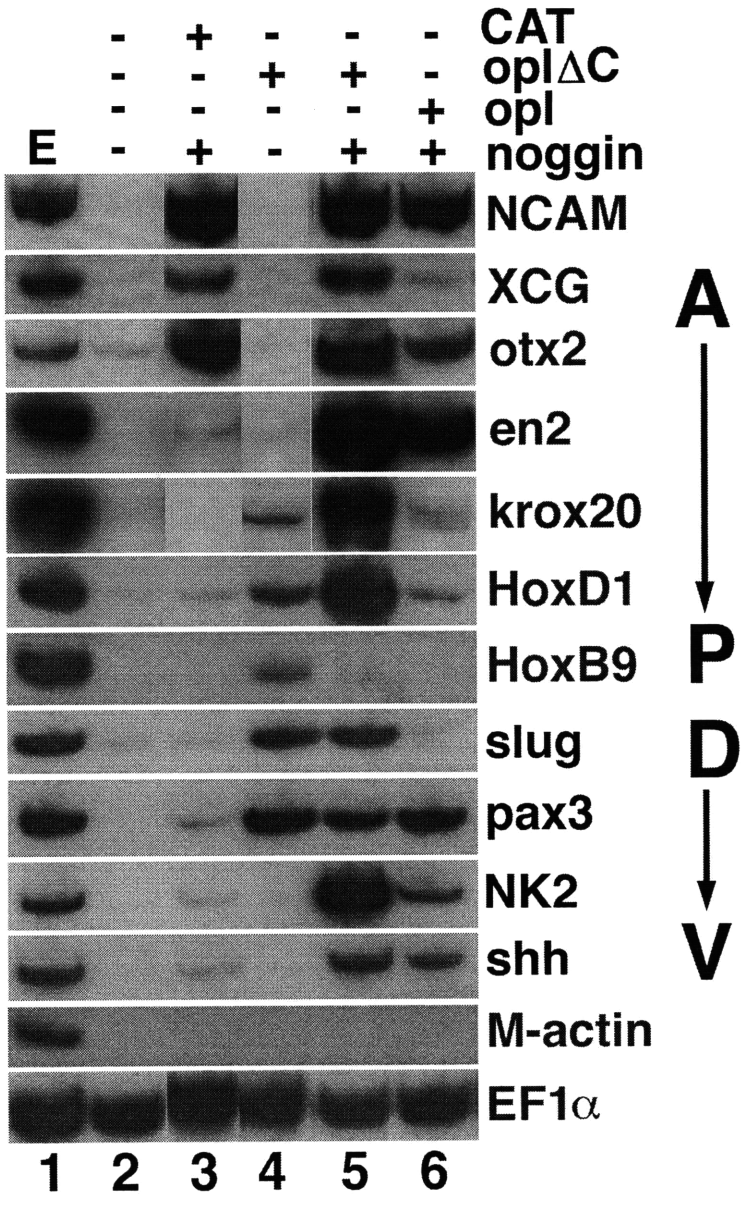

E
- + - - - CAT
- - + + - oplΔC
- - - - + opl
- + - + + noggin
NCAM
XCG
otx2
en2
krox20
HoxD1
HoxB9
slug
pax3
NK2
shh
M-actin
EF1α
A
P
D
V
1 2 3 4 5 6

Figure 3.8. opl induces cellular aggregates without cell division.
(A) Experimental scheme. Wild-type embryos were injected in one cell at the two-cell stage with 500 pg of *oplGR* or *oplΔC* RNA plus 100 pg *lacZ* RNA as lineage tracer. Injected embryos were incubated in saline alone or with dexamethasone (dex) and/or hydroxyurea plus aphidicolin (HUA) starting at early gastrula (st.10) until late neurula (st.19). See Methods for details.

(B) Morphology and histology of *opl*-induced cellular aggregates. βGal staining is obscured by pigment. In panels a-d, anterior (A) is to the left, dorsal is to the top. Panel a: embryo injected with *oplGR* RNA without dex addition; b,c: embryos injected with *oplGR* RNA and treated with dex from stage 10 until harvest. Arrows point to cellular aggregates in ventrolateral ectoderm. Panel d: embryo injected with *oplGR* RNA and treated with HUA (see Methods) plus dex from stage 10 until harvest. Panels e-g: embryos sectioned and stained after *oplΔC* injection e: +dex, transverse section of st.19 embryo through trunk region. Regions magnified in panels f and g are boxed and indicate uninjected and injected sides of the embryo respectively, as judged by βGal staining. f: uninjected side; g: injected side. The width of the ectoderm is bracketed in f and g. D: dorsal, V: ventral.

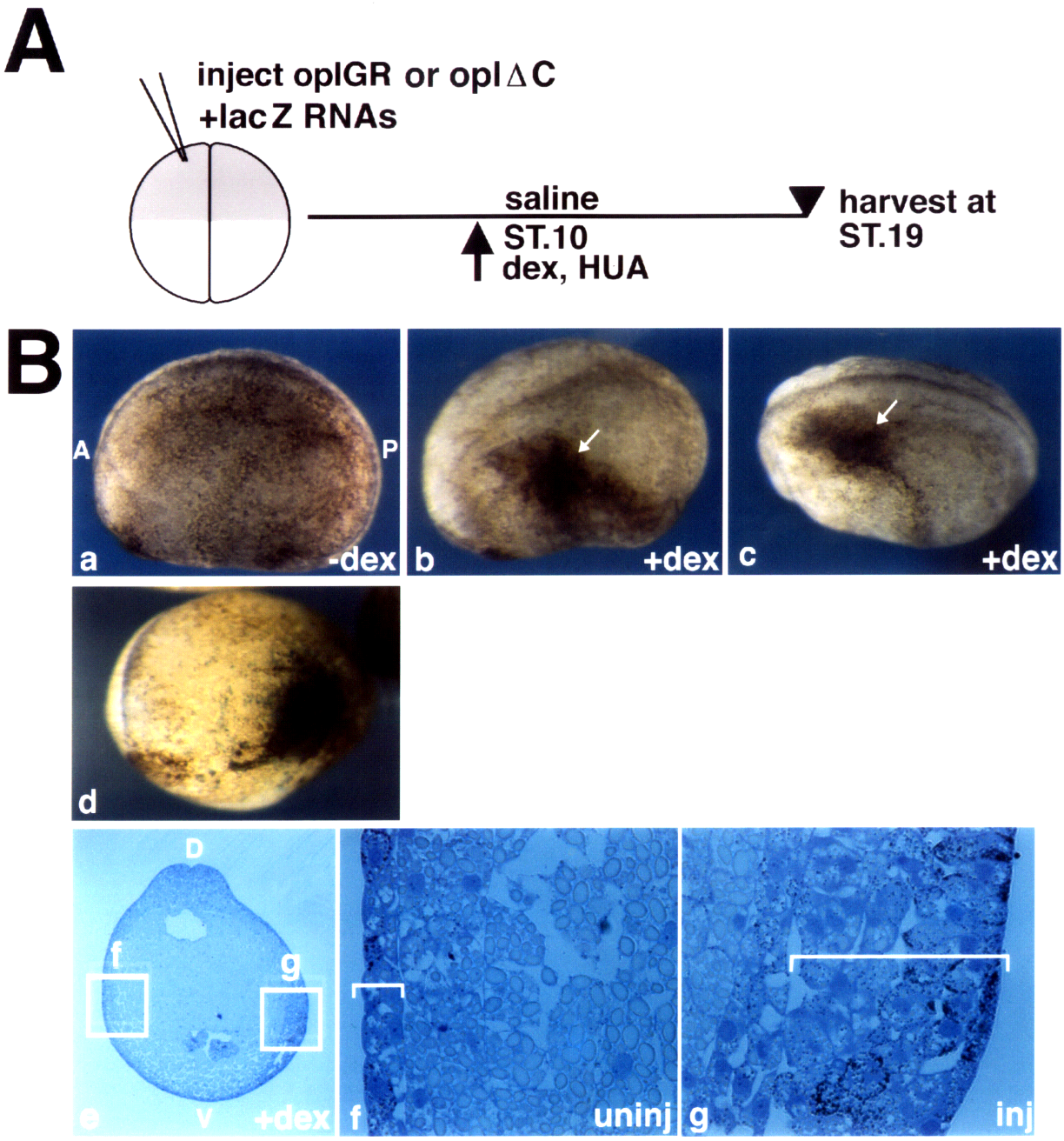

A
inject opIGR or opIΔC
+lacZ RNAs
saline
ST.10
dex, HUA
harvest at
ST.19
B
A
P
a
-dex
b
+dex
c
+dex
d
D
f
g
e
V
+dex
f
uninj
g
inj

Figure 3.9. opl induces ectopic slug and pax3 and inhibits XK81 expression in embryos.

(A) Experimental scheme. Albino embryos were injected in one cell at the two-cell stage with 500 pg *oplGR* or *oplΔC* plus 100 pg *lac*Z RNA as lineage tracer. Embryos were incubated in saline, or with addition of dexamethasone (dex) at stage 11, until embryos reached mid-neurula (st.18), when they were harvested for in situ analysis. See Methods for details.

(B) Ectopic *slug* (Mayor, et al., 1995) and *pax3* (Espeseth, et al., 1995) expression in whole embryos. Embryos were injected with *oplGR* plus *lacZ* RNAs and incubated without (panels a,c) or with dex (panels b, d) before histochemical staining for βGal (light blue) and in situ hybridization for *slug* or *pax3* (purple). Anterior is to the top in all panels. Arrowheads indicate injected side. Ectopic expression of *slug* and *pax3* is indicated by brackets. Panel a: -dex, stained for *slug*; panel b: +dex, stained for *slug*; panel c: -dex, stained for *pax3*; panel d: +dex, stained for *pax3*. A: anterior, P: posterior.

(C) *oplΔC* suppressed expression of the epidermal cytokeratin *XK81* (Jonas, et al., 1985). Embryos were injected with 500 pg *oplΔC* or *globin* RNAs mixed with 100 pg *lacZ* RNA, incubated in saline until st.18, before histochemical staining for βGal (light blue) and in situ hybridization for *XK81* (purple). Anterior is to the top in both panels. Endogenous *XK81* is expressed throughout the ventrolateral epidermis and excluded from the neural plate (white regions). White arrow indicates *oplΔC*-expressing cells (marked by βGal staining) do not express *XK81*. Panel a: *globin* injected; panel b: *oplΔC* injected. A: anterior, P: posterior.

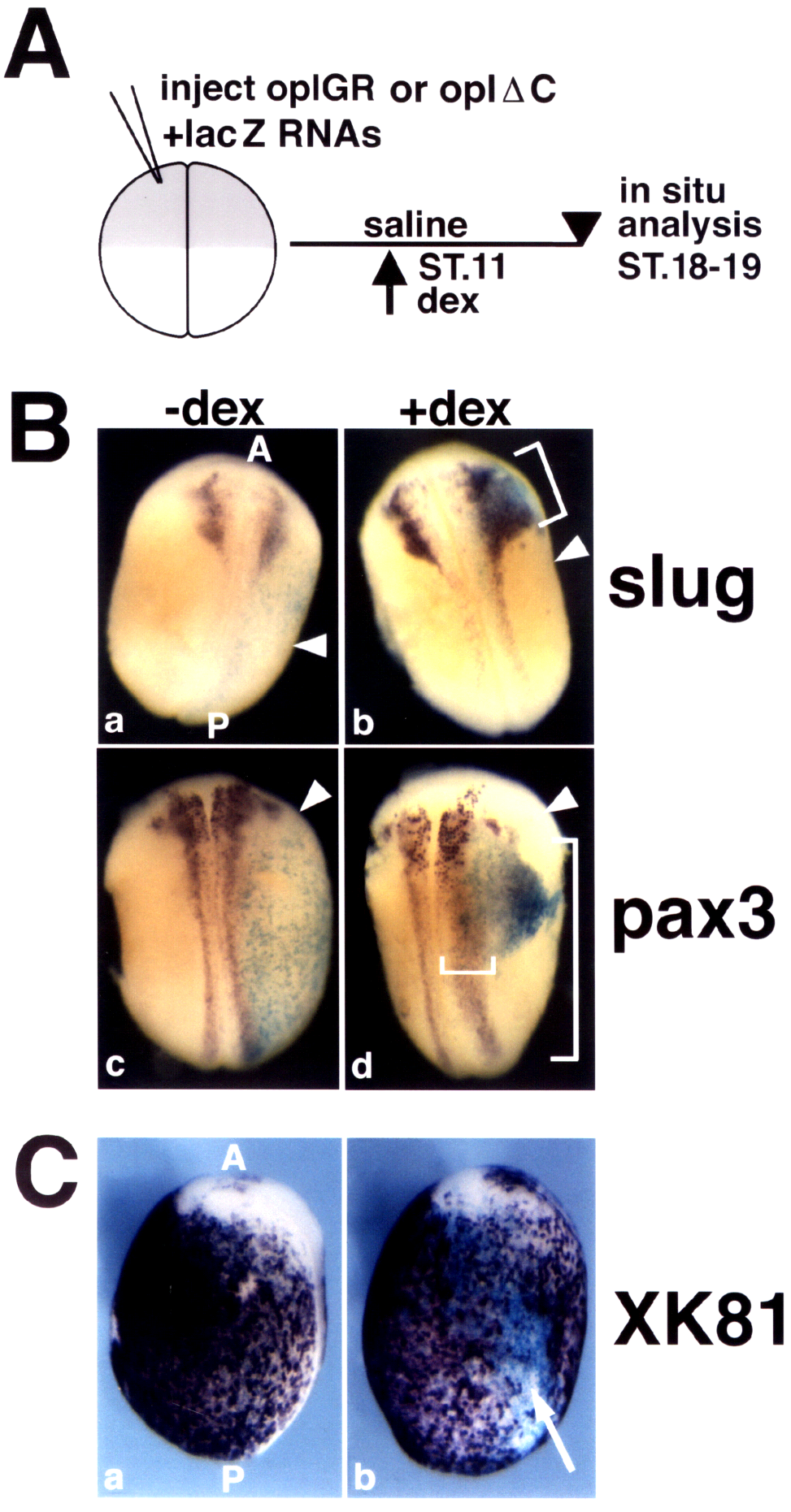

A
inject oplGR or oplΔC
+lacZ RNAs
saline
ST.11
dex
in situ
analysis
ST.18-19
B
-dex
+dex
A
a
P
b
slug
c
d
pax3
C
A
a
P
b
XK81

Figure 3.10. Model of the roles opl plays in early neural determination.

Schematics of whole embryos during gastrulation are shown. Dorsal views, Anterior is to the top in each schematic. Arrow, dorsal lip of blastopore. A: anterior, P: posterior. High level *opl* expression (spots) is activated in the dorsal ectoderm by early gastrula. By mid-gastrula *opl* expression has sensitized dorsal ectodermal cells for later induction and patterning by neural inducers such as *noggin* (light stripes). *opl* also acts by suppressing ventral ectodermal fates, such as epidermis (T bar). By late gastrula, *opl* expression has become restricted to the presumptive dorsal neural tube and neural crest, where it activates expression of neural markers in these regions. A dorsally restricted synergizing activity (dark stripes) is necessary for activation of neural crest fates. See text for further discussion.

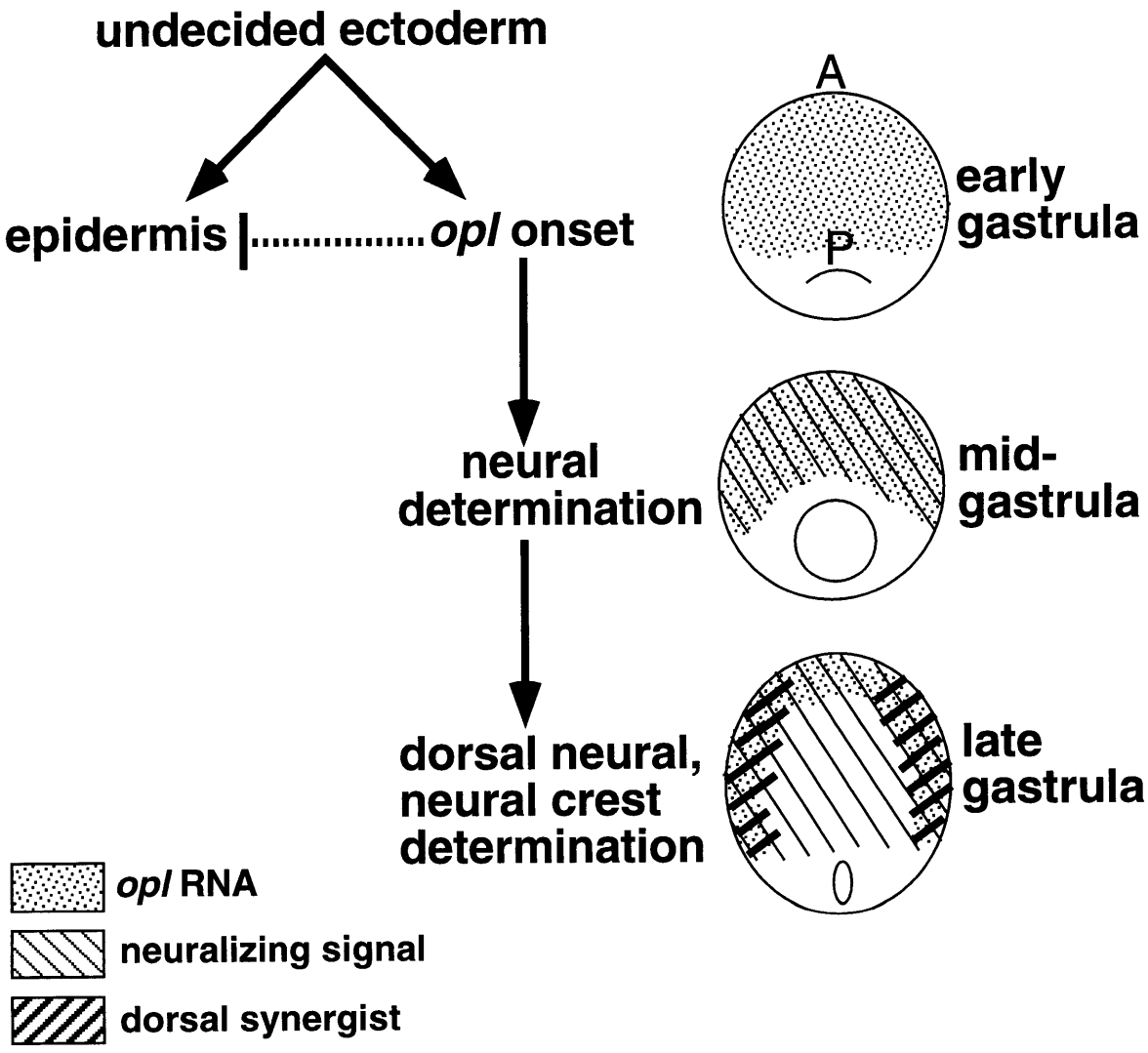

undecided ectoderm
epidermis
opl onset
neural determination
dorsal neural, neural crest determination
A
P
early gastrula
mid-gastrula
late gastrula
opl RNA
neuralizing signal
dorsal synergist

Table 3.1. Frequency of ectopic cellular aggregates in injected embryos.

Treatment[a]	Ectopic aggregates (%)[b]	Number of embryos[c]	Number of experiments[d]
opl	35 (15)	263	7
oplΔC	232 (64)	361	17
oplΔC +HUA	77 (57)	135	4
oplGR			
-dex	0 (0)	72	2
+dex	26 (50)	52	2
oplGR			
-HUA+dex	19 (66)	29	1
+HUA+dex	18 (58)	31	1
globin	0 (0)	244	12

Embryos were prepared as described in Figure 3.8A; pigmented cellular aggregates were scored at st.19.

[a] One blastomere of two-cell embryos was injected with *opl*, *oplΔC*, *oplGR* or *globin* RNAs. Embryos were incubated in saline or with 10 μM dexamethasone (dex) until harvest at st.19. See Methods for details.

[b] Number and frequency of embryos showing pigmented cellular aggregates in the region of injected RNA (marked by βGal blue staining).

[c] Total number of embryos examined.

[d] Number of independent experiments.

Table 3.2. Induction of cellular aggregates depends on time of addition and duration of dex treatment in *oplGR*-injected embryos.

Stage of dex addition[a]	Stage of maximal phenotype (elapsed hours)[b]	Ectopic aggregates (%)[c]	Number of embryos[d]	Number of experiments[e]
8	15 (12.5)	19 (59)	32	2
10	15 (8)	28 (85)	33	2
11.5	14 (4.5)	20 (59)	34	2
12	20 (8)	22 (71)	31	2
15	24 (9)	14 (78)	18	1
18	25 (9)	3 (15)	20	2
24	36 (24)	0 (0)	10	1

Embryos were prepared as described in Figure 3.6A; pigmented cellular aggregates were scored over time after addition of dexamethasone (dex).

[a] Stage when dex treatment was started.
[b] Stage and elapsed hours since start of dex treatment when maximal size and extent of pigmented cellular aggregates was observed in embryos.
[c] Number and frequency of embryos with ectopic pigmented cellular aggregates.
[d] Total number of embryos examined.
[e] Number of independent experiments.

Table 3.3. Frequency of ectopic *slug* and *pax3* expression in *oplGR*-injected embryos.

Treatment[a]	Marker examined	Ectopic expression(%)[c]	Number of embryos[d]	Number of experiments[e]
oplGR	*slug*	1 (3)	35	3
- dex	*pax3*	0 (0)	38	4
+ dex	*slug*	18 (42)	43	3
	pax3	58 (75)	77	4

Embryos were prepared as described in Figure 3.5A; *slug* and *pax3* expression was scored by in situ hybridization on neurula (st. 18) embryos.

[a] One blastomere of two-cell embryos was injected with *oplGR* RNA. Embryos were incubated in saline or with 10 μM dexamethasone (dex) starting at st.11 until harvested at st.18. See Methods for details.
[b] *slug* and *pax3* expression in embryos was examined by in situ hybridization as described in Methods.
[c] Number and frequency of embryos with ectopic *slug* or *pax3* expression, of the total number with βGal staining anywhere in the embryo. In the +dex group examined with *slug*, none showed ectopic expression in ventrolateral ectoderm not contiguous with the endogenous *slug* domain. In the +dex group examined with *pax3*, 55 (71%) embryos showed ectopic expression that was broad and contiguous with the endogenous *pax3* expression, 3 (4%) embryos showed ectopic expression in ventrolateral ectoderm that was non-contiguous with the endogenous *pax3* domain.
[d] Total number of embryos examined.
[e] Number of independent experiments.

Table 3.4. Frequency of *XK81* inhibition in *opl*ΔC-injected embryos.

Treatment[a]	*XK81* inhibition (%)[b]	Number of embryos[c]	Number of experiments[d]
*opl*ΔC	43 (51)	84	3
globin	0 (0)	85	3

Embryos were prepared as described in Figure 5A; *XK81* expression was scored by in situ hybridization on neurula (st.18) embryos.

[a] One blastomere of two-cell embryos was injected with *opl*ΔC or *globin* and embryos were incubated in saline until harvest. See Methods for details.
[b] Number and frequency of embryos with *XK81* expression inhibited when *opl* expression (marked by βGal blue staining) was located in ventrolateral ectoderm.
[c] Total number of embryos examined.
[d] Number of independent experiments.

Chapter 4. Future Directions

4.1 Summary

The molecular basis of early neural determination and patterning in *Xenopus* embryos is largely unknown. Many previous efforts to identify genes involved in neural development relied on homology with known genes, therefore potentially missing many novel neural-specific genes. In Chapter 2, I described a PCR-based subtraction strategy to identify genes that are differentially expressed in *Xenopus* gastrula dorsal ectoderm. 75 different clones were identified by differential blot analysis and DNA sequencing out of 800 cDNAs randomly chosen from the subtracted dorsal ectoderm cDNA library. In situ analysis showed that 44 different clones were specifically expressed in gastrula ectoderm, with 26 of the 44 being dorsal-specific; 30 other clones were undetectable in gastrula, but expressed later in restricted neural domains. In situ assays with these markers on early gastrula ectoderm show that the dorsal ectoderm is already committed to the neural lineage, and that there exists a rudimentary ectodermal pattern by mid-gastrula.

Many of the isolated clones were novel sequences, but some contain DNA-binding motifs, suggesting that they encode transcriptional regulatory proteins. Five isolated clones (including the known anterior patterning homeobox gene *otx2*) are candidate genes for involvement in early neural determination and patterning because they are highly expressed in restricted neurectodermal domains at gastrula stage, and later persist in various parts of the nervous system.

In Chapter 3, I reported on the analysis of one candidate gene, *opl* (*odd-paired*-like), which resembles the *Drosophila* pair-rule gene *odd-paired* and encodes a zinc finger protein that belongs to the *Zic* gene family. At the onset of gastrulation, *opl* is expressed throughout the presumptive neural plate, indicating that neural determination has begun at this stage; by neurula, *opl* expression is restricted to the dorsal neural tube and neural crest. *opl* encodes a transcriptional activator, with a carboxy terminal regulatory domain, that when removed increases *opl* activity. *opl* both sensitizes animal cap ectoderm to low levels of the neural inducer *noggin*, and alters the spectrum of genes induced by *noggin*, allowing activation of the midbrain marker *engrailed*. Consistent with the later dorsal neural expression of *opl*, the activated form of *opl* is able to induce neural crest and dorsal neural tube markers both in animal caps and whole embryos. In

ventral ectoderm, *opl* induces formation of loose cell aggregates that may indicate commitment to the neural crest lineage. Aggregates do not express an epidermal marker, indicating that *opl* suppresses ventral fates. Together, these data suggest that *opl* may mediate neural competence early, and later be involved in activation of midbrain, dorsal neural and neural crest fates.

4.2 Future directions

The subtraction screen yielded a large number of still uncharacterized neural-specific genes. All are expressed in specific regions of the developing nervous system, and fall broadly into two classes based on their onset of expression. Genes expressed at high level in the gastrula ectoderm, such as *opl, otx2*, and *fkh5* have potential functions in mediating early neural determination and patterning. Many other uncharacterized genes are detected at low level during gastrula stages, or only found at high level in specific neural domains at later stages. This second class may contain genes important for specifying neural tissues that form later (i.e. eyes, otic vesicle), or for maintaining neural identity or differentiation. Due to my interest in early neural determination, I will focus my discussion of future investigations on the gastrula dorsal ectodermal genes, with *opl* being a model.

What activates these gastrula dorsal ectodermal markers in the presumptive neurectoderm? How is their initial expression domain established and regulated? How do they interact with other factors to regulate neurectodermal pattern? What functional roles do they play in the developing neurectoderm? What target genes do they regulate? Answering these questions will yield better understanding of the molecular mechanisms in early neural development.

4.2A What regulates opl expression?

opl has a complex early expression profile in the ectoderm. First it is expressed throughout the late blastula ectoderm, then becomes dorsal ectoderm-specific at early gastrula, and concentrated at the anterior and lateral neural plate margins by the end of gastrulation, finally located in the dorsal nervous system. To examine *opl* regulation, genomic *opl* clones containing 5' and 3' flanking regions have been isolated from *Xenopus* and zebrafish (Gamse et al, unpublished).

4.2A1 What activates early opl expression?

Dorsal ectodermal *opl* expression at early gastrula extends to the animal pole, probably too far away from the organizer to be initiated by neural inducers. One possibility is to test whether maternal dorsalizing activity is required for *opl* specification in animal caps. UV treatment or depolymerizing agents can be used to prevent cortical rotation and dorsal nuclear localization of β-catenin. Another possibility is that the chromatin structure of *opl* 's regulatory region is similar to that of ubiquitous housekeeping genes, and so *opl* is activated together with housekeeping genes at the mid-blastula transition.

4.2A2 How is opl expression regulated after the gastrula stage?

Consistent with opl's later roles in activating dorsal neural tube and neural crest fates, its expression is cleared from the middle and localized to the margins of the presumptive neural plate by the end of gastrulation. I have already shown that noggin activates *opl*, and may maintain endogenous *opl* expression in the dorsal neural tube because they are co-localized in the dorsal neural tube into tailbud stages (Kuo, unpublished; (Smith and Harland, 1992). It is also reasonable to hypothesize that factor(s) in the presumptive floor-plate (medial gastrula neurectoderm) override noggin (also secreted from chordamesoderm under the medial gastrula neurectoderm) and block *opl* expression. HNF-3β, other shh-activated factors, or the short-range shh gradient may contribute to *opl* inhibition in the medial region of gastrula neurectoderm.

Detailed analysis to define and study the regulatory regions of the genomic *opl* sequence could yield clues to the identity of regulatory factors. A non-biased approach to identifying factors that interact with *opl*'s regulatory regions is to perform a yeast one-hybrid screen: *opl*'s regulatory region is linked to a selectable marker and used as 'bait' to screen a gastrula-stage library of cDNAs fused to a transcriptional activator domain. Comparison of the non-coding *opl* sequences between *Xenopus*, zebrafish and mouse may also suggest what sequences account for the similarities and differences in onset of expression between the species. Zebrafish *opl* is not expressed until mid-gastrula stage, but is also later localized to the dorsal nervous system. Mouse *zic1* is expressed in a similar dorsal neural location as *Xenopus opl*, and in addition, is also present in the dorso-medial somites (Aruga, et al., 1996; Nagai, et al., 1997).

4.2B What are opl's functional roles in neural development?

4.2B1 Ablation of opl function in vivo

The current understanding of *opl's* multiple roles in early neural determination comes from misexpression analysis. There is a distinct possibility that opl overexpression could mimic the effects of another *Zic* family member, or even due to binding of related zinc finger binding sites (i.e. DNA binding sites of the Gli protein family). To define precisely developmental processes that require opl, it is necessary to ablate opl function in vivo. There are several different strategies for genetic ablation.

In *Xenopus*, the technique of creating transgenic embryos has been achieved recently (Kroll and Amaya, 1996). It is conceivable that an antisense construct introduced into a transgenic embryo will ablate *opl* function, thereby revealing which pathways require opl activity. This would be more reliable and efficient than the injection of antisense constructs into embryos that result in mosaic expression. One can pursue gene targeting studies in mouse; but there still remains the possibility that related proteins or redundant activities will mask the effects of the genetic ablation (i.e. as in the case of the myogenic bHLH factors). Transgenesis has the advantage of universally ablating endogenous opl activity; but may be most informative for dissecting multiple functions if expression of the transgenic construct was made tissue-specific or stage-specific.

Another strategy is to create dominant-interfering opl proteins, since opl is a transcriptional activator in vitro. Possible dominant interfering constructs are opl fusions with known repressors, and truncated opl proteins that sequester co-factors or opl DNA binding sites. One expects to observe a mutant phenotype that can be rescued to wildtype by co-expression of wildtype opl. An alternative approach is the creation and injection of appropriate ribozyme constructs to target *opl* transcripts in vivo.

4.2B2 What factors interact with opl to mediate its functions?

Opl's multiple functional roles and diverse spatial expression domains suggest that it interacts with other factors to mediate different effects.

It would be interesting to examine how opl interacts with other known neural competence factors such as the translation initiation factor eIF4AII (Morgan and Sargent, 1997), protein kinase-C activity (Otte, 1992a; Otte, et al., 1991) , and Xotch (Coffman, et al., 1993). *eIF4AII* is expressed

throughout the gastrula dorsal ectoderm and later restricted to the lateral neurectodermal margins; this expression is preceded by *opl* expression in those same domains. Does opl activate eIF4AII expression? Does opl interact with the eIF4AII-PKC regulatory loop? Is *opl* modulated by or regulating the other competence factors?

Retinoic acid, wnt, FGF signaling and Opl have been implicated in posteriorization of induced neurectoderm. There are many experimental reagents (dominant negative ligands and receptors, modulators of intracellular signaling) that could be useful for perturbing each of these pathways in conjunction with opl expression. It would be interesting to ask whether opl-mediated activation of the midbrain marker, *engrailed*, is affected or dependent on the above signaling pathways.

Opl's role in determining dorsal neural tube and neural crest fates should be explored further. Does opl directly activate the dorsal neural tube marker, *pax3*, and the neural crest marker, *slug*? What opl-activated effectors mediate the apparent change in cell adhesion that results in cellular aggregates? It would be relevant to ask which cell adhesion components are altered by opl to result in differential cell adhesion? This may be an important step in neural crest determination.

Finally, possible interactions with other factors expressed in overlapping neurectodermal regions could be explored further. *opl* and *otx2* partially overlap at the anterior margin of the gastrula neurectoderm, and preliminary data show that in animal caps, *otx2* activates *opl* expression, but *opl* does not activate *otx2* (Gammill, unpublished). This is curious because *opl* activation precedes *otx2* in this region, and speculation is that this interaction may have consequences for anterior neurectodermal patterning.

4.2C What downstream target genes are regulated by opl?

One major goal of this thesis was to identify gastrula dorsal ectodermal genes expressed mediating ectodermal response to neural induction. An important question remains: how are these gastrula dorsal ectodermal genes linked to known neurogenesis genes?. I showed that opl activates the dorsal neural tube and neural crest markers (*pax3*, *slug*, and *snail*) in isolated ectoderm and ectopically in embryos (Chapter 3, (Kuo, et al., 1998) . Are these direct targets of *opl* ? One can answer that question by checking

whether cycloheximide treatment (inhibits protein synthesis) still allows activation of the potential downstream target genes in opl-expressing tissue.

An unbiased approach to identify opl's downstream target genes involves a subtractive cloning strategy. To enrich for opl-activated genes, a cDNA library made from animal caps injected with opl RNA would be subtracted against a cDNA library made from uninjected animal caps. Selection for direct downstream targets would be enhanced by harvesting animal caps after activating an inducible opl for a short time (i.e. 60 min), or to treat with cycloheximide concurrent with opl activation.

A more involved approach is to first identify an optimal opl DNA binding site (i.e. reiterated selection of random oligonucleotides by gel mobility shift assays), then 1) use the sequence in a computer search through the known database for genes, or 2) utilize the opl DNA binding sequence to probe for genomic DNA fragments that may contain candidate target genes.

Another strategy to consider is to use the powerful yeast 'one-hybrid' screen to identify genomic DNA fragments that bind opl. Sheared genomic DNA would be subcloned next to a selectable marker, and opl-yeast activator fusions would serve as 'bait'. Selected DNA would then be used to probe a genomic library to isolate candidate opl target genes which are linked to opl-binding sequences.

What regulates opl expression? What genes are regulated by opl? Answering these questions will better define opl's functional roles in neural development. These are strategies also useful for studying other identified gastrula dorsal ectodermal genes, and will lead to a better understanding of the molecular mechanisms of early neural determination.

Appendix I. Materials and Methods

Growth, dissection and culture of embryos and explants

Xenopus laevis eggs were collected, fertilized and cultured as described in (Sive, et al., 1989) . Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop, et al., 1967) . For animal caps, late blastula (stage 9) animal hemisphere ectoderm was isolated and incubated in 0.5X MBS alone, or with other reagents as indicated. In experiments with inducible *opl* proteins, embryos and animal caps were treated with dexamethasone (dex, Sigma, 10μM) freshly diluted in 0.1X or 0.5X MBS as appropriate. To inhibit cell proliferation, embryos were allowed to develop to early gastrula (stage 10), then placed in hydroxyurea (Sigma, 20 mM) and aphidicolin (Sigma, 150 μM) (Harris and Hartenstein, 1991) . Sibling embryos were allowed to develop to hatching stages to confirm treatment efficacy.

IB.Subtractive cloning of opl

PCR-based subtractive cloning modified from (Wang, et al., 1991) and described in detail by (Patel and Sive, 1996) was used to identify *opl*. Briefly, RNA was isolated from dissected mid-gastrula (st.11.5) dorsal ectoderm and blastula (st.9 - 9.5) animal caps aged to mid-gastrula equivalent (st.11.5) using the proteinase K-phenol extraction method (Condie, et al., 1987) , followed by DNAseI treatment to remove any contaminating DNA. polyA$^+$ RNA was prepared using oligo-dT cellulose columns. cDNA was prepared by oligo-dT primed first strand cDNA synthesis, then using the Pharmacia Timesaver cDNA synthesis kit according to manufacturer's instructions. cDNA products were digested with AluI and RsaI restriction enzymes, then ligated with oligo adaptors. Oligo 1 (5'-TAGTCCGAATTCAAGCAAGAGCACA-3') was kinased, then annealed to oligo 2 (5'-CTCTTGCTTGAATTCGGACTA-3'), and ligated to the dorsal ectoderm (D) cDNA, thereby introducing flanking EcoRI sites (underlined). Oligo 3 (5'-ATGCTGGATATCTTGGTACTCTTCA-3') was kinased and annealed to oligo 4 (5'-GAGTACCAAGATATCCAG*CAT*-3'), and ligated to the animal cap (uninduced) ectoderm (U) cDNA, thereby introducing flanking EcoRV sites (underlined). Before each round of subtraction, tracer cDNA (from the D population) was PCR-amplified in the presence of ^{32}-P-dCTP, and driver cDNA (from the U population) was PCR-amplified in the presence of biotinylated-11-dUTP (Enzo Diagnostics). Subtractive hybridization was carried out at 68^{o}C in a Perkin-Elmer thermocycler, with long hybridizations (40 hours) alternating with short

hybridizations (2 hours) to remove rare common sequences and abundant common sequences, respectively. Biotinylated duplexes were removed by treatment with streptavidin and phenol extraction. The enriched tracer and driver cDNAs were amplified for the next round of subtraction. Ten rounds of subtraction were performed to produce D_{10} and U_{10} cDNAs. At intermediate steps, subtraction efficiency was monitored by measuring removal of ^{32}-P-labeled tracer cDNA, enrichment of known dorsal ectodermal genes expressed at mid-gastrula and removal of the ubiquitous translation factor *EF1α*. Mesodermal contamination was assessed by analysis of *brachyury* RNA, and was undetectable in the D cDNA pools. The resulting D_{10} cDNA products with average size of 300 bp (range 150 to 1000 bp) were cut with EcoRI and subcloned into CS2+ vector (Rupp, et al., 1994) . 800 clones were randomly selected and screened by dot blot analysis with probe prepared from D_{10} and U_{10} cDNAs to identify cDNAs expressed more strongly in the dorsal pool. 194 dorsal clones were sequenced and grouped into 75 sequences. By in situ hybridization, 44 were of these were detected in gastrula stage dorsal ectoderm: 26 were expressed more strongly in dorsal ectoderm, with 18 being equally expressed in both dorsal and ventral ectoderm. Many of these sequences contained putative DNA binding motifs and their analysis will be reported elsewhere. Details of the subtraction screen are presented and discussed in Chapter 2.

One of the most abundant clones isolated encoded a zinc finger protein, *opl* (Fig.1). A full length *opl* cDNA clone was isolated by screening clones from a mid-gastrula (st.11.5) λZap phage library (prepared by C. Nocente-McGrath) using standard methods. Two cDNA clones were identified, the larger one called TG6.11 contained a 2.6 kb insert. Conceptual translation of the largest open reading frame yielded a 443 aa protein (Figure 1B). BLAST searching with the protein sequence revealed high similarity to *Drosophila opa* in the putative zinc finger regions (Figure 1A).

IC opl constructs

Constructs are diagrammed in Fig.3. All techniques are as described in (Sambrook, et al., 1989), unless otherwise indicated. Enzymes were obtained from Stratagene or New England Biolabs. All constructs are in pCS2+ or pCS2+MT vectors (Rupp, et al., 1994; Turner, et al., 1994) unless otherwise indicated. First, the *3F* plasmid was created by ligating the 5' UTR-*opl* open reading frame (ORF) fragment (699 bp) from TG6.11 into pCS2+ vector. The 5'

end of *opl* was modified using the primers 5'ORF (5'GTGAATTCGCCTCCATGGCGATGCTGCTGGACACCGGA-3') and opl-D2 (5'-GTTGGCCAACTGCTCGG-3'). This introduces an EcoRI site and an optimal translation initiation site (underlined; (Kozak, 1987)) upstream of the *opl* initiation ATG (underlined) and results in two extra aa (M-A) at the NH2-end of translated peptide. The amplified product was digested with EcoRI and SacI, then ligated into an EcoRI+SacI digested *3F* plasmid to create the *opl* plasmid. To create *oplDC*, a PstI (end-filled)-XhoI (blunted) opl plasmid backbone was self-ligated. Klenow activity was used to remove 16 extra bp distal to the XhoI site (see Fig.1). Myc-tagged *opl* (*MTopl*) was made by ligating an EcoRI (end-filled)-XbaI (end-filled) fragment of *opl* into the XbaI (end-filled) site of CS2+MT that contains 6 copies of the myc epitope. *MTopl*Δ*C* was made by ligating a NotI-NotI *opl*Δ*C* fragment into the NotI-digested MT*opl* vector backbone. To make constructs encoding inducible opl proteins (*oplGR*), the ligand binding domain of the human glucocorticoid receptor was PCR-amplified from the *MyoD*GR plasmid (Hollenberg, et al., 1993) with the primers MP-GR1-XhoI (5'-GGCGCCGAGCTCGAGCCTCTGAAAATCCT-3') and MP-GR2-XhoI (5'-GGCGGGCACTCGAGACTTTTGATGAAAC-3'). Products were then digested with XhoI (underlined) and inserted into an XhoI-digested *opl* plasmid. The integrity of all PCR products were verified by sequencing.

ID Microinjection

Embryos were dejellied in 2% cysteine pH 8.0, 15-30 minutes after fertilization and rinsed in 0.1X MBS. At the two-cell stage, embryos were placed in 1X MBS + 4% Ficoll (Sigma) and injected with 5-10 nl of RNA in the animal hemisphere (whole embryo experiments) or near the animal pole (for animal cap experiments). 200 to 500 pg of *opl*, *opl*Δ*C* or *oplGR* RNAs were injected per embryo. 0.1 to 10 pg *noggin* RNA (derived from the CS2+*noggin* plasmid) per embryo was co-injected in the *opl* plus *noggin* synergy experiments. 100 pg *lacZ* RNA was co-injected as tracer in whole embryo assays. Embryos recovered in 1x MBS + 4% Ficoll for 1 to 4 hours, then were transferred to 0.1 x MBS and incubated at 15°C until the desired stage.

IE In vitro transcription

Capped sense RNAs for microinjection were synthesized as in (Krieg, et al., 1984) . *lacZ* sense RNA was made according to Gammill and Sive, 1997. All *opl*-derived sense RNAs (*opl*, *opl*ΔC, *oplGR*, MT*opl*, MT*opl*ΔC) were made by SP6 transcription of a NarI-NarI fragment from CS2+derived vectors. Other templates for sense RNAs are: *CAT* (p64TCAT; (Kuo, et al., 1996)), SmaI linearized, SP6 transcribed; *globin* (pXbM), PstI linearized, SP6 transcribed; *noggin* (pCS2+x*noggin*), NotI linearized, SP6 transcribed. Antisense probes for in situ hybridization were transcribed in the presence of digoxigenin-11-UTP or fluorescein-11-UTP as follows: *XCG* (Sive, et al., 1989) , *otx2* (Pannese, et al., 1995) , *en2* (Hemmati-Brivanlou and Harland, 1989) , *krox20* (Bradley, et al., 1993), *slug* (Mayor, et al., 1995) , *pax3* (Espeseth, et al., 1995) , *XK81* (Jonas, et al., 1985).

IF In situ hybridization and sectioning

Whole mount in situ hybridization was performed on albino embryos essentially as described in (Harland, 1991) with the following modifications. Antibody (anti-DIG-AP) binding was carried out in MAB (100 mM maleic acid, 150 mM NaCl, pH 7.5) containing 2% Boehringer Mannheim Blocking Reagent. Alkaline phosphatase reaction was performed with NBT and BCIP in AP buffer (Tris pH 9.5, 10% polyvinyl alcohol (PVA; 98-99% hydrolyzed; Mw 31,000-50,000; Aldrich), MgCl2, NaCl, Tween20), producing a purple stain. For double in situ hybridization, a second probe was synthesized using fluorescein-UTP and detected by binding with anti-fluorescein-AP antibody. The first color reaction, using BCIP alone in the AP buffer (light blue staining), was followed by AP inactivation (MAB, EDTA, 65°C, 20 min), dehydration in methanol and binding of the anti-DIG-AP antibody. The second color reaction was with NBT and BCIP as described above. β-galactosidase staining was performed as in (Kolm and Sive, 1995a). To examine details of internal or faint expression, embryos were refixed, then dehydrated in methanol before mounting in 2:1 benzyl benzoate/benzyl alcohol clearing solution and examined under Normarski optics (Cold Spring Harbor Laboratory Xenopus Course manual, 5th edition). For sections, embryos were embedded in JB4 resin (Polysciences 00226) and 5 to 10 μM sections were cut on a microtome using a dry glass knife and mounted in Crystal mount (Biomeda M03). Sections were stained with toluidine blue.

IG Isolation of RNA and Northern analysis

RNA was prepared from explants and embryos by proteinase-K treatment and phenol extraction (Condie and Harland, 1987). RNA was analyzed by Northern blotting (Sambrook, et al., 1989). *opl* antisense probe was made by asymmetric PCR (Sive, et al., 1991). with the opl-D2 primer described above.

IH Relative quantitative RT-PCR

RNA from pools of 15-30 explants or 2-4 embryos were treated with DNaseI (2 µl, 37°C for 60 minutes in 1x New England Biolabs Buffer #4). Analysis of gene expression by RT-PCR was essentially as described in (Sagerström, et al., 1996) . RNA from 5 animal cap equivalents or 0.25 embryo equivalents was annealed to random hexamers (0.5 µM in final reaction). First strand cDNA was synthesized with Superscript II Reverse Transcriptase (Gibco BRL) according to manufacturer's instructions. The linear range of amplification was determined for all primer pairs using cDNA from 0.25 animal caps or 0.0125 embryo equivalents (representing an equal amount of template for each sample). Cycles of 1 minute, 94°C; 1 minute., 55°C ; 2 minutes. 72°C were used. PCR reaction mixes contained 1X Taq buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 5 uCi [^{32}P]-adCTP (800Ci/mmol), 2.5 U Taq polymerase and 0.4 mM of each primer. Expression was analyzed by separation on a 6% acrylamide gel and autoradiography. *NCAM*, *en2*, *krox20* are described in (Hemmati-Brivanlou, et al., 1994a). *EF1a* and *XCG* primers are described in (Gammill and Sive, 1997). Other primers yielding product sizes as indicated are: *otx2*- 485 bp [X*otx2*.C (5'-GCAACAGCAGCAGCAGAATG-3'), X*otx2*.D (5'-TGTAATCCAGGCAGTCAGTG-3')] (Pannese, et al., 1995); *HoxD1*- 245 bp [PJK3 (5'-CAGCCCCGATTACGATTATTATGG-3'), PJK4 (5'-CCGGGGAGGCAGGTTTTG-3')] (Kolm and Sive, 1995a); *HoxB9* - 201 bp [HLS51 (5'-GCCCCTGCGCAATCTGAAC-3'), HLS52 (5'-CAGCAGCGGCTCAGACTTGAG-3')] (Wright, et al., 1990); *slug* - 220 bp [(Xslug1 (5'-GGTTCGATTTGCAGTATGG-3'), Xslug2 (5'-GAGCTGCTTCGTAAAGCAC-3')] (Mayor, et al., 1995) ; *pax3* - 181 bp [pax3A (5'-CAGCCGAATTTTGAGGAGCA*CAT*-3'), pax3b (5'-GGGCAGGTCTGGTTCGGAGTC-3')] (Espeseth, et al., 1995); *NK2* - 406 bp [*NK2*.1 (5'-AGAAACCGTCGGCTGATGAGTC-3'), *NK2*.2 (5'-

TGGGCGCTATAGGCAGAGAAAG-3')] (Saha, et al., 1993); *shh* - 191 bp [shh1 (5'-GGTTGACCGCGGCCCATCTAC-3'), shh2 (5'-AGGCGCATAAGCTCCAGTGTCC-3')] (Ekker, et al., 1995); *Xash3*- 227 bp [forward (5'-AAGCGGCTCCTGTCAAACACTAT-3'), reverse (5'-CCCCCGCGCTCCTCTCC-3')] (Zimmerman, et al., 1993); *neurogenin*- 263 bp [forward (5'-CGCCGCAACCCGACTCACCT-3'), reverse (5'-CCTGCATCGCGGGCTGTTCTC-3')](Ma, et al., 1996); *XK81*- 308 bp [forward (5'-T*CAT*TCCGTTCCAGCTCTTCTTAC-3'), reverse (5'-TCCAGGGCTCTTACTTTCTCCAG-3')] (Jonas, et al., 1985); *GATA2*- 415 bp [forward (5'-CTAAACAGAGGAGCAAGAGC-3'), reverse (5'-CCTGGAAAGTTCCTCAAAAC-3')] (Walmsley, et al., 1994); *m-actin*-222 bp [forward (5'-GCTGACAGAATGCAGAAG-3'), reverse (5'-TTGCTTGGAGGAGTGTGT-3')] (Stutz, et al., 1986)

IIWestern analysis and immunocytochemistry

Western blotting (Kolm and Sive, 1995b) and whole-mount immunostaining (Hemmati-Brivanlou and Harland, 1989) were performed using the c-myc 9E10 antibody (Santa Cruz Biotechnology) diluted 1:1000 (0.1 mg/ml) and the ECL Vector Elite kit from Amersham.

IJ Transient transfection reporter assays

A reporter plasmid (pGL15) containing optimal Gli3 DNA binding sites (Vortkamp, et al., 1995) inserted upstream of the luciferase reporter gene in pGL2-promoter vector (Promega) (a kind gift from A. Vortkamp) was mixed with CS2+ or CS2+*opl* ORF or CS2+*oplΔC* plasmids for transfections at the ratio of 5 mg to 1 mg. Human pancreatic tumor cell line BXPC (ATCC #CRL 1687) were transfected using the DEAE-dextran/chloroquine method (Aruffo, et al., 1987) , and cell lysates were assayed 48 hours after transfection for luciferase activity using the Promega Luciferase Assay System (E1501) on a Analytical Luminescence Laboratory Monolight 2010 machine. Duplicate transfections were done for each DNA mix and duplicate readings were taken for each lysate sample.

Appendix II. References

Akazawa, C., Ishibashi, M., Shimizu, C., Nakanishi, S. and Kageyama, R. (1995). A mammalian helix-loop-helix factor structurally related to the product of *Drosophila* proneural gene atonal is a positive transcriptional regulator expressed in the developing nervous system.*J. Biol. Chem.*,**270**, 8730-8738.

Albers, B. (1987). Competence as the main factor determining the size of the neural plate.*Dev. Growth Diff.*,**29**, 535-545.

Amaya, E., Stein, P., Musci, T. and Kirschner, M. (1993). FGF signalling in the early specification of mesoderm in Xenopus.*Development.*,**118**, 477 - 487.

Ang, S., Conlon, R., Jin, O. and Rossant, J. (1994). Positive and negative signals from mesoderm regulate the expression of mouse *Otx2* in ectoderm explants.*Development.*,**120**, 2979-2989.

Aruffo, A. and Seed, B. (1987). Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system.*Proc Natl Acad Sci USA.*,**84**, 8573-8577.

Aruga, J., Yokota, N., Hashimoto, M., Furuichi, T., Fukuda, M. and Mikoshiba, K. (1994). A novel zinc finger protein, zic, is involved in neurogenesis, especially in the cell lineage of cerebellar granule cells.*J Neurochem.*,**63**, 1880-90.

Aruga, J., Nagai, T., Tokuyama, T., Hayashizaki, Y., Okazaki, Y., Chapman, V. M. and Mikoshiba, K. (1996). The mouse zic gene family. Homologues of the Drosophila pair-rule gene odd-paired.*J Biol Chem.*,**271**, 1043-7.

Aruga, J., Minowa, O., Yaginuma, H., Kuno, J., Nagai, T., Noda, T., Mikoshiba, K. (1998). Mouse Zic-1 is involved in cerebellar development. *J. Neurosci.* **18 (1)**, 284-293.

Bang, A., Papalopulu, N., Kintner, C. and Goulding, M. (1997). Expression of *Pax-3* is initiated in the early neural plate by posteriorizing signals produced by the organizer and by posterior non-axial mesoderm.*Development.*,**124**, 2075-2085.

Bartholoma, A. and Nave, K.-A. (1994). Nex-1: A novel brain-specific helix-loop-helix protein with autoregulation and sustained in mature mature cortical neurons.*Mech. Dev.*,**48**, 217-228.

Basler, K., Edlund, T., Jessell, T. M. and Yamada, T. (1993). Control of cell pattern in the neural tube: regulation of cell differentiation by *dorsalin-1*, a novel TGF-ß family member.*Cell.*,**73**, 687-702.

Benedyk, M., Mullen, J. and DiNardo, S. (1994). *odd-paired*: a zinc finger pair-rule protein required for the timely activation of *engrailed* and *wingless* in *Drosophila* embryos.*Genes & Development.*,**8**, 105-117.

Benezra, R., Davies, R., Lockshon, D., Turner, D. and Weintraub, H. (1990). The protein Id: A negative regulator of helix-loop-helix DNA binding proteins.*Cell.*,**61**, 49-59.

Blitz, I. and Cho, K. (1994). Anterior neurectoderm is progressively induced during gastrulation: the role of the *Xenopus* homeobox gene *orthodenticle*.*Development.*,**121**, 993-1004.

Blitz, I. L. and Cho, K. W. Y. (1995). Anterior neurectoderm is progressively induced during gastrulation: the role of the *Xenopus* homeobox gene *orthodenticle*.*Development.*,**121**, 993-1004.

Blumberg, B., Bolado, J., Moreno, T., Kintner, C., Evans, R. and Papalopulu, N. (1997). An essential role for retinoid signaling in anteroposterior neural patterning.*Development.*,**124**, 373-379.

Bouwmeester, T., Kim, S.-H., Sasai, Y., Lu, B. and DeRobertis, E. (1996). Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer.*Nature.*,**382**, 595-601.

Bradley, L., Snape, A., Bhatt, S. and Wilkinson, D. (1993). The structure and expression of the Xenopus Krox-20 gene: conserved and divergent patterns of expression in rhombomeres and neural crest.*Mech Dev.*,**40**, 73-84.

Brannon, M., Gomperts, M., Sumoy, L., Moon, R. and Kimelman, D. (1997). A β-catenin/XTcf3 complex binds to the *siamois* promoter to regulate dorsal axis specification in *Xenopus*.*Genes Dev.*,**11**, 2359-2370.

Bronner-Fraser, M. (1995). Patterning of the vertebrate neural crest.*Perspectives on Developmental Neurobiology.*,**3**, 53-62.

Carnac, G., Kodjabachian, L., Gurdon, J. and Lemaire, P. (1996). The homeobox gene *Siamois* is a target of the Wnt dorsalisation pathway and triggers organiser activity in the absence of mesoderm.*Development.*,**122**, 3055-3065.

Champagne, A., Dufresne, C., Viney, L. and Gueride, M. (1997). Cloning, sequencing and expression of the two genes encoding the mitochondrial single-stranded DNA-binding protein in *Xenopus laevis*.*Gene.*,**184**, 65-71.

Chang, C. and Hemmati-Brivanlou, A. (1998). Neural crest induction by Xwnt7B in *Xenopus*.*Dev. Bio.*,**194**, 129-134.

Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D. and Kintner, C. (1995). Primary neurogenesis in *Xenopus* embryos regulated by a homologue of the *Drosophila* neurogenic gene *Delta.Nature.*,**375**, 761-766.

Christian, J. and Moon, R. (1993). Interactions between the Xwnt-8 and Spemann organizer signalling pathways generate dorsoventral pattern in the embryonic mesoderm of Xenopus.*Genes Dev.*,**7**, 13 - 28.

Cimbora, D. M. and Sakonju, S. (1995). *Drosophila* midgut morphogenesis requires the function of the segmentation gene *odd-paired. Dev. Bio.* **169**, 580-595.

Coffman, C., Skoglund, P., Harris, W. and Kintner, C. (1993). Expression of an extracellular deletion of Xotch diverts cell fate of Xenopus embryos.*Cell.*,**73**, 659 - 671.

Coffman, C. R., Skoglund, P., Harris, W. A. and Kintner, C. R. (1993). Expression of an extracellular deletion of *Xoth* diverts cell fate in *Xenopus* embryos.*Cell.*,**73**, 659-671.

Condie, B. G. and Harland, R. M. (1987). Posterior expression of a homeobox gene in early *Xenopus* embryos.*Development.*,**101**, 93-105.

Cornell, R. and Kimelman, D. (1994a). Activin-mediated mesoderm induction requires FGF.*Development.*,**120**, 453 - 462.

Cox, W. and Hemmati-Brivanlou, A. (1995). Caudalization of neural fate by tissue recombination and bFGF.*Development.*,**121**, 4349-4358.

Dale, L., Howes, G., Price, B. and Smith, J. (1992). Bone morphogenetic protein 4: a ventralizing factor in early Xenopus development.*Development.*,**115**, 573 - 585.

Dale, L. and Slack, J. (1987b). Regional specification within the mesoderm of early embryos of *Xenopus laevis.Development.*,**100**, 279-295.

Darras, S., Marikawa, Y., Elinson, R. and Lemaire, P. (1997). Animal to vegetal pole cells of early *Xenopus* embryos respond differently to maternal dorsal determinants: implications for the patterning of the organiser.*Development.*,**124**, 4275-4286.

Davidson, D. and RE, H. (1991). Msh-like genes: a family of homeobox genes with wide ranging expression during vertebrate development.*Semin. Dev. Biol.*,**2**, 405-412.

Dawid, I., Toyama, R. and Taira, M. (1995). LIM domain proteins.*C. R. Acad. Sci.*,**318**, 295-306.

Dickenson, M., Selleck, M., McMahon, A. and Bronner-Fraser, M. (1995). Dorsalization of the neural tube by the non-neural ectoderm.*Development.*,**121**,

Dirksen, M. and Jamrich, M. (1995). Differential expression of fork head genes during early *Xenopus* and zebrafish development.*Dev. Genet.*,**17**, 107-116.

Dirksen, M. and Jamrich, M. (1992). A novel, activin-inducible, blastopore lip-specific gene of Xenopus laevis contains a fork head DNA-binding domain.*Genes Dev.*,**6**, 599 - 608.

Dirksen, M., Morasso, M., Sargent, T. and Jamrich, M. (1994). Differential expression of a Distal-less homeobox gene Xdll-2 in ectodermal cell lineages.*Mech. Dev.*,**46**, 63-70.

Doniach, T. (1992). Induction of anteroposterior neural pattern in *Xenopus* by planar signals.*Development.*,**1992**, 183-193.

Doniach, T. (1993). Planar and vertical induction of anteroposterior pattern during the development of the amphibian central nervous system.*J. Neurobiol.*,**24**, 1256-1275.

Doniach, T. and Musci, T. (1995). Induction of anteroposterior pattern in *Xenopus*: evidence for a quantitative mechanism.*Mech. Dev.*,**53**, 403-413.

Echelard, Y., Epstein, D., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. and McMahon, A. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity.*Cell.*,**75**, 1417-1430.

Ekker, S. C., McGrew, L. L., Lai, C.-J., Lee, J. J., von Kessler, D. P., Moon, R. T. and Beachy, P. A. (1995). Distinct expression and shared activities of members of the *hedgehog* gene family of *Xenopus* laevis.*Development.*,**121**, 2337-2347.

Elinson, R. and Pasceri, P. (1989). Two UV-sensitive targets in dorsoanterior specification of frog embryos.*Development.*,**106**, 511 - 518.

Elinson, R. and Rowning, B. (1988). A transient array of microtubules in frog eggs: potential tracks for a cytoplasmic rotation that specifies the dorso-ventral axis.*Dev. Biol.*,**128**, 185-197.

Epstein, D., Vekemans, M. and Gruss, P. (1991). splotch, a mutation affecting development of the mouse neural tube, show a deletion within the paired homedomain of Pax-3.*Cell.*,**67**, 767-774.

Espeseth, A., Johnson, E. and Kintner, C. (1995). Xenopus F-cadherin, a novel member of the cadherin family of cell adhesion molecules, is expressed at boundaries in the neural tube.*Mol Cell Neurosci.*,**6**, 199-211.

Eyal-Giladi, H. (1954). Dynamic aspects of neural induction in amphibia.*Archives de Biologie.*,**65**, 179-259.

Ferreiro, B., Skoglund, P., Bailey, A., Dorsky, R. and Harris, W. (1992). XASH1, a Xenopus homolog of achaete-scute: a proneural gene in anterior regions of the vertebrate CNS.*Mech. Dev.*,**40**, 25 - 36.

Fredieu, J., Cui, Y., Maier, D., Danilchik, M. and Christian, J. (1997). Xwnt-8 and lithium can act upon either dorsal mesodermal or neurectodermal cells to cause a loss of forebrain in *Xenopus* embryos.*Dev. Biol.*,**186**, 100-114.

Fredieu, J., Cui, Y., Maier, D., Danilchik, M. and Christian, J. (1997). Xwnt-8 and lithium can act upon either dorsal mesodermal or neurectodermal cells to cause a loss of forebrain in *Xenopus* embryos.*Dev. Biol.*,**186**, 100-114.

Fujisue, M., Kobayakawa, Y. and Yamana, K. (1993). Occurrence of dorsal axis-inducing activity around the vegetal pole of an uncleaved *Xenopus* egg and displacement to the equatorial region by cortical rotation.*Development.*,**118**, 163-170.

Gammill, L. and Sive, H. (1997). Identification of *otx2* target genes and restrictions in ectodermal competence during *Xenopus* cement gland formation.*Development.*,**124**, 471-481.

Gerhart, J. and Keller, R. (1986). Region-specific cell activities in amphibian gastrulation.*Ann. Rev. Cell Biol.*,**2**, 201 -229.

Gilbert, S. and Saxen, L. (1993). Spemann's organizer: models and molecules.*Mech. Dev.*,**41**, 73 -89.

Gimlich, R. and Cooke, J. (1983). Cell lineage and the induction of second nervous systems in ambphibian development.*Nature.*,**306**, 471-473.

Glinka, A., Wu, W., Delius, H., Monaghan, A., Blumenstock, C. and Niehrs, C. (1998). Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction.*Nature.*,**391**, 357-362.

Godsave, S. F. and Slack, J. M. W. (1989). Clonal analysis of mesoderm induction in *Xenopus laevis*.*Dev. Biol.*,**134**, 486-490.

Green, J., New, J. and Smith, J. (1992). Responses of embryonic Xenopus cells to activin and FGF are separated by multiple dose thresholds and correspond to distinct axes of the mesoderm.*Cell.*,**71**, 731 - 739.

Grinblat, Y., Gamse, J., Patel, M. and Sive, H. (1998). Determination of the zebrafish forebrain: induction and patterning.*Development.*,in press.

Grunz, H. and Tacke, L. (1989). Neural differentiation of *Xenopus laevis* ectoderm takes place after disaggregation and delayed reaggregation without inducer.*Cell Differ. Dev.*,**28**, 211-218.

Gruss, P. and Walther, C. (1992). Pax in development.*Cell.*,**69**, 719-722.

Hansen, C., Marion, C., Steele, K., George, S. and Smith, W. (1997). Direct neural induction and selective inhibition of mesoderm and epidermis inducers by Xnr3.*Development.*,**124**, 483-492.

Harland, R. and Gerhart, J. (1997). Formation and function of Spemann's organizer.*Annu. Rev. Cell Dev. Biol.*,**13**, 611-667.

Harland, R. M. (1991). In situ Hybridization: An Improved Whole-Mount Method for Xenopus Embryos.*Methods in Cell Biology.*,**36**, 685-695.

Harris, W. and Hartenstein, V. (1991). Neuronal determination without cell division in *Xenopus* embryos.*Neuron.*,**6**, 499-515.

Hawley, S., Wünnenberg-Stapleton, K., Hashimoto, C., Laurent, M., Watabe, T., Blumberg, B. and Cho, K. (1995). Disruption of BMP signals in embryonic *Xenopus* ectoderm leads to direct neural induction.*Genes Dev.*,**9**, 2923-2935.

Heasman, J., Crawford, A., Goldstone, K., Garner-Hamrick, P., Gumbiner, B., McCrea, P., Kintner, C., Noro, C. and Wylie, C. (1994). Overexpression of cadherins and underexpression of β-catenin inhibit dorsal mesoderm induction in early *Xenopus* embryos.*Cell.*,**79**, 791-803.

Hemmati-Brivanlou, A. and Harland, R. (1989). Expression of an *engrailed*-related protein in the anterior neural ectoderm of early Xenopus embryos.*Development.*,**106**, 611-617.

Hemmati-Brivanlou, A., Kelly, O. and Melton, D. (1994). Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity.*Cell.*,**77**, 283 - 295.

Hemmati-Brivanlou, A. and Melton, D. (1994). Inhibition of activin receptor signaling promotes neuralization in Xenopus.*Cell.*,**77**, 273 - 281.

Hemmati-Brivanlou, A. and Melton, D. (1997). Vertebrate neural induction.*Annu. Rev. Neurosci.*,**20**, 43-60.

Hemmati-Brivanlou, A. and Melton, D. A. (1994a). Inhibition of activin receptor signaling promotes neuralization in *Xenopus*.*Cell.*,**77**, 273-281.

Hemmati-Brivanlou, A., Stewart, R. and Harland, R. (1990). Region-specific neural induction of an *engrailed* protein by anterior notochord in *Xenopus*.*Science.*,**250**, 800-802.

Hollenberg, S. M., Cheng, P. F. and Weintraub, H. (1993). Use of a conditional MyoD transcription factor in studies of MyoD trans-activation and muscle determination.*Proc. Natl. Acad. Sci. USA.*,**90**, 8028-8032.

Hoppler, S., Brown, J. and Moon, R. (1996). Expression of a dominant-negative Wnt blocks induction of MyoD in *Xenopus* embryos.*Genes Dev.*,**10**, 2805-2817.

Hopwood, N. D., Pluck, A. and Gurdon, J. B. (1989). A *Xenopus* mRNA related to *Drosophila twist* is expressed in response to induction in the mesoderm and the neural crest.*Cell.*,**59**, 893-903.

Horb, M. E. and Thomsen, G. H. (1997). A vegetally localized T-box transcription factor in Xenopus eggs specifies mesoderm and endoderm and is essential for embryonic mesoderm formation.*Development.*,**124**, 1689-98.

Isaacs, H., Pownall, M. and Slack, J. (1994). eFGF regulates *Xbra* expression during *Xenopus* gastrulation.*EMBO J.*,**13**, 4469-4481.

Isaacs, H., Tannahill, D. and Slack, J. (1992). Expression of a novel FGF in the Xenopus embryo: A new candidate inducing factor for mesoderm formation and antero-posterior patterning.*Development.*,**114**, 711 - 720.

Jacobson, M. (1984). Cell lineage analysis of neural induction: origins of cells forming the induced nervous system.*Dev. Biol.*,**102**, 122-129.

Johnson, J., Birren, S. and Anderson, D. (1990). Two rat homologies of *Drosophila* achaete-scute specifically expressed in neuronal precursors.*Nature.*,**346**, 858-861.

Jonas, E., Sargent, T. D. and Dawid, I. B. (1985). Epidermal keratin gene expressed in embryos of *Xenopus laevis*.*Proceedings of the National Academy of Science, USA.*,**82**, 5413-5417.

Jones, C., Lyons, K., Lapan, P., Wright, C. and Hogan, B. (1992). DVR-4 (Bone Morphogenetic Protein - 4) as a posterior-ventralizing factor in Xenopus mesoderm induction.*Development.*,**115**, 639 - 647.

Jones, E. A. and Woodland, H. R. (1987). The development of animal cap cells in *Xenopus*: a measure of the start of animal cap competence to form mesoderm.*Development.*,**101**, 557-563.

Jones, E. A. and Woodland, H. R. (1987). The development of animal cap cells in *Xenopus*: the effects of environment on the differentiation and the migration of grafted ectodermal cells.*Development.*,**101**, 23-32.

Kaestner, K. H., Schutz, G. and Monaghan, A. P. (1996). Expression of the winged helix genes fkh-4 and fkh-5 defines domains in the central nervous system.*Mech Dev.*,**55**, 221-30.

Kengaku, M. and Okamoto, H. (1995). bFGF as a possible morphogen for the anteroposterior axis of the central nervous system in *Xenopus*.*Development.*,**121**, 3121-3130.

Kintner, C. and Dodd, J. (1991). Hensen's node induces neural tissue in *Xenopus* ectoderm. Implications for the action of the organizer in neural induction.*Development.*,**113**, 1495 - 1505.

Kintner, C. R. and Melton, D. A. (1987). Expression of *Xenopus* N-CAM RNA in ectoderm in an early response ot neural induction.*Development.*,**99**, 311-325.

Knecht, A., Good, P., Dawid, I. and Harland, R. (1995). Dorsal-ventral patterning and differentiation of noggin-induced neural tissue in the absence of mesoderm.*Development.*,**121**, 1927-1936.

Knecht, A. and Harland, R. (1997). Mechanisms of dorsal-ventral patterning in noggin-induced neural tissue.*Development.*,**124**, 2477-2488.

Knöchel, S., Lef, J., Clement, J., Klocke, B., Hille, S., Köster, M. and Knöchel, W. (1992). Activin A induced expression of a fork head related gene in posterior chordamesoderm (notochord) of Xenopus laevis embryos.*Mech. Dev.*,**38**, 157-165.

Kolm, P., Apekin, V. and Sive, H. (1997). *Xenopus* hindbrain patterning requires retinoid signaling.*Dev. Biol.*,**192**, 1-16.

Kolm, P. and Sive, H. (1994). Complex regulation of *Xenopus* HoxA1 and HoxD1.*Biochem. Soc. Trans.*,**22**, 579-584.

Kolm, P. and Sive, H. (1995b). Efficient hormone-inducible protein function in *Xenopus laevis*.*Dev. Biol.*,**171**, 267-272.

Kolm, P. and Sive, H. (1995a). Regulation of the Xenopus labial homeodomain genes, HoxA1 and HoxD1: activation by retinoids and peptide growth factors.*Dev. Biol.*,**167**, 34-49.

Kolm, P. and Sive, H. (1997) Retinoids and posterior neural inducton: a reevaluation of Nieuwkoops two-step hypothesis. In *Cold Spring Harbor Symposia on Quantitative Biology.* (ed. pp. 511-521. Cold Spring Harbor Laboratory Press.

Kozak, M. (1987). An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs.*Nuc. Acids. Res.*,**15**, 8125-8148.

Krieg, P. A. and Melton, D. A. (1984). Functional messenger RNAs are produced by SP6 *in vitro* transcription of cloned cDNAs.*Nucleic Acids Res.*,**12**, 7057-7070.

Krieg, P. A., Varnum, S. M., Wormington, W. M. and Melton, D. A. (1989). The mRNA Encoding Elongation Factor 1-α (EF-1α) Is a Major Transcript at the Midblastula Transition in *Xenopus*.*Developmental Biology.*,**133**, 93-100.

Kroll, K. and Amaya, E. (1996). Transgenic *Xenopus* embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation.*Development.*,**122**, 3173-3183.

Kuo, J. S., Patel, M., Gamse, J., Merzdorf, C., Liu, X., Apekin, V. and Sive, H. (1998). opl: a zinc finger protein that regulates neural determination and patterning in *Xenopus*.*Development.*,in press.

Kuo, J. S., Veale, R., Maxwell, B. and Sive, H. (1996). Translational inhibition by 5' polycytidine tracts in Xenopus embryos and in vitro.*Gene.*,**176**, 17-21.

LaBonne, C. and Whitman, M. (1994). Mesoderm induction by activin requires FGF-mediated intracellular signals.*Development.*,**120**, 463 - 472.

Lamb, T. and Harland, R. (1995). Fibroblast growth factor is a direct neural inducer, which combined with noggin generates anterior-posterior neural pattern.*Development.*,**121**, 3627-3636.

Lamb, T., Knecht, A., Smith, W., Stachel, S., Economides, A., Stahl, N., Yancopolous, G. and Harland, R. (1993). Neural induction by the secreted polypeptide noggin.*Science.*,**262**, 713 - 718.

Lamb, T. M., Knecht, A. K., Smith, W. C., Stachel, S. E., Economides, A. N., Stahl, N., Yancopolous, G. D. and Harland, R. M. (1993). Neural induction by the secreted polypeptide noggin.*Science.*,**262**, 713-718.

Larabell, C., Torres, M., Rowning, B., Yost, C., Miller, J., Wu, M., Kimelman, D. and Moon, R. (1997). Establishment of the dorso-ventral axis in *Xenopus* embryos is presaged by early asymmetries in β-catenin that are modulated by the wnt signaling pathway.*J. Cell Biol.*,**136**, 1123-1136.

Lee, J., Hollenberg, S., Snider, L., Turner, D., Lipnick, N. and Weintraub, H. (1995). Conversion of *Xenopus* ectoderm into neurons by NeuroD, a basic helix-loop-helix protein.*Science.*,**268**, 836-844.

Lemaire, P., Garrett, N. and Gurdon, J. (1995). Expression cloning of *Siamois*, a *Xenopus* homeobox gene expressed in dorsal-vegetal cells of blastulae and able in induce a complete secondary axis.*Cell.*,**81**, 85-94.

Leyns, L., Bouwmeester, T., Kim, S.-H., Piccolo, S. and DeRobertis, E. (1997). Frzb-1 is a secreted antagonist of Wnt signaling expressed in the Spemann organizer.*Cell.*,**88**, 747-756.

Liang, P. and Pardee, A. B. (1995). Recent advances in differential display.*Curr Opin Immunol.*,**7**, 274-80.

Liem, K., Jr., Tremml, G., Roelink, H. and Jessell, T. M. (1995). Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm.*Cell.*,**82**, 969-79.

Liem, K. J., Tremml, G., Roelink, H. and Jessell, T. (1995). Dorsal differentiation of neural plate cells induced by BMP-mediated signals from the epidermal ectoderm.*Cell.*,**82**, 969-979.

Lindsell, C., Shawber, C., Boulter, J. and Weinmaster, G. (1995). Jagged: A mammalian ligand that activates Notch1.*Cell.*,**80**, 909-917.

London, C., Akers, R. and Phillips, C. (1988). Expression of Epi 1, and epidermis-specific marker in Xenopus laevis embryos, is specified prior to gastrulation.*Dev. Biol.*,**129**, 380 -389.

Lustig, K., Kroll, K., Sun, E. and Kirschner, M. (1996). Expression cloning of a xenopus T-related gene (Xombi) involved in mesodermal patterning and blastopore lip formation.*Development.*,**122**, 4001-4012.

Ma, Q., Kintner, C. and Anderson, D. (1996). Identification of *neurogenin,* a vertebrate neuronal determination gene.*Cell.*,**87**, 43-52.

Mancilla, A. and Mayor, R. (1996). Neural crest formation in *Xenopus laevis.* Mechanism of *Xslug* induction.*Dev. Biol.*,**177**, 580-589.

Marshall, H., Morrison, A., Studer, M., Pöpperl, H. and Krumlauf, R. (1996). Retinoids and Hox genes.*FASEB J.*,**10**, 969-978.

Marti, E., Takada, R., Bumcrot, D., Sasaki, H. and McMahon, A. (1995). Distribution of Sonic hedgehog peptides in the developing chick and mouse embryo.*Development.*,**121**, 2537-2547.

Mathers, P., Miller, A., Doniach, T., Dirksen, M. and Jamrich, M. (1995). Initiation of anterior head-specific gene expression in uncommitted ectoderm of *Xenopus laevis* by ammonium chloride.*Dev. Biol.*,**171**, 641-654.

Mattioni, T., Louvion, J. and Picard, D. (1994) Regulation of protein activities by fusion to steriod binding domains. In *Methods Cell Biol.* (ed. M. Roth). pp. 335-352. San Diego: Academic Press.

Mayor, R., Morgan, R. and Sargent, M. (1995). Induction of the prospective neural crest of *Xenopus.Development.*,**121**, 767-777.

McGrew, L., Hoppler, S. and Moon, R. (1997). Wnt and FGF pathways cooperatively pattern anteroposterior neural ectoderm in *Xenopus.Mech of Development.*,**69**, 105-114.

McGrew, L., Hoppler, S. and R, M. (1997). Wnt and FGF pathways cooperatively pattern anteroposterior neural ectoderm in *Xenopus.Mech of Development.*,**69**, 105-114.

McGrew, L., Lai, C. and Moon, R. (1995). Specification of the anteroposterior neural axis through synergistic interaction of the Wnt signaling cascade with *noggin* and *follistatin.Dev. Biol.*,**172**, 337-342.

McKendry, R., Hsu, S.-C., Harland, R. and Grosschedl, R. (1997). LEF-1/TCF proteins mediate wnt-inducible transcription from the Xenopus nodal-related 3 promoter.*Dev. Biol.*,**192**, 420-431.

McMahon, A. (1992). The *Wnt* family of developmental regulators.*Trends Genet.*,**8**, 236-242.

Mizuseki, K., Kishi, M., Matsui, M., Nakanishi, S. and Sasai, Y. (1998). *Xenopus* Zic-related-1 and Sox-2, two factors induced by chordin, have distinct activities in the initiation of neural induction.*Development.*,**125**, 579-587.

Mohun, T., Brennan, S., Dathan, N., Fairman, S. and Gurdon, J. B. (1984). Cell type-specific activation of actin genes in the early amphibian embryo.*Nature.*,**511**, 716-721.

Molenaar, M., Wetering, M. v. d., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destrée, O. and Clevers, H. (1996). XTcf-3 transcription factor mediates β-catenin-induced axis formation in Xenopus embryos.*Cell.*,**86**, 391-399.

Moon, R. (1993). In pursuit of the functions of the *Wnt* family of developmental regulators: Insights from *Xenopus laevis.BioEssays.*,**15**, 91-97.

Morgan, R. and Sargent, M. G. (1997). The role in neural patterning of translation initiation factor elF4All; induction of neural fold genes.*Development.*,**124**, 2751-2760.

Moury, J. and Jacobson, A. (1990). The origins of neural crest cells in the axolotl.*Dev. Biol.*,**141**, 243-253.

Myat, A., Henrique, D., Ish-Horowicz, D. and Lewis, J. (1996). A chick homologue of Serrate and its relationship with notch and delta homologues during central neurogenesis.*Dev. Biol.*,**174**, 233-247.

Nagai, T., Aruga, J., Takada, S., Gunther, T., Sporle, R., Schughart, K. and Mikoshiba, K. (1997). The expression of the mouse Zic1, Zic2, and Zic3 gene suggests an essential role for Zic genes in body pattern formation.*Dev Biol.*,**182**, 299-313.

Nakata, K., Nagai, T., Aruga, J. and Mikoshiba, K. (1997). Xenopus Zic3, a primary regulator both in neural and neural crest development.*Proc. Natl. Acad. Sci. USA.*,**94**, 11980 - 11985.

Nieuwkoop, P. (1952b). Activation and organization of the central nervous system in amphibians. II. Differentiation and organization.*J. Exp. Zool.*,**120**, 33-81.

Nieuwkoop, P. (1952c). Activation and organization of the central nervous system in amphibians. III. Synthesis of a new working hypothesis.*J. Exp. Zool.*,**120**, 83-108.

Nieuwkoop, P. (1985). Inductive interactions in early amphibian development and their general nature.*J. Embryol. exp. Morph.*,**89**, 333-347.

Nieuwkoop, P. and Faber, J. (1967).*Normal table of Xenopus laevis (Daudin)*.. Amsterdam: North-Holland Publishing Co.

Nieuwkoop, P. and Faber, J. (1994).*Normal Tables of Xenopus laevis (Daudin)*.. New York and London: Garland Publishing, Inc.

Nieuwkoop, P. D. (1952a). Activation and organization of the central nervous system in amphibians. I. Induction and activation.*J. Exp. Zool.*,**120**, 1-32.

Nieuwkoop, P. D. (1969). The formation of the mesoderm in urodelean amphibians (I. Induction by the endoderm).*Wilhelm Roux' Archiv.*,**162**, 341-373.

Northrop, J. and Kimelman, D. (1994). Dorsal-ventral differences in Xcad-3 response to FGF mediated induction in Xenopus.*Dev. Biol.*,**161**, 490 - 503.

Otte, A. (1992a). Protein kinase C isozymes have distint roles in neural induction and competence in xenopus.*Cell.*,**68**, 1021 - 1029.

Otte, A., Kramer, I. and Durston, A. (1991). Protein kinase C and regulation of the local competence of Xenopus ectoderm.*Science.*,**251**, 570 - 573.

Pannese, M., Polo, C., Andreazzoli, M., Vignali, R., Kablar, B., Barsacchi, G. and Boncinelli, E. (1995). The *Xenopus* homologue of *Otx2* is a maternal homeobox gene that demarcates and specifies anterior body regions.*Development.*,**121**, 707-720.

Pannese, M., Polo, C., Andreazzoli, M., Vignali, R., Kablar, B., Barsacchi, G. and Boncinelli, E. (1995). The Xenopus homologue of *Otx2* is a maternal homeobox gene that demarcates and specifies anterior body regions.*Development.*,**121**, 707-720.

Papalopulu, N. and Kintner, C. (1996). A posteriorising factor, retinoic acid, reveals that anteroposterior patterning controls the timing of neuronal differentiation in *Xenopus* neuroectoderm.*Development.*,**122**, 3409-3418.

Papalopulu, N. and Kintner, C. (1993). *Xenopus Distal-less* related homeobox genes are expressed in the developing forebrain and are induced by planar signals.*Development.*,**117**, 961-975.

Patel, M. and Sive, H. L. (1996) PCR-based subtractive cDNA cloning. In *Current Protocols in Molecular Biology.* (ed. F. Ausubel, R. Brent, R. Kingston, D. Moore, J. Seidman, J. Smith and K. Struhl). pp. Unit 5.9. John Wiley and Sons, Inc.

Pfaff, S., Mendelson, M., Stewart, C., Edlund, T. and Jessell, T. (1996). Requirement for Lim homeobox gene isl-1 in motor neuron generation reveals a motor neuron-dependent step in interneuron differentiation.*Cell.*,**84**, 309-320.

Piccolo, S., Sasai, Y., Lu, B. and De Robertis, E. M. (1996). Dorsoventral patterning in Xenopus: inhibition of ventral signals by direct binding of chordin to BMP-4.*Cell.*,**86**, 589-598.

Placzek, M. and Furley, A. (1996). Neural development: Patterning cascades in the neural tube.*Curr. Biol.*,**6**, 526-529.

Placzek, M., Yamada, T., Tessier-Lavigne, M., Jessell, T. and Dodd, J. (1991). Control of dorsoventral pattern in vertebrate neural development: induction and polarizing properties of the floor plate.*Development.*,**2**, 1105 - 122.

Pownall, M., Tucker, A., Slack, J. and Isaacs, H. (1996). *eFGF*, *Xcad3*, and Hox genes form a molecular pathway that establishes the anteroposterior axis in *Xenopus*.*Development.*,**122**, 3881-3892.

Price, M. (1993). Members of the Dlx- and Nkx2-gene families are regionally expressed in the developing forebrain.*J. Neurobiol.*,**24**, 1385-1399.

Re'em-Kalma, Y., Lamb, T. and Frank, D. (1995). Competition between noggin and bone morphogenetic protein 4 activities may regulate

dorsalization during *Xenopus* development.*Proc. Natl. Acad. Sci.*,**92**, 12141-12145.

Rebagliati, M., Weeks, D., Harvey, R. and Melton, D. (1985). Identification and cloning of localized maternal RNAs from *Xenopus* eggs.*Cell.*,**42**, 769-777.

Riddle, R., Johnson, R., Laufer, E. and Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA.*Cell.*,**75**, 1401 - 1416.

Rissi, M., Wittbrodt, J., Delot, E., Naegeli, M. and Rosa, F. (1995). Zebrafish Radar: A new member of the TGFβ superfamily defines dorsal regions of the neural plate and the embryonic retina.*Mech. Dev.*,**49**, 223-234.

Roelink, H., Augsburger, A., Heemskerk, J., Korzh, V., Norlin, S., Ruiz-i-Altaba, A., Tanabe, Y., Placzek, M., Edlund, T., Jessell, T. and Dodd, J. (1994). Floor plate and motor neuron induction by vhh-1, a vertebrate homolog of hedgehog expressed by the notochord.*Cell.*,**76**, 761 - 775.

Roelink, H., Augsburger, A., Jeemskerk, J., Korzh, V., Norlin, S., Ruiz i Altaba, A., Tanabe, Y., Placzek, M., Edlund, T., Jessell, T. and Dodd, J. (1994). Floor plate and motor neuron induction by vhh-1, a vertebrate homolog of hedgehog expressed by the notochord.*Cell.*,**76**, 761 - 775.

Rowning, B., Wells, J., Wu, M., Gerhart, J., Moon, R. and Larabell, C. (1997). Microtubule-mediated transport of organelles and localization of β-catenin to the future dorsal side of *Xenopus* eggs.*PNAS.*,**94**, 1224-1229.

Ruiz i Altaba, A. and Jessell, T. (1992b). Pintallavis, a gene expressed in the organizer and midline cells of frog embryos: involvement in the development of the neural axis.*Development.*,**116**, 81 - 93.

Ruiz i Altaba, A. and Jessell, T. (1991a). Retinoic acid modifies mesodermal patterning in early *Xenopus* embryos.*Genes Dev.*,**5**, 175 - 187.

Ruiz i Altaba, A., Jessell, T. M. and Roelink, H. (1995). Restrictions to floor plate induction by hedgehog and winged-helix genes in the neural tube of frog embryos.*Mol Cell Neurosci.*,**6**, 106-21.

Ruiz i Altaba, A., Prezioso, V., Darnell, J. and Jessell, T. (1993). Sequential expression of HNF-3β and HNF-3α by embryonic organizing centers: the dorsal lip/ node, notochord and floor plate.*Mech. Dev.*,**44**, 91-108.

Rupp, R., Snider, L. and Weintraub, H. (1994). *Xenopus* embryos regulate the nuclear localization of XMyoD.*Genes Dev.*,**8**, 1311-1323.

Ruppert, J. M., Kinzler, K. W., Wong, A. J., Bigner, S. H., Kao, F. T., Lax, M. L., Seuanez, H. N., O'Brien, S. J. and Vogelstein, B. (1988). The GLI-kruppel family of human genes.*Mol. Cell Biol.*,**8**, 3104 - 3113.

Sadaghiani, B. and Thiebaud, C. H. (1987). Neural Crest Development in the *Xenopus laevis* Embryo, Studied by Interspecific Transplantation and Scanning Electron Microscopy.*Developmental Biology.*,**124**, 91-110.

Sagerström, C., Grinblat, Y. and Sive, H. (1996). Anteroposterior patterning in the zebrafish, *Danio rerio*: an explant assay reveals inductive and suppressive cell interactions.*Development.*,**122**, 1873-1883.

Sagerström, C., Sun, B. and Sive, H. L. (1997). Subtractive cDNA cloning.*Ann. Rev. Biochem.*,**66**, 751-783.

Saha, M. and Grainger, R. (1992). A labile period in the determination of the anterior-posterior axis during early neural development in *Xenopus*.*Neuron.*,**8**, 1003-1014.

Saha, M., Michel, R., Gulding, K. and Grainger, R. (1993). A *Xenopus* homebox gene defines dorsal-ventral domains in the developing brain.*Development.*,**118**, 193-202.

Saha, M., Miles, R. and Grainger, R. (1997). Dorso-ventral patterning during neural induction in *Xenopus*: assessment of spinal cord regionalization with HB9, a marker for the motor neuron region.*Dev. Biol.*,**187**, 209-223.

Saha, M. S., Michel, R. B., Gulding, K. M. and Grainger, R. M. (1993). A Xenopus homebox gene defines dorsal-ventral domains in the developing brain.*Development.*,**118**, 193-202.

Saint-Jeannet, J., He, X., Varmus, H. and Dawid, I. (1997). Regulation of dorsal fate in the neuraxis by Wnt-1 and Wnt-3a.*Proc Natl Acad Sci (USA).*,**94**, 13713-13718.

Sambrook, J., Fritsch, E. and Maniatis, T. (1989).*Molecular Cloning: A Laboratory Manual*.. Plainview, NY: Cold Spring Harbor Laboratory Press.

Sasai, Y. and DeRobertis, E. (1997). Ectodermal patterning in vertebrate embryos.*Dev. Biol.*,**182**, 5-20.

Sasai, Y., Kageyama, R., Tagawa, Y., Shigemoto, R. and Nakanishi, S. (1992). Two mammalian helix-loop-helix factors structurally related to *Drosophila* hairy and enhancer of split.*Genes Dev.*,**6**, 2620-2634.

Sasai, Y., Lu, B., Piccolo, S. and DeRobertis, E. (1996). Endoderm induction by the organizer-secreted factors chordin and noggin in Xenopus animal caps.*EMBO J.*,**15**, 4547-4555.

Sasai, Y., Lu, B., Steinbeisser, H. and DeRobertis, E. (1995). Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in *Xenopus.Nature.*,**376**, 333-336.

Sasai, Y., Lu, B., Steinbeisser, H., Geissert, D., Gont, L. and DeRobertis, E. (1994). *Xenopus chordin*: a novel dorsalizaing factor activated by organizer-specific homeobox genes.*Cell.*,**79**, 779-790.

Sasaki, H. and Hogan, B. L. (1994). HNF-3 beta as a regulator of floor plate development.*Cell.*,**76**, 103-15.

Savage, R. and Phillips, C. (1989). Signals from the dorsal blastopore lip region during gastrulation bias the ectoderm toward a nonepidermal pathway of differentiation in Xenopus laevis.*DevBiol.*,**133**, 157 - 168.

Savage, R. and Phillips, C. R. (1989). Signals from the Dorsal Blastopore Lip Region during Gastrulation Bias the Ectoderm toward a Nonepidermal Pathway of Differentiation in *Xenopus laevis.Developmental Biology.*,**133**, 157-168.

Scharf, S. and Gerhart, J. (1983). Axis determination in eggs of *Xenopus laevis:* a critical period before first cleavage, identified by the common effects of cold, pressure and ultraviolet irradiation.*Dev. Biol.*,**99**, 75-87.

Schneider, S., Steinbeisser, H., Warga, R. and Hausen, P. (1996). β-catenin translocation into nuclei demarcates the dorsalizing centers in frog and fish embryos.*Mech. Dev.*,**57**, 191-198.

Selleck, M. and Bronner-Fraser, M. (1995). Origins of the avian neural crest: the role of neural plate-epidermis interactions.*Development.*,**121**, 525-538.

Servetnick, M. and Grainger, R. (1991). Changes in neural and lens competence in *Xenopus* ectoderm: evidence for an autonomous developmental timer.*Development.*,**112**, 177-188.

Servetnick, M. and Grainger, R. M. (1991). Homeogenetic neural induction in *Xenopus.Dev. Biol.*,**147**, 73-82.

Sharpe, C., Fritz, A., DeRobertis, E. and Gurdon, J. (1987). A homeobox-containing marker of posterior neural differentiation show the importance of predetermination in neural induction.*Cell.*,**50**, 749 - 758.

Sharpe, C. and Gurdon, J. (1990). The induction of anterior and posterior neural genes in *Xenopus laevis.Development.*,**109**, 765-774.

Simeone, A., Avantaggiato, V., Moroni, M., Mavilio, F., Arra, C., Cotelli, F., Nigro, V. and Acampora, D. (1995). Retinoic acid induces

stage-specific antero-posterior transformation of rostral central nervous system.*Mech. Dev.*,**51**, 83-98.

Sirad, C., Pompa, J. d. l., Elia, A., Itie, A., Mirtsos, C., Cheung, A., Hahn, S., Wakeham, A., Schwartz, L., Kern, S., Rossant, J. and Mak, T. (1998). The tumor suppressor gene *Smad4/Dpc4* is required for gastrulation and later for anterior development of the mouse embryo.*Genes Dev.*,**12**, 107-119.

Sive, H. (1993). The frog prince-ss: a molecular formula for dorsoventral patterning in *Xenopus*.*Genes Dev.*,**7**, 1 - 12.

Sive, H. and Cheng, P. (1991). Retinoic acid perturbs the expression of Xhox.lab genes and alters mesodermal determination in *Xenopus laevis*.*Genes Dev.*,**5**, 1321 - 1332.

Sive, H., Draper, B., Harland, R. and Weintraub, H. (1990). Identification of a retinoic acid-sensitive period during primary axis formation in *Xenopus laevis*.*Genes Dev.*,**4**, 932 - 942.

Sive, H., Hattori, K. and Weintraub, H. (1989). Progressive determination during formation of the anteroposterior axis in *Xenopus laevis*.*Cell.*,**58**, 171-180.

Sive, H., Hattori, K. and Weitraub, H. (1989). Progressive determination during formation of the anteroposterior axis in *Xenopus laevis*.*Cell.*,**58**, 171 - 180.

Sive, H. L. and Cheng, P. F. (1991). Retinoic acid perturbs the expression of *Xhox.lab* genes and alters mesodermal determination in *Xenopus laevis*.*Genes & Development.*,**5**, 1321-1332.

Sive, H. L. and St John, T. (1988). A simple subtractive hybridization technique employing photoactivatable biotin and phenol extraction.*Nucleic Acids Res.*,**16**, 10937.

Smith, J., Price, B., Green, J., Weigel, D. and Herrmann, B. (1991). Expression of a Xenopus homolog of Brachyury (T) is an immediate-early response to mesoderm induction.*Cell.*,**67**, 79 - 87.

Smith, W. and Harland, R. (1992). Expression Cloning of noggin, a New Dorsalizing Factor Localized to the Spemann Organizer in Xenopus Embryos.*Cell.*,**70**, 829-840.

Smith, W., Knecht, A., Wu, M. and Harland, R. (1993). Secreted *noggin* protein mimics the Spemann organizer in dorsalizing *Xenopus* mesoderm.*Nature.*,**361**, 547-549.

Sokol, S. (1996). Analysis of dishevelled signalling pathways during *Xenopus* development.*Current Biology.*,**6**, 1456-1467.

Sokol, S. and Melton, D. A. (1991). Pre-existent pattern in Xenopus animal pole cells revealed by induction with activin.*Nature.*,**351**, 409-411.

Stennard, F., Carnac, G. and Gurdon, J. B. (1996). The Xenopus T-box gene, Antipodean, encodes a vegetally localised maternal mRNA and can trigger mesoderm formation.*Development.*,**122**, 4179-88.

Stewart, R. and Gerhart, J. (1990). The anterior extent of dorsal development of the Xenopus embryonic axis depends on the quantity of organizer in the late blastula.*Development.*,**109**, 363 - 372.

Stutz, F. and Spohr, G. (1986). Isolation and characterization of sarcomeric actin genes expressed in Xenopus laevis embryos.*J Mol Biol.*,**187**, 349 - 361.

Sun, B., Bush, S., Collins-Racie, L., LaVallie, E., DiBlasio-Smith, E., Wolfman, N., McCoy, J. and Sive, H. (1998). *derriere*: a TGFβ family member required for posterior determination in *Xenopus*.submitted.

Taira, M., Otani, H., Saint-Jeannet, J. and Dawid, I. (1994). Role of the LIM class homeodomain protein Xlim-1 in neural and muscle induction by the Spemann organizer in *Xenopus*.*Nature.*,**372**, 677-679.

Taira, M., Saint-Jeannet, J. and Dawid, I. (1997). Role for the *Xlim-1* and *Xbra* genes in anteroposterior patterning of neural tissue by the head and trunk organizer.*Proc. Natl. Acad. Sci.*,**94**, 895-900.

Tsuchida, T., Ensini, M., Morton, S., Baldassare, M., Edlund, T., Jessell, T. and Pfaff, S. (1994). Topographic organization of embryonic motor neurons defined by the expression of LIM homebox genes.*Cell.*,**79**,

Turner, D. and Weintraub, H. (1994). Expression of achaete-scute homolog 3 in *Xenopus* embryos converts ectodermal cells to a neural fate.*Genes Dev.*,**8**, 1434-1447.

Turner, D. L. and Weintraub, H. (1994). Expression of achaete-scute homolog 3 in *Xenopus* embryos converts ectodermal cells to a neural fate.*Genes & Development.*,**8**, 1434-1447.

von Dassow, G., Schmidt, J. and Kimelman, D. (1993). Induction of the Xenopus organizer: expression and regulaton of Xnot, a novel FGF and activin-regulated homeo box gene.*Genes Dev.*,**7**, 355 - 366.

Vortkamp, A., Gessler, M. and Grzeschik, K. H. (1995). Identification of optimized target sequences for the Gli3 zinc finger protein.*DNA Cell Biol.*,**14**, 629 - 634.

Walmsley, M., Guille, M., Bertwistle, D., Smith, J., Pizzey, J. and Patient, R. (1994). Negative control of *Xenopus* GATA-2 by activin and noggin with eventual expression in precursors of the ventral blood islands.*Development.*,**120**, 2519-2529.

Wang, S., Krinks, M., Lin, K., luyten, F. and Jr, M. M. (1997). Frzb, a secreted protein expressed in the Spemann organizer, binds and inhibits Wnt-8.*Cell.*,**88**, 757-766.

Wang, Z. and Brown, D. D. (1991). A gene expression screen.*Proc Natl. Acad. Sci. (USA).*,**88**, 11505-11509.

Weinstein, D. C., Ruiz i Altaba, A., Chen, W. S., Hoodless, P., Prezioso, V. R., Jessell, T. M. and Darnell, J., Jr. (1994). The winged-helix transcription factor HNF-3 beta is required for notochord development in the mouse embryo.*Cell.*,**78**, 575-88.

Werner, M., JR, H., Gronenborn, A. and Clore, G. (1995). Molecular basis of human 46X, Y sex reversal revealed from the three-dimensional solution structure of the human SRY-DNA complex.*Cell.*,**81`**, 705-714.

Wilson, P. and Hemmati-Brivanlou, A. (1995). Induction of epidermis and inhibition of neural fate by Bmp-4.*Nature.*,**376**, 331-333.

Wilson, P., Lagna, G., Suzuki, A. and Hemmati-Brivanlou, A. (1997). Concentration-dependent patterning of the *Xenopus* ectoderm by BMP4 and its signal transducer Smad1.*Development.*,**124**, 3177-3184.

Wilson, P. A. and Hemmati-Brivanlou, A. (1995). Induction of epidermis and inhibition of neural fate by Bmp-4.*Nature.*,**376**, 331-333.

Wolda, S., Moody, C. and Moon, R. (1993). Overlapping expression of *Xwnt-3A* and *Xwnt-1* in neural tissue of *Xenopus laevis* embryos.*Dev. Biol.*,**155**, 46-57.

Wright, C. V. E., Morita, E. A., Wilkin, D. J. and De Robertis, E. M. (1990). The *Xenopus* XIHbox 6 homeo protein, a marker of posterior neural induction, is expressed in proliferating neurons.*Development.*,**109**, 229-234.

Wylie, C., Kofron, M., Payne, C., Anderson, R., Hosobuchi, M., Joseph, E. and Heasman, J. (1996). Maternal beta-catenin establishes a 'dorsal signal' in early Xenopus embryos.*Development.*,**122**, 2987-2996.

Yamada, T., Pfaff, S. L., Edlund, T. and Jessell, T. M. (1993). Control of cell pattern in the neural tube: motor neuron induction by diffusible factors from notochord and floor plate.*Cell.*,**73**, 673-86.

Yamada, T., Placzek, M., Tanaka, H., Dodd, J. and Jessell, T. M. (1991). Control of cell pattern in the developing nervous system: polarizing activity of the floor plate and notochord.*Cell.*,**64**, 635-47.

Yokota, N., Aruga, J., Takai, S., Yamada, K., Hamazaki, M., Iwase, T., Sugimura, H. and Mikoshiba, K. (1996). Predominant expression of human *zic* in cerebellar granule cell lineage and medulloblastoma.*Cancer Res.*,**56**, 377-83.

Yost, C., Torres, M., Miller, J., Huang, E., Kimelman, D. and Moon, R. (1996). The axis-inducing activity, stability, and subcellular distribution of β-catenin is regulated in *Xenopus* embryos by glycogen synthase kinase 3.*Genes Dev.*,**10**, 1443-1454.

Zaraisky, A., Ecochard, V., Kazanskaya, O., Lukyanov, S., Fesenko, I. and Duprat, A. (1995). The homeobox-containing gene *XANF-1* may control development of the Spemann organizer.*Development.*,**121**, 3839-3847.

Zaraisky, A., Lukyanov, S., Vasiliev, O., Smirnov, Y., Belyavsky, A. and Kazanskaya, O. (1992). A novel homeobox gene expressed in the anterior neural plate of the Xenopus embryo.*Dev. Biol.*,**152**, 373 - 382.

Zhang, J. and King, M. L. (1996). Xenopus VegT RNA is localized to the vegetal cortex during oogenesis and encodes a novel T-box transcription factor involved in mesodermal patterning.*Development.*,**122**, 4119-29.

Zimmerman, K., Shih, J., Bars, J., Collazo, A. and Anderson, D. (1993). XASH-3, a novel Xenopus achaete-scute homolog, provides an early marker of planar neural induction and position along the mediolateral axis of the neural plate.*Development.*,**119**, 221 - 232.

Zimmerman, K., Shih, J., Bars, J., Collazo, A. and Anderson, D. J. (1993). *XASH-3,* a novel Xenopus *achaete-scute* homolog, provides an early marker of planar neural induction and position along the mediolateral axis of the neural plate.*Development.*,**119**, 221-232.

Zimmerman, L. B., De Jesus-Escobar, J. M. and Harland, R. M. (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4.*Cell.*,**86**, 599-606.

Zoltewicz, J. and Gerhart, J. (1997). The Spemann organizer of *Xenopus* is patterned along its anteroposterior axis at the earliest gastrula stage.*Dev. Biol.*,**192**, 482-491.

Appendix III. Translational inhibition by 5' polycytidine tracts in *Xenopus* embryos and in vitro

C.1 Preface

This appendix was published in its entirety in *Gene* as:

Kuo, J.S., Veale, R., Mazwell, B., and Sive H. (1996) "Translational inhibition by 5'-polycytidine tracts in *Xenopus* embryos and in vitro." *Gene* 176, 17-21

C.2 Abstract

Introduction of in vitro transcribed mRNA into *Xenopus* embryos is a useful technique for analyzing gene function. In order to optimize expression of cDNA constructs from in vitro transcribed mRNAs, we examined the translational efficiency of reporter genes that simulated cDNAs synthesized by tailing with deoxyguanosine (dG) before second strand cDNA synthesis. When transcribed in vitro, these cDNAs give rise to RNAs containing a 5'-homopolymeric cytidine (poly (C)) stretch. We observed that the presence of a 5' poly(C) tract depressed translation of a CAT reporter gene at least 100-fold in *Xenopus* embryos and up to 5-fold *in vitro*. This effect was not seen when a 5' polyadenosine tract was tested. Translational depression was dependent on the phage polymerase used for in vitro transcription reaction: RNAs transcribed by T7 polymerase transcribed translated far more poorly than those transcribed by SP6 polymerase. These results have general implications for optimizing expression of cDNA constructs.

C.3 Introduction

Embryos of the frog *Xenopus laevis* are invaluable for defining principles of vertebrate development. In most studies, gene function in embryos has been analyzed by misexpression of mRNAs transcribed in vitro by phage polymerases (Melton et al, 1984; reviewed in Vize et al., 1991). For this approach to be effective, it is essential that injected mRNAs are efficiently translated, since a high concentration of exogenous nucleic acid is toxic to the embryos.

The 5' untranslated region (UTR) of an mRNA can have profound effects on translation during development or under various metabolic conditions (reviewed in Kozak, 1991; Melefors and Hentze, 1993; Richter, 1993). Although anecdotal evidence for the deleterious effects of 5' leader sequences on translation in *Xenopus* embryos has been reported (see Vize et al., 1991), no systematic study of the effects of leader sequences, or phage polymerase promoters, on translational efficiency has been made.

It is generally desirable that cDNAs tested for their function contain full length coding regions. The most efficient method for obtaining full length cDNAs involves tailing first strand cDNA products with deoxyguanosine triphosphate (dGTP), then priming second strand synthesis with oligodeoxycytidine (oligo-dC) (Sambrook et al., 1989). This method allows the

extreme 5' end of an mRNA to be copied into cDNA, whereas the other commonly used method (using RNAse H) does not (Sambrook et al., 1989). Tailing is also used in PCR-based RACE cloning to isolate the 5' end of mRNAs (Frohman et al., 1988). Tailing is usually performed with dGTP rather than another nucleotide since dG-tailing reactions self-limit at 15-30 nucleotides per cDNA molecule by formation of quadruple helices (Okayama et al., 1987). mRNAs that are transcribed from dG-tailed cDNA templates will therefore contain a stretch of 15-30 cytidine (poly-C) residues 5' to the original mRNA start site. In this study, we asked whether 5' poly-C leader sequences would alter translational efficiency of a linked reporter gene in *Xenopus* embryos and in vitro.

C.4 Experimental Design and Discussion

A series of clones was constructed that contained various leader sequences linked to a chloramphenicol acetyltransferase (CAT) reporter gene (Figure C.1). When transcribed in vitro, resulting RNAs either had no homopolymeric stretch, a stretch of 15 cytosine (C) or 15 adenosine (A) residues in the 5'-UTR. Constructs were cloned so that in vitro transcription could be driven by SP6, T7 or T3 phage RNA polymerases (Melton et al., 1984); and capped mRNA was synthesized in vitro by the inclusion of GpppG in the transcription reaction (Krieg and Melton, 1984).

C.3A Poly(C) leader sequence depresses translation in Xenopus embryos

We tested each RNA for relative CAT activity after injection into *Xenopus* embryos. As detailed in Table 1, RNA transcribed from a T7 (or T3, not shown) promoter in pBS.CAT, which contains no homopolymeric leader sequence, was translated at least 100-fold more efficiently than RNA from pBS.5'C.CAT, which contains a homopolymeric C_{15} leader sequence. This effect was not simply due to a longer 5' UTR, since pBS.5'A.CAT, containing a 5' homopolymeric A_{15} sequence was translated about 2-fold more efficiently than pBS.CAT. Surprisingly, the translational suppresion by a 5' C_{15} stretch was much less pronounced when RNAs were transcribed by SP6 polymerase from pGEM.5'C.CAT, with only a 5-fold suppression relative to RNAs lacking the 5'-poly(C) leader (transcribed from pSP65.CAT; Table C.1).

The differences in CAT activity that we observed after transcribing CAT RNAs with different phage polymerases did not result from differential

mRNA stability; as shown by Northern analysis (Figure C.2). Another possibility was that RNAs transcribed by different polymerases had leader sequences different in length than predicted by the DNA template due to slippage of the polymerase during transcription, and that these different length leaders influenced translational efficiency. We therefore examined the lengths of transcripts prepared from various templates relative to the sizes of the transcripts predicted by the DNA template. Templates were linearized to generate short "run-off" transcripts that can be more accurately sized (by denaturing PAGE) than full length transcripts. As shown in Figure C.3B, RNAs transcribed from pBS.CAT, pBS.5'C.CAT, pBS.5'A.CAT and pGEM.5'C.CAT had leader sequences close to the length predicted by the DNA template sequdnce (Figure C.3A); however, the gel system used could not exclude small size discrepancies of 5 or fewer nt between the templates and transcripts. These data indicated that the greater translational inhibition we observed from T7-transcribed RNAs relative to SP6-transcribed RNAs was not because the T7-derived RNAs had a significantly longer poly(C) tract.

For expression in *Xenopus* embryos, cDNAs are often cloned into a vector containing 5'- and 3'-untranslated sequences from the β-globin gene that can enhance translation (Krieg and Melton, 1984). We therefore asked whether cloning a 5'-poly(C).CAT construct into such a vector (pSP64T.5'C.CAT) would increase the translational efficiency of this construct compared to one lacking the 5'-poly(C) tract (pSP64T.CAT). As shown in Table C.1, RNA derived from pSP64T.5'C.CAT was translated at levels equivalent to RNA derived from pSP64T.CAT. These data indicate that for this CAT reporter gene, 5'- and 3'-β-globin sequences could overcome the inhibitory effect of the poly(C) leader. Although it is not clear whether this compensation would be observed for T7-derived transcripts, the data suggest that cDNAs made after dG-tailing can be effectively expressed in embryos from vectors that contain translation-enhancing sequences.

C.3B A poly(C) leader sequence depresses translation in vitro

To characterize further the translational suppression conferred by a 5'-poly(C) tract, we also examined the levels of CAT protein produced in reticulocyte lysates using the various RNA constructs. At high RNA concentrations (15 ng/ml), qualitatively similar results were obtained to those

in embryos. Using RNAs transcribed by T7 (or T3, not shown) polymerase, we observed up to a 5-fold decrease in protein levels when RNAs contained a 5'-C_{15} tract (Table C.2) relative to RNAs without a 5'-poly(C) stretch. However, at 10-fold lower RNA concentration (1.5 ng/ml), T7-transcribed RNAs with or without a 5'-poly(C) tract were translated to similar levels. At both RNA concentrations, RNAs containing a 5'-A_{15} stretch were translated 2- to 3-fold more efficiently than the parental constructs. The inhibitory effect of a 5'-poly(C) tract was not observed when uncapped transcripts were used in the in vitro reaction. No inhibitory effects of 5'-poly(C) tracts were observed when SP6-transcribed RNAs were translated in vitro even at the high (15 ng/ml) concentration, and these therefore not tested at lower concentrations.

Taken together, these results suggest that some component that is limiting in the reticulocyte lysate is involved in translational inhibition by poly(C) tracts, and that this effect is dependent on the presence of a 5' cap. We hypothesize that the polymerase-specific effect may involve formation of secondary structures at the 5' end of the RNA. In particular, T7 and T3 promoters initiate transcription with 5'-GGG as the first three bases, while SP6 transcripts initiate with 5' GAA (Figure C.3A). We suggest that "loop back" secondary structure between the 5' end of T7 transcripts and downstream poly(C) tracts may inhibit interaction of the mRNA with cap-binding protein, eIF-4E, or some other component of the eIF-4F initiation complex, to prevent ribosome assembly and translation. This interpretation is consistent with limiting amounts of eIF-4E in HeLa cell extracts (see Frederickson and Sonenberg, 1992). This factor may also be limiting in *Xenopus* embryos since its overexpression has profound effects on development (Klein and Melton, 1994).

Our results show quantitatively that 5' homopolymeric-C tracts can severely attenuate translational efficiency in *Xenopus* embryos and in vitro. These results are important because dG-tailing is routinely used to optimize recovery of full length cDNAs and results in the addition of 5' poly(C) leader sequence to mRNAs. This could occur during conventional cDNA synthesis or RACE cloning techniques (Okayama et al., 1987; Sambrook et al., 1989). It is feasible to employ other nucleotides in the tailing reaction (Frohman et al., 1988). Tailing with dTTP would result in poly(A) stretches at the 5' end of RNAs, which our results show can enhance translation. These data also

indicate that, in the context of certain 5' UTRs, the choice of phage polymerase for in vitro transcription is important for efficient RNA translation.

C.5 Acknowledgments

The authors would like to thank members of the Sive lab for critical comments on the manuscript, especially Peggy Kolm for help with figure graphics. J.S.K. was supported by a Medical Scientist Training Program Fellowship (PHS grant 2-T32-GM07753) from the Harvard MD-PhD Program. H.L.S. acknowledges Ken Hattori for help with initial experiments in Harold Weintraub's laboratory. Funded by a grant from the Council for Tobacco Research and by the NSF. H.L.S. was a Searle Scholar and was supported by the Searle Scholars Program/Chicago Community Trust.

Figure C.1. CAT reporter constructs

pBS.CAT was constructed by subcloning an EcoRI-Bam HI fragment (after partial EcoRI digest) derived from pSP65.CAT (Harland and Weintraub, 1985) into the EcoRI and BamHI sites of pBS+ (Stratagene). pBS.5'C.CAT yields an RNA with a stretch of 15 C's upstream of the CAT coding sequence. This plasmid was constructed by ligating an oligonucleotide containing a 5' EcoRI site and dC_{15} stretch into a blunted SacI cut-pSP65.CAT. After digestion with BamHI, this insert was subcloned into the EcoRI and BamHI sites of pBS+. pBS.5'A.CAT yields an RNA with a stretch of 15 A's upstream of the CAT coding sequence. This plasmid was constructed using a similar method, using an oligo with a stretch of A_{15}. pGEM.5'C.CAT was constructed by subcloning a blunted NotI-BamHI insert from pBS.5'C.CAT into the SalI (blunted) and BamHI sites of pGEM3 (Promega). pSP64T.CAT was constructed by subcloning a blunted CAT fragment from a partial EcoRI-BamHI digest into the blunted BglII site of pSP64T (Krieg and Melton, 1984). pSP64T.5'C.CAT was constructed by subcloning the blunted NotI-BamHI insert from pBS.5'C.CAT into the blunted BglII site of pSP64T. These two constructs have 5'- and 3'-β-globin untranslated regions (βG-UTR) flanking the CAT reporter construct.

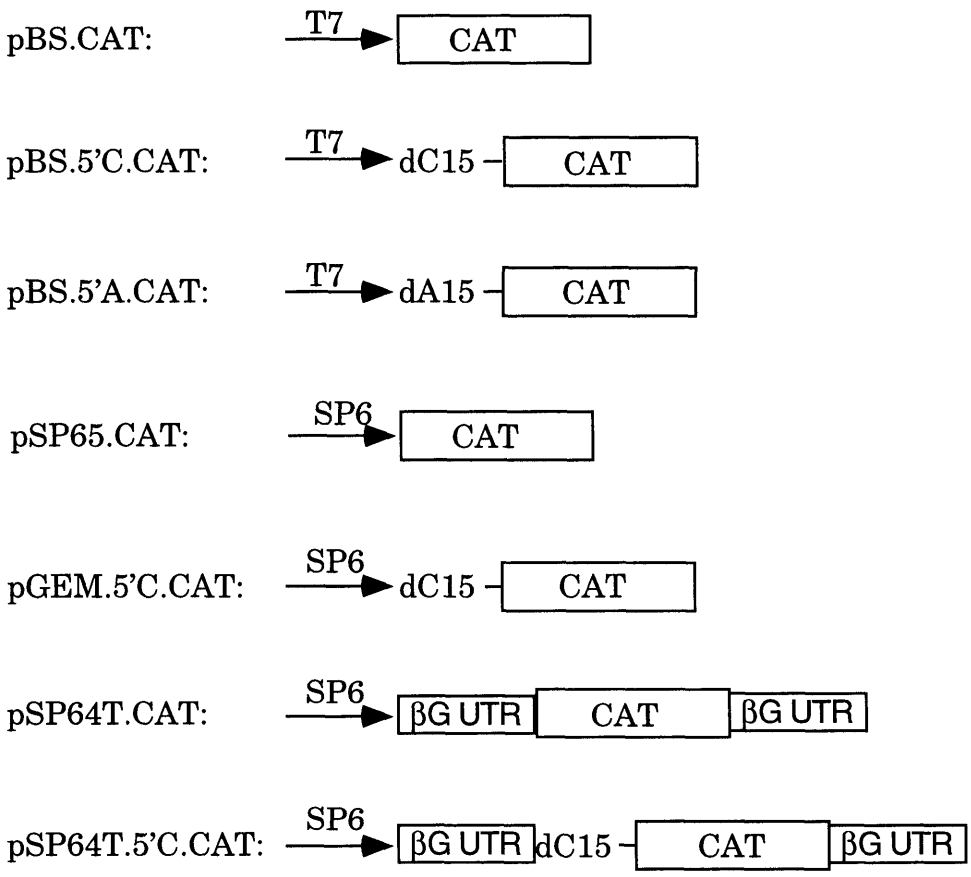

pBS.CAT:
T7
CAT
pBS.5'C.CAT:
T7
dC15
CAT
pBS.5'A.CAT:
T7
dA15
CAT
pSP65.CAT:
SP6
CAT
pGEM.5'C.CAT:
SP6
dC15
CAT
pSP64T.CAT:
SP6
βG UTR
CAT
βG UTR
pSP64T.5'C.CAT:
SP6
βG UTR
dC15
CAT
βG UTR

Figure C.2. Similar stability in Xenopus embryos of RNAs with various 5' leaders

Each cell of a two cell embryo was injected with 0.5 ng of RNA as indicated. Embryos were harvested immediately after injection or 5 h. later (mid-blastula stages). Total RNA was prepared from embryos by treatment with proteinase-K and phenol extraction (Condie and Harland, 1987). Northern analysis of RNA samples were done by standard methods, using a ^{32}P-labeled random primed probe prepared from CAT insert (Sambrook et al., 1989). Representative data are shown, with each lane containing RNA derived from a pool of three injected embryos. Lanes 1-3, CAT RNA levels immediately after injection; 1, pBS.CAT RNA; 2, pBS.5'A.CAT RNA; 3, pBS.5'C.CAT RNA; 4-6 CAT RNA levels 5 h after injection; 4, pBS.CAT RNA; 5, pBS.5'A.CAT RNA; 6, pBS.5'C.CAT RNA. An ethidium bromide stained panel of 28S rRNA is shown as loading control. The 5'-C and 5'-A RNAs are larger than the RNA with no homopolymeric leader because of sequence differences in the vector (Figure C.3A).

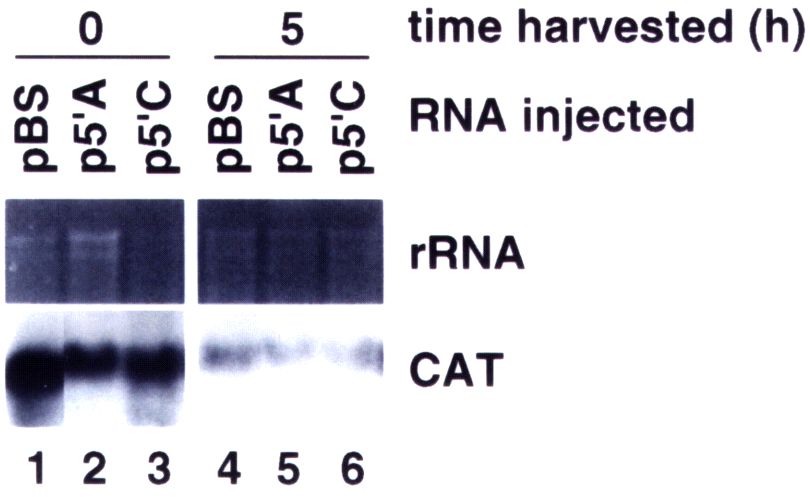

0
5
time harvested (h)
pBS
p5'A
p5'C
pBS
p5'A
p5'C
RNA injected
rRNA
CAT
1 2 3 4 5 6

Figure C.3. Similar sizes of CAT RNAs transcribed by different polymerases

(A) Nucleotide sequences for the 5' leader to the CAT reporter gene. Nt 1 is the first transcribed by the phage polymerase. The 5' leader lengths vary due to differences in the subcloning: pBS.CAT, 20nt; pBS.5'C.CAT, 30nt; pBS.5'A.CAT, 34nt; pGEM.5'C.CAT, 55nt. Subsequent to the last nt shown, the sequences of these constructs are identical.

(B) Sizes of run-off transcripts. DNA templates were digested with BsmI to generate short "run-off" CAT transcripts for size analysis by 6% denaturing PAGE. The length of the CAT transcripts is predicted to be 241 bp plus the 5' leader sequence. Transcription was carried out using standard methods in the presence of ^{35}S-CTP to label synthesized RNAs (T7 polymerase: pBS.CAT, pBS.5'C.CAT, pBS.5'A.CAT; SP6 polymerase: pGEM.5'C.CAT). Lanes:1, pBS.CAT; 2, pBS.5'C.CAT; 3, pBS.5'A.CAT; 4, pGEM.5'C.CAT. The sizes of the RNAs observed after transcription were as predicted from the nt sequence (Part A), indicating that differential translational efficiencies of these RNAs were not due to different length leader sequences.

A.

pBS.CAT

```
          10        20
5' GGGCGAATTCGAGCTCGCCC
```

pBS.5'C.CAT

```
          10        20        30
5' GGGCGAATTCCCCCCCCCCCCCCCCCCGCCC
```

pBS.5'A.CAT

```
          10        20        30
5' GGGCGAATTCAAAAAAAAAAAAAAAAGAGCTCTAG
```

pGEM.5'C.CAT

```
          10        20        30        40        50
5' GAATACAAGCTTGCATGCCTGCAGGTCGAGGCCGCCCCCCCCCCCCCCCCCCGCCC
```

B.

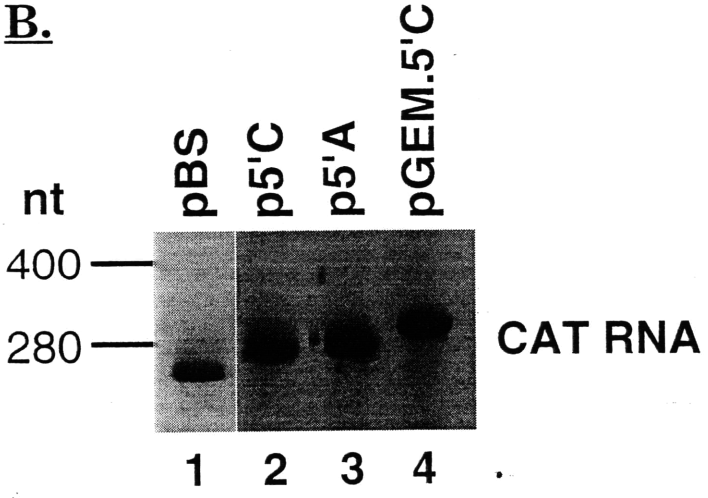


Table C.1. Relative CAT activity in Xenopus embryos

Each cell of a two-cell embryo was injected with 0.5 ng of RNAs indicated. After 5 h incubation at room temperature (to mid-blastula), embryos were collected and assayed for CAT activity as detailed below. Within each experiment, the cpm obtained after CAT assay for each construct were compared. CAT activity of RNA lacking a 5'-poly(C) tract was set at 100% (Expts. 1, 2 = pBS.CAT; Expts. 3-5 = pSP65.CAT; Expts. 6, 7 = pSP64T.CAT) and values obtained from expression of other constructs are expressed comparatively. Absolute counts could not be compared from experiment to experiment because of variability in the translational efficiency of different clutches of frog embryos and in the specific activity of [^{3}H]-acetyl-CoA. However, results between normalized values were highly consistent between experiments, as shown. To determine CAT activity, embryos were lysed in 10 µl PBS+0.5% NP40/embryo and incubated at 65C for 10 minutes; lysates were collected after centrifugation at 14,000 rpm for 10 minutes. Lysates were incubated with [^{3}H]-acetylCoA and chloramphenicol at 37C for 2 hours, extracted with ethyl acetate and CAT activity assayed by scintillation counting of ^{3}H-acetylated chloramphenicol. Multiple dilutions of each lysate were tested to determine the linear range. Pools of three to five embryos were collected for each experiment and the results shown represent the mean of the collective pools. Pool to pool variation was small indicating that RNA injections were uniform. Northern analyses of injected embryos also indicated uniform RNA dosage injected (Figure C.2).

a) Refer to Figure C.1.
b) Each independent experiment was numbered.
c) Number of injected embryos analyzed for a particular construct.
d) For each parameter tested, the CAT activity derived from RNA made from pBS.CAT (Expts. 1, 2), pSP65.CAT (Expts. 3-5) or pSP64T (Expts. 6, 7) was set at 100% , and used for normalizing the other measured values.
e) Average of normalized values ± S.D.

Construct (Promoter)[a]	Exp. #[b]	n[c]	%CAT[d] activity	avg.[e] %CAT activity
pBS.CAT (T7)	1	25	100	**100**
	2	15	100	
pBS.5'C.CAT (T7)	1	25	0	**0**
	2	20	0	
pBS.5'A.CAT (T7)	1	12	171	**174 ± 4**
	2	15	177	
pSP65.CAT (SP6)	3	10	100	**100**
	4	40	100	
	5	8	100	
pGEM.5'C.CAT (SP6)	3	8	15	**17 ± 2**
	4	50	19	
	5	9	19	
pSP64T.CAT (SP6)	6	25	100	**100**
	7	25	100	
pSP64T.5'C.CAT (SP6)	6	25	77	**94 ± 24**
	7	25	111	

Table C.2. Relative CAT activity in vitro

In vitro translation was carried out by incubation of RNAs in reticulocyte lysates according to the manufacturer (Promega) in the presence of ^{35}S-methionine. RNAs were added at 50 ng (1.5 ng/ml) or 500 ng (15 ng/ml) as indicated, and were capped except where indicated. Translation products were analyzed by SDS-PAGE using standard methods (Sambrook et al., 1989). CAT protein levels were quantitated by scanning autoradiograms on a Molecular Dynamics Computing densitometer (Model 300A) using ImageQuant Software v3.0; multiple autoradiographic exposures were scanned to ensure that quantitation was in the linear range. Within each experiment, the amount of CAT protein translated from RNAs transcribed from pBS.CAT (Expts. 1-6) or pSP65.CAT (Expts. 7,8) was set at 100%, and used for normalizing the other measured values. In Expts. 4a and 4b, capped and uncapped pBS.CAT-derived RNAs injected at 15 ng/ml gave essentially the same amounts of CAT protein. Quantitatively similar results were obtained when CAT activity was monitored by efficiency of chloramphenicol conversion (not shown).

a) See Figure C.1.
b) All RNAs were capped except where indicated.
c) Each independent experiments was numbered.
d) For each parameter tested, the amount of CAT protein derived from RNA made from pBS.CAT (Expts. 1-6) or pSP65.CAT (Expts. 7, 8) was set at 100%, and used for normalizing the other measured values.
e) Average of normalized values $\pm$ S.D.

Construct (Promoter)[a]	RNA[b] (ng/ml)	Exp. #[c]	%CAT[d] protein	ave.[e] %CAT protein
pBS.CAT (T7)	15	1	100	**100**
		2	100	
		3	100	
		4a	100	
	uncapped 15	4b	100	**100**
	1.5	5	100	**100**
		6	100	
pBS.5'C.CAT (T7)	15	1	15	**25 ± 11**
		2	38	
		3	33	
		4a	16	
	uncapped 15	4b	98	**98**
	1.5	5	126	**111 ± 20**
		6	97	
pBS.5'A.CAT (T7)	15	3	196	**196**
	1.5	5	367	**324 ± 60**
		6	282	
pSP65.CAT (SP6)	15	7	100	**100**
		8	100	
pGEM.5'C.CAT (SP6)	15	7	90	**106 ± 23**
		8	123	

C.5 References

Condie, B. G., and Harland, R. M.: Posterior expression of a homeobox gene in early Xenopus embryos. Development 101 (1987) 93-105.

Frederickson, R. M., and Sonenberg, N.: Signal transduction and regulation of translation initiation. Seminars in Cell Biology 3 (1992) 107-115.

Frohman, M. A., Dush, M. K., and Martin, G. R.: Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc Natl Acad Sci USA 85 (1988) 8998-9002.

Harland, R., and Weintraub, H.: Translation of mRNA injected into Xenopus oocytes is specifically inhibited by antisense RNA. J Cell Biol 101 (1985) 1094-1099.

Klein, P. S., and Melton, D. A.: Induction of mesoderm in Xenopus laevis embryos by translation initiation factor 4E. Science 265 (1994) 803-806.

Kozak, M.: An analysis of vertebrate mRNA sequences: intimations of translational control. J. Cell Bio 115 (1991) 887-903.

Krieg, P. A., and Melton, D. A.: Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNAs. Nucleic Acids Res 12 (1984) 7057-7070.

Melefors, O., and Hentze, M. W.: Translational regulation by mRNA/protein interactions in eukaryotic cells: ferritin and beyond. BioEssays 15 (1993) 85-90.

Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K., and Green, M. R.: Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res 12 (1984) 7035-7056.

Okayama, H., Kawaichi, M., Brownstein, M., Lee, F., Yokota, T., and Arai, K.: High-efficiency cloning of full-length cDNA; construction and screening of cDNA expression libraries for mammalian cells. Methods Enzymol 154 (1987) 3-28.

Richter, J.: Translational control in development: a perspective. Developmental Genetics 14 (1993) 407-411.

Sambrook, J., Fritsch, E. F., and Maniatis, T.: Molecular Cloning. A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

Vize, P. D., Melton, D. A., Hemmati-Brivanlou, A., and Harland, R. M. Assays for gene function in developing *Xenopus* embryos. In: B. K. Kay, and Peng, H. B. (Eds.), Methods in Cell Biology (36): *Xenopus laevis*: Practical Uses in Cell and Molecular Biology. Academic Press, Inc., San Diego, CA, 1991, pp. 367-387.