Patterning the *Xenopus* neurectoderm: the role of *otx2* in the determination of cement gland and anterior neural fates.

by

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Abstract

During gastrulation, the dorsal ectoderm of the embryo is induced and patterned to give rise to an anteroposterior array of structures. My thesis research has specifically addressed how anterior neurectodermal derivatives (midbrain and forebrain) and associated non-neural cement gland are appropriately positioned in the *Xenopus* embryo during this process. The cement gland is a mucus-secreting organ that forms from the anterior-most ectoderm at the dorsal-ventral border, which is equivalent to the ectoderm overlying the mammalian chin. The presumptive cement gland and anterior neurectoderm form an anterior domain defined by expression of the homeobox gene *otx2*, the vertebrate homologue of the *Drosophila* anterior gap gene *orthodenticle*. In mouse, *otx2* is essential for development of the rostral head, however, since *otx2* is expressed in both ectoderm and mesendoderm, the requirement and function of *otx2* in the ectoderm are not known. I have analyzed the role of *otx2* in anterior ectodermal development in *Xenopus* in the following manner. First, I used a hormone inducible *otx2*-glucocorticoid receptor fusion to demonstrate that *otx2* is sufficient for cement gland formation, but that it can activate cement gland development only in ventrolateral ectoderm after mid-gastrula. Second, I have shown that coexpression of *otx2* and BMP4 promotes cement gland formation and that this unique juxtaposition normally occurs only in the cement gland primordium. This allows cement gland development exclusively from the ventral aspect of the *otx2* expression domain and not dorsally in the neurectoderm. Third, I have found that *otx2* activates anterior and represses posterior neural gene expression in embryos and in isolated ectoderm. I have created a dominant negative *otx2*-engrailed repressor fusion, which ablates cement gland and head formation, phenocopying the *otx2* mouse mutant. Together these data have led to a model in which *otx2* acts in the ectoderm to promote anterior neural and cement gland fates while repressing posterior neural gene expression, with *otx2* activity spatially and temporally modulated by overlap with additional factors.

Thesis advisor: Hazel Sive
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To Mom and Dad

for giving me legs to walk and footsteps to follow,

and to York

for joining me along the path.

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The rest of the wonderfully interesting and quirky people with whom I shared my days in the Sive lab have also made life educational and fun. I will always remember John's lightening-legs, Jenya's giggles, Mary Ellen's sarcasm, Ben's profound silence, Leila's strip tease act, Dan's insightful seriousness, Vladimir's sweet tooth, Sara's devious mind, Liz's swinging red ponytail, Betsy's love story, Jacqui's funkin' groovin', Charles' stoicism, Bertha's purple gloves, and Bridey's quest for the perfect man.

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Finally, my family has always cheered me on. My parents never stopped saying, "keep going, you can do it, its worth it, we're proud of you." They spoke from their own experience and made me believe it. My brothers, Rich, who has always expected me to want the best for myself, and Dave, who thinks that I am "cool", have helped me to hold my head high. The amazing women in my family have always been an inspiration to me. Throughout my life, both of my grandmothers have always emphasized the importance of my education, each in her own way, while Aunt Jean and my Great Aunts Elsie and Lil taught me that a woman can do anything, even get a Ph.D.

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Figures and Tables

Abbreviations

A	anterior
A/P	anteroposterior
CG	cement gland
CHX	cycloheximide
D	dorsal
D/V	dorsoventral
DEL	zebrafish deep layer of the ectoderm
dex	dexamethasone
En	engrailed repressor domain
EVL	zebrafish enveloping layer of the ectoderm
GR	glucocorticoid receptor ligand binding domain
LBD	ligand binding domain
MBS	Modified Barth's Saline
mg	milligram
µg	microgram
µl	microliter
nl	nanoliter
otd	*orthodenticle*
P	posterior
pg	picogram
RA	retinoic acid
V	ventral

Chapter 1. Heads or tails: when does the A/P axis form?

The signals and tissue interactions responsible for patterning the vertebrate embryo have been studied for over half a century in amphibians, including the frog *Xenopus laevis*. Early embryologists chose amphibians for their large, and externally and rapidly developing embryos. These advantages, plus a long history of embryological studies and defined staging (Fig. 1.1; Nieuwkoop, et al., 1994), make *Xenopus* an excellent model system for studying early development. In the past ten years, great progress has been made in isolating some of the molecules involved in early embryonic induction, patterning, and differentiation. How these signals are coordinated to give rise to an embryo with structures in appropriate dorsoventral (D/V) and anteroposterior (A/P) patterns, however, is only minimally understood. For my thesis, I have examined how the anterior-most ectoderm of the *Xenopus* gastrula embryo is patterned and subdivided. I have studied the function of one particular gene, *otx2*, addressing its role in instructing anterior ectodermal fates and considering how it interacts with other elements of axial pattern to lead to region-specific differentiation in the embryo.

But how is anterior identity established? When does the A/P axis form? It is conventionally believed A/P patterning takes place during gastrulation, as the ectoderm is induced by the involuting mesoderm. Gastrulation is the time when the embryonic tissue layers acquire their ultimate A/P organization, however, establishment of A/P polarity in the embryo takes place gradually, beginning long before the onset of gastrulation. To consider A/P pattern formation, we must follow development from its beginnings at fertilization, through D/V patterning and mesoderm induction, to gastrulation, when complicated interactions refine and elaborate A/P pattern. Together these events give rise to the A/P axis of the embryo.

1.1 Early patterning of the *Xenopus* embryo.

Although not typically viewed as part of A/P patterning, early development establishes the polarities on which the A/P axis is built. The animal-vegetal axis of the fertilized egg prefigures the A/P axis of the embryo. Meanwhile, A/P pattern is created during D/V axis formation and patterning of the mesoderm.

1.1A The egg.

The mature amphibian oocyte is radially symmetrical about an animal-vegetal axis, apparent as a darkly pigmented animal pole and yolky vegetal pole

(Fig. 1.2A). The animal pole is fated to become the anterior ectoderm later in development, and to the extent that this region is relatively isolated from early signaling events in the embryo, this position is established early on. The distribution and size of yolk platelets are asymmetric along the animal-vegetal axis, with yolk concentrated in the vegetal hemisphere and non-yolk cytoplasm and the germinal vesicle in the animal hemisphere (reviewed in Gerhart, et al., 1986). In addition, maternal RNAs are localized to both the animal and vegetal poles (King, et al., 1985; Rebagliati, et al., 1985). Although the activity of the egg animal cytoplasm has not been defined, vegetally localized maternal factors have been implicated in mesoderm determination. Factors localized along the animal vegetal axis may be important for establishment of polarity in the mesoderm as well (see below).

1.1B Fertilization and dorsoventral axis determination.

Radial symmetry of the oocyte is broken at fertilization as the sperm meets the egg in the animal hemisphere. The sperm entry point defines the future ventral side of the embryo, initiating a 30° rotation of the cortical cytoplasm to the dorsal side (Fig. 1.2A; reviewed in Slack, 1991). The vegetal cortex of the oocyte contains a maternally localized dorsalizing activity that is translocated dorsally during rotation (Fujisue, et al., 1993; Holowacz, et al., 1993). This displacement of dorsal determinants takes place along a parallel array of microtubules established by rotation beneath the cortex (Elinson, et al., 1988; Rowning, et al., 1997). The importance of this process is illustrated by treatments that depolymerize microtubules, leading to formation of ventralized embryos (reviewed in Slack, 1991) and activation of dorsal-specific gene expression in the vegetal pole (Darras, et al., 1997).

Due to their dorsalizing activity, the *wnt* genes were originally identified as candidates for the dorsal determinant (Smith, et al., 1991; Sokol, et al., 1991). However, factors that inhibit upstream events in the wnt signaling cascade do not prevent dorsal axis formation, indicating that a wnt ligand is not normally involved (Hoppler, et al., 1996; Sokol, 1996). Rather, it has recently been suggested that cortical rotation leads to activation of the target of the wnt signaling pathway, β-catenin, specifically on the dorsal side through an unknown, wnt-independent mechanism (Fig. 1.2B; see Harland, et al., 1997). In support of this view, depletion of *β-catenin* RNA leads to ventralized embryos (Heasman, et al., 1994), and β-catenin protein, although ubiquitously expressed, is

stabilized and nuclear only in dorsal cells of the embryo at early cleavage stages (Larabell, et al., 1997; Schneider, et al., 1996; Yost, et al., 1996). Through an interaction with another factor Xtcf3, activated β-catenin initiates transcription of dorsal-specific genes to establish dorsal fate (see below; Brannon, et al., 1997; Molenaar, et al., 1996).

Interestingly, activation of β-catenin may create asymmetry important for A/P pattern as well. β-catenin accumulates in both dorsal vegetal and dorsal animal blastomeres at early cleavage stages (Larabell, et al., 1997), and at later blastula stages, animal caps and vegetal masses both contain a β-catenin-dependent ability to dorsalize mesoderm (Wylie, et al., 1996). β-catenin localization likely creates D/V pattern in the animal hemisphere, since dorsal animal blastomeres at the 8-cell stage are only weakly specified to express a ventral epidermal antigen compared to their ventral animal counterparts (London, et al., 1988). β-catenin dorsalizing activity is much less in animal than vegetal cells (Wylie, et al., 1996), and it is possible that a gradient of active β-catenin exists into the animal hemisphere, although this has not been examined. However, β-catenin/Xtcf3 signaling is critical for establishment of A/P polarity in the dorsal animal cap during blastula stages (Gamse et al., in preparation; see below). This observation creates an intriguing connection between earliest dorsal fate in the embryo and A/P identity in the ectoderm later on.

1.1C Mesoderm determination.

As cell division takes place, dorsal determination is superimposed upon generation of the three embryonic tissue layers. Mesoderm arises at the interface of animal and vegetal cells in the equatorial region of the embryo, the marginal zone, while remaining animal cells form ectoderm and vegetal cells form endoderm (Fig. 1.2C). Mesoderm determination takes place as a result of localized cytoplasmic determinants (Lemaire, et al., 1994) and signaling between cells in the animal and vegetal poles (Nieuwkoop, 1969), known as mesoderm induction. The cell-autonomous portion of mesoderm determination may be due in part to a localized T-box transcription factor, known by several different names (Xombi, Lustig, et al., 1996; Veg-T, Zhang, et al., 1996; Antipodean, Stennard, et al., 1996; and Brat, Horb, et al., 1997), that is required for mesoderm formation (Horb and Thomsen, 1997). Meanwhile, a number of secreted factors have been identified that mediate mesoderm induction. Several different TGF-β family members, including the most often emphasized, activin, as well as Vg-1

(in a form that can be processed), nodal-related Xnr-1 and -2, and a novel TGFβ, derrière (Sun et al., submitted), are able to induce mesoderm of dorsal character (reviewed in Kessler, et al., 1994). Another TGF-β, BMP4, induces ventral mesoderm. Although the specific function and requirement of each of these signals is not clear (see Harland and Gerhart, 1997), some combination of TGF-β signaling is required for mesoderm formation (Hemmati-Brivanlou, et al., 1992). Another factor, FGF, was originally thought to induce ventral mesoderm, however, inhibition of FGF signaling blocks all mesoderm formation, suggesting that FGF may be required as a competence or maintenance factor for mesoderm induction (Amaya, et al., 1993; Cornell, et al., 1994a; LaBonne, et al., 1994).

By blastula stage, a ring of mesoderm exists around the margin of the embryo as a consequence of these events. The overlap of this ring and β-catenin defines the dorsal-most mesoderm, the organizer (Fig. 1.2C). However, within the organizer (Zoltewicz, et al., 1997) and around the D/V circumference of the marginal zone, mesodermal specification (Dale, et al., 1987b) and fate (Keller, 1991) are not equivalent. Different regions of the mesoderm will later occupy diverse A/P and D/V positions in the embryo and give rise to distinct mesodermal structures. More importantly, the inductive capacity of the mesoderm varies, and will impart A/P pattern to the ectoderm during gastrulation. Pattern in the mesoderm is thus of critical importance for formation of the A/P axis. How are different positional values established in the mesoderm?

1.1D Pattern in the organizer.

The organizer consists of a 60° arc of the dorsal marginal zone (Stewart, et al., 1990). Characterization of several molecules responsible for formation and activity of the organizer indicates that the dorsal, β-catenin signal and activity of mesoderm inducing factors converge to define organizer mesoderm (Fig. 1.3A; reviewed in Harland and Gerhart, 1997). Two genes, the homeobox gene *siamois* and the TGF-β *Xnr-3*, contain β-catenin/Xtcf-3 sites in their promoters (Brannon, et al., 1997; McKendry, et al., 1997) and are expressed in the anterior mesoderm and endoderm containing the activated dorsal determinant (Darras, et al., 1997; Lemaire, et al., 1995). In the mesoderm, siamois activates expression of the homeobox gene *goosecoid* and the secreted factors *noggin* and *chordin* (Carnac, et al., 1996; Darras, et al., 1997). siamois expression in the endoderm and the dorsalizing effect (see below) of noggin and chordin secretion from the mesoderm

in turn activate expression of the secreted factor *cerberus* in the deep, anterior endoderm (Bouwmeester, et al., 1996; Darras, et al., 1997). Two other secreted factors, *dickkopf* and *frz-b1*, are also expressed in the anterior endoderm and could be similarly regulated (Glinka, et al., 1998; Leyns, et al., 1997; Wang, et al., 1997).

The most obvious A/P pattern in the mesoderm occurs in the organizer, which is crudely arranged along the inverted future A/P axis of the embryo (Figs. 1.2E, 1.3). This was determined from the experiments of early embryologists such as Spemann, who defined separate head and trunk/tail inducing activities within the dorsal mesoderm (reviewed in Slack, 1991). More recently it has been shown that anterior (vegetal) dorsal marginal zone, or "head organizer", activates only anterior gene expression when apposed to uninduced ectoderm, while posterior (animal) marginal zone, or "trunk/tail organizer", induces anterior and posterior gene expression (Doniach, et al., 1995; Zoltewicz and Gerhart, 1997). The endoderm has a different effect and is considered separately below. A/P pattern in the organizer is also evident in regionally restricted expression of transcription factors before gastrulation begins (Fig. 1.3B): *Xbra* and *Xnot2* in posterior dorsal mesoderm , the future notochord (Smith, et al., 1991; von Dassow, et al., 1993; Zoltewicz and Gerhart, 1997); *otx2*, *Xlim-1*, and *goosecoid* in anterior dorsal mesoderm , the presumptive prechordal plate (Blitz, et al., 1995; Pannese, et al., 1995; Taira, et al., 1992; Zoltewicz and Gerhart, 1997); and *otx2* and *Xlim-1* in the anterior endoderm.

In contrast, very little A/P pattern is apparent in the expression of secreted factors that make up the organizer, discussed above (Fig. 1.3A). It is interesting to note, however, that ectopic marginal zone expression of organizer-derived factors such as noggin leads to the formation of secondary axes with A/P polarity (Smith, et al., 1992). This suggests that there is asymmetry in the marginal zone that cooperates with organizer activity to create an A/P axis. Strikingly, a secreted factor not discussed above, *eFGF*, is restricted to the animal half of the entire marginal zone, including the posterior organizer (Fig. 1.3A; Isaacs, et al., 1992). *eFGF* expression is thus indicative of asymmetry in the marginal zone, and, intriguingly, may be regulated in part by the maternally localized T-box transcription factor involved in mesoderm determination (see above; B. Sun and H. Sive, unpublished). This presents the possibility that the A/P axis of the organizer is defined using information localized along the animal-vegetal axis of the egg. Although speculative, this idea bears similarity to the recent proposal

that the A/P axis of a mouse embryo is initially evident in the proximal-distal axis of the egg cylinder (Thomas, et al., 1998). Further consideration of these early events will be important for understanding A/P axis formation, since A/P differences in the organizer mesoderm will provide the basis for primary induction and A/P patterning of the ectoderm during gastrulation.

1.1E The organizer and dorsalization.

Although the remainder of the marginal zone may to some extent be regionalized by different doses or types of inducers at the time of mesoderm induction (Green, et al., 1992), this is unlikely to be the driving force behind pattern in the mesoderm. In the absence of the blastula organizer (Dale and Slack, 1987b; Stewart and Gerhart, 1990), or in *β-catenin* depleted embryos (Heasman, et al., 1994), the remaining marginal zone adopts ventral fates. Rather, intermediate fates within the mesoderm appear to be specified by opposing signals from the organizer and the ventral mesoderm (Fig. 1.2D; Dale and Slack, 1987b; reviewed in Harland and Gerhart, 1997). This process, termed dorsalization, is carried out by organizer secreted factors noggin, chordin, and Xnr3 and affects all three tissue layers of the embryo: the mesoderm (Hansen, et al., 1997; Sasai, et al., 1994; Smith, et al., 1993), the endoderm (leading to cerberus and dickkopf expression and formation of liver and pancreas; Bouwmeester, et al., 1996; Sasai, et al., 1996) and the ectoderm (leading to neural induction; Hansen, et al., 1997; Lamb, et al., 1993; Sasai, et al., 1995). The effect of dorsalizing signals is opposed by ventralizing influences. Within the marginal zone, *BMP4* (Fainsod, et al., 1994; Schmidt, et al., 1995) and *Xwnt8* (Smith and Harland, 1991) are expressed in lateral mesoderm but excluded from dorsal mesoderm. Overexpression of *BMP4* or *Xwnt8* in dorsal mesoderm inhibits organizer function, indicating that the absence of these factors in the dorsal marginal zone is critical to normal development (Christian, et al., 1993; Dale, et al., 1992; Jones, et al., 1992). Both noggin and chordin act by binding to BMPs and blocking BMP signaling (Piccolo, et al., 1996; Zimmerman, et al., 1996), while another organizer derived factor, frz-b1, inhibits wnt signaling (Leyns, et al., 1997; Wang, et al., 1997). This indicates that dorsalization proceeds by repression of ventral signals.

The prevailing model for mesodermal patterning (Fig. 1.2D) maintains that dorsalization induces intermediate paraxial and lateral plate mesoderm. As a result, the marginal zone is specified as dorsal (future notochord), lateral (future somites and lateral plate), and ventral mesoderm (future blood) around its

circumference (Dale and Slack, 1987b). However, this model has de-emphasized the observation that the presumptive somites, which will lie in an A/P array after gastrulation, arise from a field encompassing the entire lateral and ventral marginal zone (Dale, et al., 1987a). Indeed, the gastrula stage fate map indicates that the circumference of the embryo is patterned along the A/P axis instead (Fig. 1.4A; reviewed in Keller, 1991). Convergent extension movements and more pronounced involution of dorsal mesoderm during gastrulation result in displacement of the mesodermal ring to the dorsal side of the embryo, converting the D/V arrangement of the marginal zone into an A/P axis (Fig. 1.4A, numbers). Meanwhile, the presumptive D/V axis of the mesoderm after gastrulation corresponds more closely to the animal-vegetal polarity of the blastula mesoderm (Fig. 1.4A, letters). The existing model for regionalization of the mesoderm does not explain this arrangement of cell types (Fig. 1.2D), and it is not known how such complex pattern is realized. It may be that D/V and A/P pattern in the ventrolateral marginal zone are specified by the same signals, since a dose range of activin produces a posterior-ventral to anterior-dorsal range of cell types (Green, et al., 1992). As would be predicted from the gastrula fate map, the lateral mesoderm is a source of lateral posterior inducers during gastrulation (see below; Kolm, et al., 1997; Woo, et al., 1997), in support of the idea that A/P positional information is encoded in the D/V arrangement of the marginal zone.

1.1F Pattern in the ectoderm.

As indicated above, the ectoderm is also subject to pre-gastrula patterning events. D/V pattern in the animal hemisphere, established at fertilization, is manifested as an increased competence of the late blastula/early gastrula dorsal ectoderm to respond to neural inducers (Fig. 1.2D; Otte, et al., 1991; Sharpe, et al., 1987) . This effect has been ascribed to differences in protein kinase C expression (Otte, 1992a; Otte, et al., 1991), and more recently to expression of a zinc-finger gene, *opl* (Kuo et al., in press; also known as *Zic-1*, Mizuseki, et al., 1998). Examination of *opl* regulation suggests that D/V pattern in the ectoderm depends upon dorsal axis determination; β-catenin/Xtcf3 activity in dorsal animal cells is required for late blastula opl expression (Gamse et al., in preparation). At blastula stages, dorsalizing signals from the organizer mesoderm also regulate *opl* expression in the ectoderm (Gamse et al., in preparation), emphasizing D/V differences and contributing to dorsal bias for neural induction (Otte, et al., 1992; Savage, et al., 1989).

Intriguingly, the combination of these early signaling events also establish a rudimentary A/P pattern in the dorsal ectoderm (Gamse et al., in preparation). At the onset of gastrulation, *opl* which will mark the anterior and lateral edges of the neural plate (Kuo et al., in press; Mizuseki, et al., 1998) is expressed throughout the dorsal ectoderm, while *fkh5*, a marker of the future diencephalon (Gamse et al., in preparation), and *sox3*, a general neural marker (Mizuseki, et al., 1998), are expressed in the ectoderm proximal to the organizer mesoderm. It is not known whether this polarity is required for A/P axis formation, but later events will expand and elaborate this early A/P ectodermal pattern.

1.2 Induction and patterning of the neurectoderm.

The various A/P polarities established during early stages are repositioned and aligned as a single A/P axis as a result of gastrulation. During this process, the mesoderm involutes and migrates beneath the ectoderm, bringing the ectoderm and dorsal mesoderm into close proximity. These movements allow neural induction and the transfer of A/P positional information from the mesoderm to the overlying dorsal ectoderm. A/P pattern in the ectoderm is gradually refined by signals from non-axial tissues.

1.2A Neural induction.

Neural tissue is induced during gastrulation as mesoderm involutes beneath the ectoderm. Gastrulation begins when deep anterior mesoderm and endoderm begin to move animally on the dorsal side (Fig. 1.4Ab; Gerhart and Keller, 1986). Externally, lateral mesoderm converges dorsally, moves inside at the blastopore lip in the vegetal pole, and extends animally. Involution of the mesoderm occurs to the greatest extent on the dorsal side, but gradually does so ventrally as well, creating a circular blastopore and the future opening of the alimentary canal (Fig. 1.4A; Nieuwkoop and Faber, 1994). Ectoderm also converges and extends dorsally, spreading from the ventral side to cover the outside of the embryo (Fig. 1.4B). As a result of these movements, the ectoderm comes in vertical contact with the involuting organizer mesoderm and neural induction occurs (Fig. 1.4C). To some extent, signals may also pass from the mesoderm through the shared plane of the ectoderm in the form of "planar" signals (reviewed in Doniach, 1993).

As alluded to above, neural induction is effected by dorsalizing signals from the organizer mesoderm. In the ectoderm, BMP4 induces ventral epidermis

and represses dorsal neural fates (Wilson, et al., 1995). As a result of gastrulation, the dorsal ectoderm is exposed to neural inducers/dorsalizing factors noggin (Zimmerman, et al., 1996), chordin (Piccolo, et al., 1996), and Xnr3 (Hansen, et al., 1997) which relieve this repression by binding BMP4 and consequently activating the formation of neural tissue. The sufficiency of BMP4 inhibition for neural induction is demonstrated by experimentally derived constructs that interfere with BMP4 processing (Hawley, et al., 1995) or block signaling by a BMP4 receptor (reviewed in Hemmati-Brivanlou, et al., 1997). Neuralization can also be achieved by dissociating animal cap cells (Grunz, et al., 1989), an effect apparently due to dilution of extracellular BMP4 (Wilson and Hemmati-Brivanlou, 1995) that seems to have a cell contact component as well (Jones, et al., 1986); Sagerström et al., submitted). The choice between epidermal and neural fates is illustrated by the epidermal cytokeratins, which are expressed in the ectoderm at the onset of gastrulation and downregulated following contact with the dorsal mesoderm (Jamrich, et al., 1987). Similarly, epidermal antigens are specified in blastula ectoderm but not in the neurectoderm at the end of gastrulation (Akers, et al., 1986; Jones and Woodland, 1986).

1.2B Anteroposterior patterning of the neurectoderm.

Early ectodermal A/P pattern is expanded greatly during the process of neural induction. The model that provides the foundation for our understanding of A/P patterning of the neurectoderm was formulated many years ago by Nieuwkoop, who implanted folds of uninduced ectoderm into the neural plate (Nieuwkoop, 1952a). The grafts were induced to form neural tissue regardless of their location along the anteroposterior axis (Nieuwkoop, 1952b). The base of the folds formed neural tissue appropriate to the position of the implant, while distal regions differentiated as progressively more anterior neural structures. Fates posterior to the implant were never observed in the folds. Meanwhile, implants in the anterior neural plate formed exclusively anterior neural structures in greater quantities than grafts elsewhere along the axis. Due to the latter observation, it was concluded that a gradient of a single inducer with a source in the posterior could not explain these results because the concentration of the inducer would be lowest in the region that induced the greatest volume of anterior tissue. Rather, Nieuwkoop proposed that an anterior neural inducer, a general "activation" signal, was produced throughout the mesoderm, while a "transformation" signal was generated in posterior mesoderm to respecify

activated tissue to more posterior fates (Nieuwkoop, 1952c). Since posterior tissue is derived from anterior, posterior fates were said to be dominant to anterior. Mechanistically, this activation-transformation model is supported by assaying specification of the ectoderm as gastrulation proceeds. Pattern is established progressively, with a wave of anterior specification passing through the posterior ectoderm as the anterior mesoderm involutes below (Fig. 1.4Cb, white asterisk; Eyal-Giladi, 1954; Sive, et al., 1989). Posterior tissue is later respecified to posterior fates as it comes into contact with posterior mesoderm (Fig. 1.4Cc), a process that can be recapitulated in vitro by conjugation of anterior neurectoderm and posterior mesoderm (Cox, et al., 1995; Kolm, et al., 1997; Sharpe, et al., 1990; Sive, et al., 1989).

1.2C Activation and transformation signals.

Isolation of the molecules that participate in the induction and patterning of the ectoderm further supports the activation-transformation model. As would be expected of an activation signal, neural inducers/BMP antagonists noggin, chordin, and Xnr3 induce neural tissue of anterior character (Hansen, et al., 1997; Lamb, et al., 1993; Sasai, et al., 1994), as do experimental methods that inhibit BMP4 signaling (Frisch, et al., 1998; Hawley, et al., 1995; Wilson, et al., 1997). Meanwhile, candidate transforming factors retinoic acid (Kolm, et al., 1997; Kolm, et al., 1995a; Papalopulu, et al., 1996; Sive, et al., 1991; Sive, et al., 1990), FGF (Cox and Hemmati-Brivanlou, 1995; Kengaku, et al., 1995; Lamb, et al., 1995; Pownall, et al., 1996), and Xwnt-3a or -8 (Fredieu, et al., 1997; McGrew, et al., 1995) inhibit expression of anterior neural markers and induce posterior gene expression. As predicted by Nieuwkoop's model, these factors posteriorize anterior neural tissue and induce posterior markers in uninduced ectoderm, but do not on their own neuralize ectoderm (meaning they do not induce general neural markers). At least in the case of retinoic acid, repression of anterior markers appears to be a direct effect, however, posteriorization is also mediated by the downstream targets of the transforming signals. The RA-responsive gene *HoxD1* (Kolm, 1997) and the FGF-responsive genes *Xcad3* and *HoxA7* (Epstein, et al., 1997; Pownall, et al., 1996) repress anterior fates as assayed by expression of the anterior homeobox gene *otx2*.

A great deal of evidence indicates that retinoic acid acts as an endogenous posteriorizing factor. Retinoic acid effectively inhibits formation of anterior structures in whole embryos due to posteriorizing activity in both the mesoderm

and ectoderm (Ruiz i Altaba, et al., 1991a; Ruiz i Altaba, et al., 1991b; Sive and Cheng, 1991; Sive, et al., 1990). Several different retinoids are present in gastrula embryos, as are the receptors and binding proteins necessary for signaling (reviewed in Kolm, et al., 1997). Dominant negative retinoic acid receptors demonstrate that retinoid signaling is necessary for appropriate patterning of the dorsal ectoderm (Blumberg, et al., 1997; Kolm, et al., 1997). Furthermore, retinoic acid response elements have been identified flanking many genes, most importantly, posteriorly expressed *Hox* genes (reviewed in Marshall, et al., 1996), while an element that mediates retinoic acid repression has been identified upstream of *otx2* (Simeone, et al., 1995).

1.2D Building an A/P axis.

Activation-transformation provides a well-tested and plausible mechanism to establish broad A/P ectodermal pattern. However, a two-step model is not sufficient to explain the complex, overlapping patterns of gene expression that occur in the neurectoderm (Fig. 1.5). The A/P axis, laid down by activation-transformation, must be further refined by other signaling events. In mice, chick, and fish, surgical or genetic removal of the organizer equivalent does not abolish induction and A/P patterning of the neurectoderm (Ang, et al., 1994; Psychoyos, et al., 1996; Shih, et al., 1996; Weinstein, et al., 1994), indicating that additional signals must arise from a source other that the organizer.

In posterior ectoderm, this problem is illustrated by *Xenopus* explant analysis. Although posterior organizer mesoderm can respecify explants of anterior dorsal ectoderm to more posterior fates (Cox and Hemmati-Brivanlou, 1995; Kolm, et al., 1997; Sharpe and Gurdon, 1990; Sive, et al., 1989), only posterior midline markers are efficiently induced by this tissue (Kolm, et al., 1997). Rather, there is significant evidence to suggest that the paraxial mesoderm, arising laterally in the marginal zone, is the source of posterior lateral inducers (Bang, et al., 1997; Kolm, et al., 1997; Muhr, et al., 1997; Woo and Fraser, 1997). As stated above, these tissues are normally apposed during gastrulation. Known posteriorizing signals are clearly produced by lateral mesoderm, as its activity is blocked by a dominant negative retinoic acid receptor (Kolm, et al., 1997). However, other elements of lateral mesodermal signaling are not mimicked by or dependent upon retinoic acid, FGF, or wnt activity, indicating that lateral posterior induction involves additional, unknown factors (Bang, et al., 1997; Muhr, et al., 1997). Further elaboration of this mediolateral pattern in the

neurectoderm, which will become the D/V axis of the neural tube, also requires intraectodermal signaling from the non-neural ectoderm and midline of the neural plate (reviewed in Tanabe, et al., 1996). Clearly this is not a simple, two-step process.

The limitations of organizer-derived, activation-transformation signaling is even more obvious in the anterior ectoderm. According to the two-step model, anterior fates are simply induced at the time of neural induction as a "dorsoanterior" entity, a term that was coined because treatments that affect formation of the dorsal mesoderm also alter anterior specification (Kao, et al., 1988; Stewart and Gerhart, 1990). The link between dorsal and anterior induction has meant that the possibility of anterior-specific inducing signals has been relatively ignored. However, existing data indicate that additional tissue interactions are required to pattern the anterior neural plate. First of all, genetic or surgical ablation of the prechordal plate, the anterior axial mesoderm, in chick, fish, and mouse does not lead to massive deletions of head structures (Belo, et al., 1998b; Pera, et al., 1997; Schier, et al., 1997). Rather, these embryos exhibit midline/ ventral forebrain deficiencies, apparent as cyclopia and mediolateral narrowing of anterior neurectoderm. Thus, in the anterior as in the posterior, axial mesoderm is required for formation of the midline; other tissues must induce remaining anterior structures. Second, although *Xenopus* early gastrula organizer grafts can induce complete secondary axes (Spemann, et al., 1924), there are indications that this effect is not due entirely to signals from the organizer mesoderm. With the exception of treatments that activate ectopic *β-catenin* or *siamois* expression to establish a new dorsal center, organizer-derived and localized factors are unable to induce complete secondary axes (reviewed in Bouwmeester, et al., 1997). The requirement of an additional signaling center is even more obvious in mouse. Although mouse node transplantation leads to posterior duplications, forebrain tissue is never induced in secondary axes (Beddington, 1994). A similar effect is noted when chicken *wnt8C* is overexpressed in transgenic mouse embryos (Pöpperl, et al., 1997).

What is the source of anterior inducers, and how do these signals coordinate head formation? Before considering emerging answers to this question, it is first necessary to be familiar with the derivatives of the anterior ectoderm. The correct localization of these structures to positions within the anterior field is the final outcome of pattern created by these signals.

1.3 The anterior neurectoderm

At the end of gastrulation, the neural plate is divided into distinct anterior and posterior domains (Bally-Cuif, et al., 1997); Sagerström and Sive, in preparation). The anterior ectoderm occupies a large region of the neurectoderm (Fig. 1.6A) and will itself go on to be subdivided into midbrain and forebrain structures, and, in *Xenopus*, the adhesive organ known as the cement gland.

1.3A Anterior neural derivatives.

The embryonic hindbrain, a posterior structure, is an intensely studied region of the neurectoderm, in part due to its obvious segmentation in units known as rhombomeres. The evolutionarily conserved *Hox* genes, which are regulated by retinoic acid and segmentally expressed in the rhombomeres, specify positional identity in the hindbrain (reviewed in Lumsden, et al., 1996). However, *Hox* genes are not expressed anterior to the hindbrain, thus regional identity in the anterior neurectoderm is regulated through an independent mechanism and has been only minimally addressed. This domain will go on to give rise to the forebrain (telencephalon and diencephalon) and midbrain (mesencephalon) of the adult brain. How and when these divisions are established in the anterior neurectoderm is not clear. Comparison of gene expression patterns, obvious morphological landmarks, and axon tracts suggests that the anterior neural plate is also segmentally organized into transverse (Figdor, et al., 1993; Puelles, et al., 1993; Rubenstein, et al., 1994) and longitudinal (Rubenstein, et al., 1994) domains, dubbed neuromeres or prosomeres. However, these units may not be functionally relevant to regionalization within the brain since retroviral lineage analysis demonstrates that neuromeres are not clonally restricted populations (Golden, et al., 1996; Szele, et al., 1996). Furthermore, neuromeres form relatively late during brain morphogenesis and are not helpful to an analysis of early determination events. Thus, an understanding of earliest pattern formation in the anterior neural plate is best achieved by studying the regulation and function of genes expressed in prospective anterior regions of the brain according to the early neurula fate map (Fig. 1.6A; Eagleson, et al., 1990). For example, expression of zebrafish *opl* and *fkh5*, which mark telencephalon and diencephalon, respectively, indicate that the major subdivisions of the forebrain have been induced by mid-gastrula (Grinblat et al., in press).

1.3B The cement gland.

Along with the midbrain and forebrain, the anterior neurectoderm of a frog embryo gives rise to an additional, non-neural structure, the cement gland. The cement gland is a pigmented, conical shaped structure that secretes a sticky mucus that allows the tadpole to attach to a solid substrate before it can swim well or feed (Nieuwkoop and Faber, 1994). The cement gland forms from ectoderm overlying the positional equivalent of the mammalian chin, at the dorsal-ventral border and anterior-most extent of the neurectoderm (Fig. 1.6B). This position at the intersection of the axes and several other features make the cement gland a convenient model for studying ectodermal patterning (reviewed in Sive and Bradley, 1996). First, the cement gland is specified during gastrulation by the same signals that induce anterior neural tissue, such as noggin and chordin (Lamb, et al., 1993; Sasai, et al., 1995). Cement gland determination therefore illustrates the problem of pattern formation within the anterior ectodermal domain. Second, neighboring tissues that stimulate and repress cement gland formation have been characterized. The anterior neural plate, head endoderm, and prechordal plate stimulate cement gland formation, while posterior mesoderm and ventral ectoderm inhibit cement gland (Bradley, et al., 1996; Drysdale, et al., 1993; Sive, et al., 1989; Yamada, 1938). Third, the cement gland is a fairly simple structure that differentiates early and is easy to score morphologically by its pigment and stickiness (Nieuwkoop and Faber, 1994). Fourth, the molecular markers *XCG* and *XAG*, which are expressed exclusively in the cement gland and associated non-neural ectoderm, make it possible to assay specifically for cement gland determination (Jamrich, et al., 1989; Sive, et al., 1989). For these reasons, the cement gland provides an ideal system in which to study the tissue interactions and regulatory molecules that induce and regionalize the anterior ectoderm. For example, what makes anterior neural determination different from cement gland?

1.4 Getting a head.

The above examination of the requirements for anterior induction suggested that multiple signals were involved in creating pattern evident in the diverse structures of the anterior neural plate. Characterization of these events indicates that anterior ectodermal pattern is refined gradually by successive inductive interactions. A gradient of BMP4 inhibitors activate an initial anterior

pattern onto which signals from the endoderm are superimposed. Meanwhile, organizing centers within the ectoderm promote regional identities.

1.4A A BMP4 gradient.

Anterior neural fates are specified by BMP4 inhibitors, but does this induction create pattern within the anterior domain? One possibility that is still consistent with Nieuwkoop's observations is that neural inducers are not evenly distributed throughout the anterior ectoderm. Indeed, graded levels of BMP4 activity can induce the appropriate dorsal to ventral range of anterior markers in isolated ectoderm (Knecht, et al., 1997; Wilson, et al., 1997). In these assays, high levels of BMP4 signaling in the ectoderm induced epidermis, intermediate levels induced cement gland, and no BMP4 resulted in anterior neural fates. A similar effect was obtained by varying levels of the BMP4 signaling intermediate Smad1 (Wilson, et al., 1997). These experiments have led to the proposal that diffusion of noggin and chordin from the mesoderm creates a gradient of BMP4/activated Smad1 in the ectoderm to pattern the anterior of the embryo (Knecht and Harland, 1997; Wilson, et al., 1997). By analogy to activin responsive Smad2, which binds DNA with a winged-helix transcription factor FAST-1 (Chen, et al., 1996), different levels of activated Smad1 could interact with other regionally-restricted transcription factors to specify fate, though this has not been explored. Although an intriguing mechanism, a BMP4 gradient probably represents only the initiation of pattern and a prerequisite for additional anterior signaling events that are not taken into account by this model.

1.4B The anterior endoderm: source of anterior signals.

As indicated above, axial mesoderm is not required for the formation of most anterior ectodermal structures. The neural inducers noggin (Knecht and Harland, 1997) and chordin (Sasai, et al., 1995) are expressed in a domain broader than the prechordal plate and could provide neural inducing activity. But where do additional head inducers arise? Analysis of cement gland induction in *Xenopus* was one of the first indications that the endoderm might play a role. At the end of gastrulation, the anterior endoderm lies under the cement gland primordium and marks the anterior-most extent of dorsal involution. This tissue will later give rise to the liver and foregut (Nieuwkoop and Faber, 1994). Although isolated anterior endoderm cannot itself induce neural tissue or cement gland in naïve ectoderm (Sive, et al., 1989), it augments cement gland

formation in induced ectoderm (Bradley, et al., 1996). Similarly, the anterior endoderm of chick embryos can anteriorize posterior neural tissue but does not induce neural tissue de novo (Foley, et al., 1997). These explant analyses are corroborated by in vivo examination of analogous tissue interactions in mouse embryos. The visceral endoderm on the future anterior side of the embryo, also fated to form foregut (Thomas, et al., 1998) and opposite the site of node (mouse organizer) formation, is marked by specific expression of two homeobox genes, *Hex* and *Hesx/Rpx* (Hermesz, et al., 1996; Thomas, et al., 1996; Thomas, et al., 1998). This endoderm is required to induce *Hesx* expression in the anterior ectoderm (Thomas and Beddington, 1996), however, ectodermal *Hesx* expression does not appear to commence until the end of gastrulation (Hermesz, et al., 1996; Thomas and Beddington, 1996), when neural inducing signals are likely to first reach the anterior ectoderm. Together with tissue recombinants, these observations suggest that the anterior endoderm is a source of anterior inducers that act upon ectoderm in which a BMP4 gradient is already defined.

The critical importance of the endoderm for formation of anterior derivatives has been demonstrated directly in mouse. The anterior visceral endoderm expresses a number of different markers in addition to *Hex* and *Hesx*, including two broadly expressed genes, *otx2* and *nodal* . Mutations in these loci produce loss of head structures (Ang, et al., 1996; Matsuo, et al., 1995) and a failure to gastrulate (Conlon, et al., 1994), respectively. Despite these dissimilar phenotypes, in mice chimeric for *otx2* or *nodal* mutant cells, wildtype cells in the visceral endoderm are sufficient to rescue formation of anterior neural tissue (Rhinn, et al., 1998; Varlet, et al., 1997). Anterior induction by the endoderm is thus essential for anterior neural development.

The anterior inducing activity of the endoderm appears to be mediated by the secreted factors cerberus (Bouwmeester, et al., 1996) and dickkopf (Glinka, et al., 1998), which are expressed in the anterior endoderm of frogs and mice (Belo, et al., 1998a; Biben, et al., 1998). These proteins activate anterior neural and cement gland fates even in uninduced ectoderm. This activity may not be apparent in isolated endoderm because cerberus and dickkopf expression could require dorsalizing factors to maintain their expression (Bouwmeester, et al., 1996), although the physical separation of anterior endoderm and node in mice would argue against this hypothesis. Alternatively, as suggested above, these factors may be expressed at low enough levels in vivo that their effect is revealed only after prior suppression of BMP signaling in responding ectoderm. In either

case, cerberus activity is inhibited by BMP4, and may represent an anti-BMP4 effect (Bouwmeester, et al., 1996).

The anterior endoderm may also promote anterior fates is by producing signals that limit the spread of posteriorizing factors. Such signals have been proposed to restrict the effects of retinoic acid to the posterior of the embryo (Conlon, 1995), but no retinoid inhibiting factor has yet been identified. However, this possibility seems to have been realized by the identification of frz-b1, a secreted, truncated wnt receptor that inhibits wnt signaling and that is expressed in the anterior endoderm (Leyns, et al., 1997; Wang, et al., 1997). Like retinoic acid, wnts have posteriorizing activity (Christian and Moon, 1993; Fredieu, et al., 1997; McGrew, et al., 1995) that is probably manifested in Xwnt8, which is expressed in the ventrolateral marginal zone (Smith and Harland, 1991), a source of posterior inducers (see above). Intriguingly, frz-b1 overexpression produces embryos with big heads, but frz-b1 on its own does not have any activity in animal caps (Leyns, et al., 1997; Wang, et al., 1997) and does not induce heads in UV ventralized embryos (Wang, et al., 1997). This suggests that frz-b1 alters the response to inducers, perhaps by preventing the spread of posterior signals and reinforcing anterior specification produced by neural induction. This could explain why a dominant negative Xwnt8, which has the same activity as frz-b1 (Hoppler, et al., 1996), leads to head formation on incomplete secondary axes produced by inhibition of the BMP4 receptor (Glinka, et al., 1997). cerberus and dickkopf have also been shown to inhibit wnt signaling, however, it is not clear whether this is a direct effect or a secondary consequence of induction of factors such as frz-b1 (Glinka, et al., 1998; Glinka, et al., 1997).

1.4C Intraectodermal signaling centers.

Although signals from the underlying endoderm and mesoderm clearly induce and pattern the anterior neurectoderm, additional signaling sources within the ectoderm are required to further refine this pattern. One example of this is the midbrain/hindbrain boundary, which organizes neighboring midbrain and hindbrain regions (reviewed in Bally-Cuif, et al., 1995). This signaling center is established at the boundary of anterior (*otx2*; Millet, et al., 1996) and posterior (*gbx-2*; Wassarman, et al., 1997) gene expression and results in progressively restricted expression of *wnt1*, *FGF8*, *en-1*, and *en-2* in overlapping patterns within this domain (Bally-Cuif and Wassef, 1995). Correct localization and interplay between these factors is essential for midbrain and hindbrain patterning. For

example, *wnt1* and *otx2* expression domains become mutually exclusive (Bally-Cuif, et al., 1995), although both are required for the formation of the midbrain/hindbrain region and appropriate *en*-2 expression (Ang, et al., 1996; McMahon, et al., 1990; Rhinn, et al., 1998). Meanwhile, FGF8 is secreted from the anterior hindbrain and regulates the pattern and polarity of the midbrain, as ectopic expression of FGF8 results in induction of posterior midbrain fates (Crossley, et al., 1996; Lee, et al., 1997).

Another intraectodermal signaling center was recently described in zebrafish. This forebrain organizer consists of a row of cells in the anterior neural plate that are required for forebrain formation (Houart, et al., 1998). These cells exert a patterning influence in the anterior ectoderm since they affect the fate of cells throughout the presumptive forebrain and can induce ectopic forebrain when transplanted (Houart, et al., 1998). Although the signals responsible for this activity are not known, it is becoming clear that signaling within the neurectoderm after induction has occurred is important to create regional identity out of pattern.

1.5 Defining anterior: the homeobox gene *otx2*.

Gene expression in the neurectoderm is a reflection of the degree of induction and patterning that has taken place. However, positional markers are also the factors that will ultimately regulate differentiation of the nervous system later in development. How do distinct structures form at precise locations along the axes? This is the real question of pattern formation. Although most anterior neural markers are first expressed after gastrulation is complete, several genes have been identified that exhibit dynamic and restricted expression within the anterior neural plate as pattern is being established (Fig. 1.5). Of these, the homeobox gene *otx2* seems to be of critical importance for determination of anterior fates. During gastrulation, all presumptive anterior neurectodermal derivatives, including the cement gland primordium, are linked by their common expression of *otx2* (Blitz and Cho, 1995; Pannese, et al., 1995). *otx2* expression follows the anterior extent of neural induction in the ectoderm as gastrulation proceeds and is an early and general marker of anterior position in all three tissue layers of the embryo (Blitz and Cho, 1995; Pannese, et al., 1995).

1.5A An evolutionarily conserved regulator of anterior fates.

otx2 along with *otx1* are the vertebrate homologues of *Drosophila orthodenticle* (*otd*; Simeone, et al., 1992). *otd* is expressed in a circumferential anterior stripe in the *Drosophila* embryo, overlapping with the domains of two other genes, *emptyspiracles* (*ems*) and *buttonhead* (*btd*) in a staggered fashion (Cohen, et al., 1990). Together, these three genes are required for the formation of particular segments in the embryonic head and have been dubbed "anterior gap genes" due to the deletion, rather than transformation, of anterior segments in their absence (Cohen and Jurgens, 1990; Finkelstein, et al., 1990). *otd* is also required for the formation of the anterior-most neuromere of the embryonic brain, the protocerebrum (Hirth, et al., 1995; Younossi-Hartenstein, et al., 1997), and for development of neuroblasts in the midline of the ventral nerve cord (Finkelstein, et al., 1990; Klämbt, et al., 1991).

Vertebrate homologues of *ems* (*emx1* and *emx2*) have also been identified, and it has been shown that, similar to their invertebrate counterparts, *otx2*, *otx1*, *emx2*, and *emx1* are expressed in overlapping anterior domains in mouse (Simeone, et al., 1992) and frog (Kablar, et al., 1996). However, the vertebrate genes are nested and temporally sequential in their expression; *otx2* is the earliest and most broadly expressed, while *otx1*, *emx2* and *emx1* exhibit progressively later onset and more restricted expression patterns (Simeone, et al., 1992). As would be expected from these expression profiles and *otd* mutant flies, mice with *otx2* loss of function mutations exhibit the most dramatic phenotype of the four genes, lacking all head structures rostral to rhombomere 3 in the hindbrain (Acampora, et al., 1995; Ang, et al., 1996; Matsuo, et al., 1995). Meanwhile, mutations in *otx1*, *emx2*, and *emx1* produce more subtle effects, such as the loss of a particular structure in the brain (Acampora, et al., 1996; Pellegrini, et al., 1996; Qiu, et al., 1996). This demonstrates that *otx2* alone plays a central, early role in defining anterior fate in the embryo. As described above, recent analysis of mice chimeric for $otx2^{-/-}$ cells indicates that anterior deletions are due to a requirement for *otx2* in the endoderm (Rhinn, et al., 1998). However, the rescued neurectoderm was not appropriately patterned in the chimeras, indicating a role for *otx2* in the remaining mutant tissue: the neurectoderm and/or the underlying mesendoderm, which induces *otx2* expression in the ectoderm (Ang, et al., 1994). *otx2* is required specifically in the ectoderm to establish the mid/hindbrain boundary (see above; Rhinn, et al., 1998), however, beyond this region, the necessity and function of *otx2* in the neurectoderm is not clear.

Gain of function analysis in *Xenopus* embryos, however, suggests that *otx2* is involved in determining anterior neurectodermal fate. Overexpression of *otx2* RNA in the ectoderm leads to the formation of ectopic cement gland, neural tissue, and mesoderm (Blitz and Cho, 1995; Pannese, et al., 1995). Although this does not discriminate between a direct effect of *otx2* in the ectoderm or a secondary effect of ectopic anterior mesoderm, we have demonstrated that *otx2* directly regulates cement gland formation, indicating that *otx2* specifies regional identity in the ectoderm (Chapter 2; Gammill, et al., 1997). However, the activity of *otx2* has not been examined with respect to induction and patterning within the neural plate (see Chapter 4). Interestingly, *otd* overexpression in *Drosophila* also induces the formation of ectopic anterior neural structures, suggesting that regulation of neurectodermal fates is a conserved function of these genes (Leuzinger, et al., 1998).

The critical importance of *otx2* and *otd* function for anterior determination is emphasized by their evolutionary conservation. Beyond the similarity of their expression patterns and gain and loss of function phenotypes, *otd* and *otx* genes are largely functionally equivalent (Acampora, et al., 1998; Leuzinger, et al., 1998). Despite a low degree of sequence similarity outside of the homeodomain, *otx2* expression very effectively rescues the mutant phenotype of *otd* flies (Leuzinger, et al., 1998). Likewise, *otd* expression in *otx1* mutant mice rescues a number, but not all, of the loss of function phenotypes (Acampora, et al., 1998). This suggests that at least some aspects of the genetics of anterior head formation may be conserved between invertebrates and vertebrates.

What can we learn, then, from *Drosophila* about *otx2*? As in vertebrates, *Drosophila* head formation is regulated differently from patterning and segmentation in the trunk and is less well understood (Finkelstein, et al., 1994). Even the established regulatory network between *wingless* (*wg*), *hedgehog* (*hh*), and *engrailed* (*en*) is different during segmentation of the head. In the trunk, wg maintains en expression in neighboring segments, while en activates wg though secretion of hh (reviewed in Cadigan, et al., 1997). In the head, however, wg inhibits and restricts en and hh expression (Gallitano-Mendel, et al., 1997). Within this network, otd inhibits wg, maintains hh (and may also be activated by hh), and appears to act through en to specify medial segment identity (Royet, et al., 1996; Royet, et al., 1995). Strikingly, vertebrate homologues of these genes seem to be involved in a similar anterior network in the mid-brain/hindbrain region, discussed above. otd also activates expression of the proneural genes,

consistent with its ability to activate the formation of ectopic neuroblasts (Younossi-Hartenstein, et al., 1997). Could *otx2* regulate neuronal determination as well?

1.5B The paired class homeodomain.

Interestingly, *otx2* is a member of the paired class of homeobox genes. Unlike other homeodomains, which bind DNA as monomers, paired class molecules bind to palindromic TAAT sites as dimers (Wilson, 1993). Examination of the paired class protein Mix.1 has demonstrated that it can both homodimerize and heterodimerize in vitro with other paired class members goosecoid and siamois (Mead, et al., 1996; Wilson, 1993). This potential for combinatorial diversity also exists within the anterior neural plate. Several members of this family have been identified and many, including *otx2* (Blitz and Cho, 1995; Pannese, et al., 1995) and *otx1* (Kablar, et al., 1996), *Xanf* (*Hesx/Rpx* in mouse; Zaraisky, et al., 1992), *goosecoid* (Cho, et al., 1991), *Rx1* (Mathers, et al., 1997), *Prx1* (Takahashi, et al., 1998), *Otlx2* (Mucchielli, et al., 1997), and *Ptx1* (Lamoneirie, et al., 1996; Szeto, et al., 1996) are expressed in anterior neurectoderm. The combination of these factors expressed in a particular region could determine which target genes are activated or repressed and therefore regulate regional identity. goosecoid and Xanf-1 in particular contain a repressor domain and could modulate the activity of other paired class members through heterodimerization (Mailhos, et al., 1998; Smith, et al., 1996). Unfortunately, the potential for interaction essentially has not been addressed. For example, although coexpression of *otx2* and *otx1* suppresses the ability of *otx2* to induce ectopic cement gland formation, this effect has not been characterized in detail (Andreazzoli, et al., 1997). This network could be further expanded by interaction of paired class proteins with LIM homeodomain proteins (Bach, et al., 1997).

1.6 Does *otx2* regulate anterior ectodermal patterning?

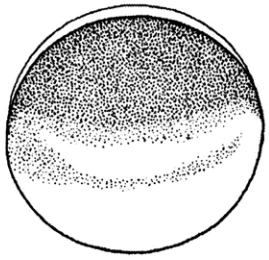
The past few years have resulted in tremendous progress in our understanding of how pattern is established within the ectoderm. But how does this pattern regulate the formation of particular structures at appropriate locations within the neurectoderm? Little has been done to explore this connection. However, in *Xenopus*, studying the formation of the cement gland versus anterior neural tissue provides a convenient system for considering this problem. As discussed above, both are induced by anterior signaling and both

express *otx2*. What makes the regions of the presumptive cement gland and anterior neural tissue molecularly unique so that they give rise to such distinctly different outcomes? Since *otx2* can induce both structures ectopically, it seemed plausible that regulation of *otx2* might be central to this problem. Analyzing the activity of *otx2* has allowed me to pose several questions: Does *otx2* actually control regional identity in the ectoderm? What modulates *otx2* activity to give rise to cement gland or neural fates? How are anterior identities maintained and protected from posteriorizing influences, and is *otx2* required in the ectoderm for this process?

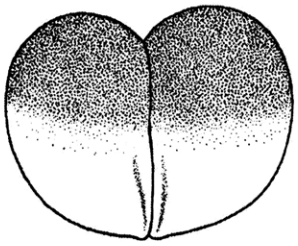
In this thesis, I have examined the role of *otx2* during anterior ectodermal patterning. Using a hormone-inducible *otx2*-glucocorticoid receptor fusion, in Chapter 2 (published as Gammill and Sive, 1997), I demonstrate that the cement gland markers, *XCG* and *XAG*, and the endogenous *otx2* gene are targets of *otx2* signaling in the ectoderm, with *XCG* a direct target. The ability of *otx2* to induce cement gland is spatially limited to the ventrolateral ectoderm of the embryo and temporally limited after mid-gastrula stages. Chapter 3 examines the molecular basis for the ventrolateral restriction of cement gland induction, demonstrating a correlation between BMP4 and cement gland formation. In chapter 4, the effect of *otx2* on ectodermal patterning is addressed. *otx2* promotes anterior and inhibits posterior and ventral gene expression, even in isolated ectoderm. A dominant negative *otx2* is also described. Experiments designed to assess the role of retinoic acid as a posteriorizing factor and a characterization of changes in sensitivity to retinoic acid are contained in Appendix A, while Appendix B provides an overview of a collaboration comparing epidermal specification in frog and in fish.

Note: A modified version of this section will be written as a review article.

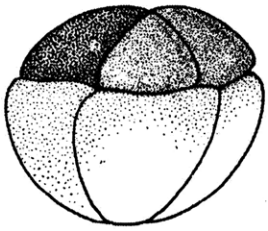
Figure 1.1 Selected stages of *Xenopus laevis* early development.
Drawings of embryos staged from fertilization at zero hours (top left) to hatching at 32.5 hours (bottom) are shown in lateral (fertilization to stage 8) and dorsal (stage 10 to stage 28) views. Taken from Nieuwkoop (1994), *Normal tables of Xenopus laevis (Daudin)*.



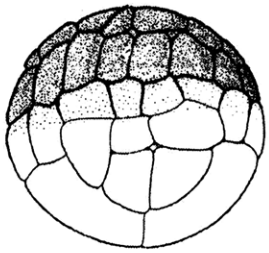
Fertilization
0 hrs



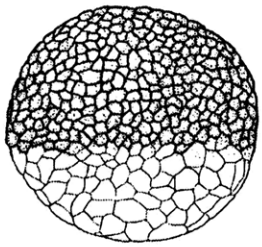
stage 2
1.5 hrs



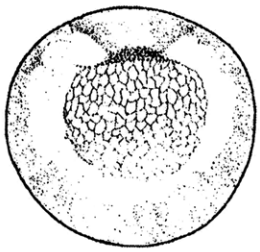
stage 4
2.25 hrs



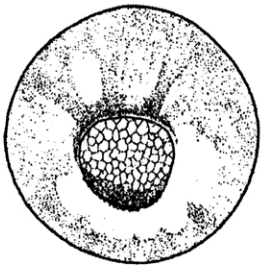
stage 6.5
3.5 hrs



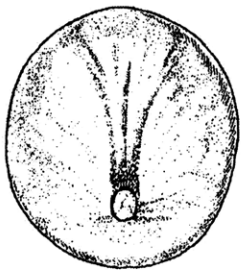
stage 8
5 hrs



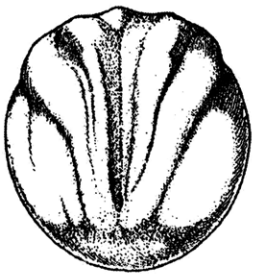
stage 10
9 hrs



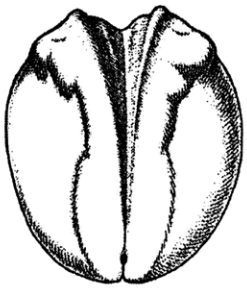
stage 11.5
12.5 hrs



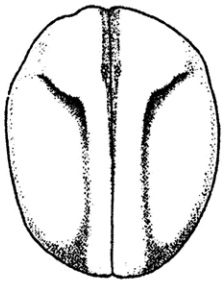
stage 12.5
14.25 hrs



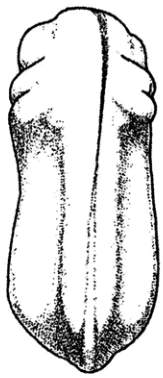
stage 14
16.25 hrs



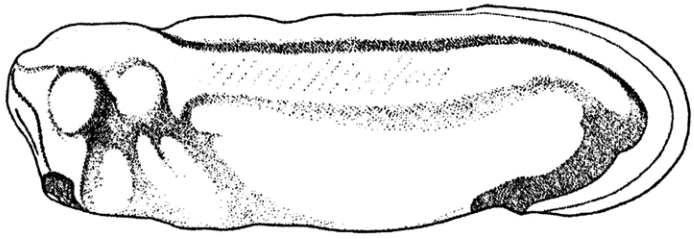
stage 16
18.25 hrs



stage 19
20.75 hrs



stage 23
24.75 hrs



stage 28
32.5 hrs

Figure 1.2 Summary of models for early pattern formation.
(A) At fertilization, the egg contains a pigmented animal hemisphere (future ectoderm; light grey) and yolky vegetal hemisphere (future endoderm; white). Sperm entry initiates a rotation of the cortical cytoplasm, including material localized in the vegetal cortex (dark grey oval), **(B)** 30° away from the site of fertilization to define the dorsal side. Some component of the vegetal cortex stabilizes β-catenin (dots) dorsally ("D"), leading to its accumulation in dorsal nuclei of cells in the animal and vegetal hemispheres by the 32-cell stage. **(C)** β-catenin stabilization in the animal hemisphere establishes asymmetry important for later anterior ("A")-posterior ("P") pattern in the ectoderm. At the same time, signaling from the vegetal hemisphere (↑) and maternally localized determinants induce mesoderm (hatched dark grey) around the circumference of the embryo ("marginal zone"). The overlap of β-catenin and mesoderm determination defines the organizer ("O"). **(D)** Dorsalizing signals (black arrows) from the organizer modify the ectoderm, mesoderm, and endoderm. In the ectoderm, dorsalization along with β-catenin appear to alter the competence to respond to neural inducers (stippling) and create anteroposterior pattern in the ectoderm. In the mesoderm, it has been proposed that dorsalizing factors and opposing ventralizing factors (white arrow) pattern the mesoderm to a dorsal (darkest grey) to ventral (lightest grey) range of fates. **(E)** However, this model does not explain the organization apparent in the fate map, which exhibits a large degree of anteroposterior pattern in the marginal zone and organizer.

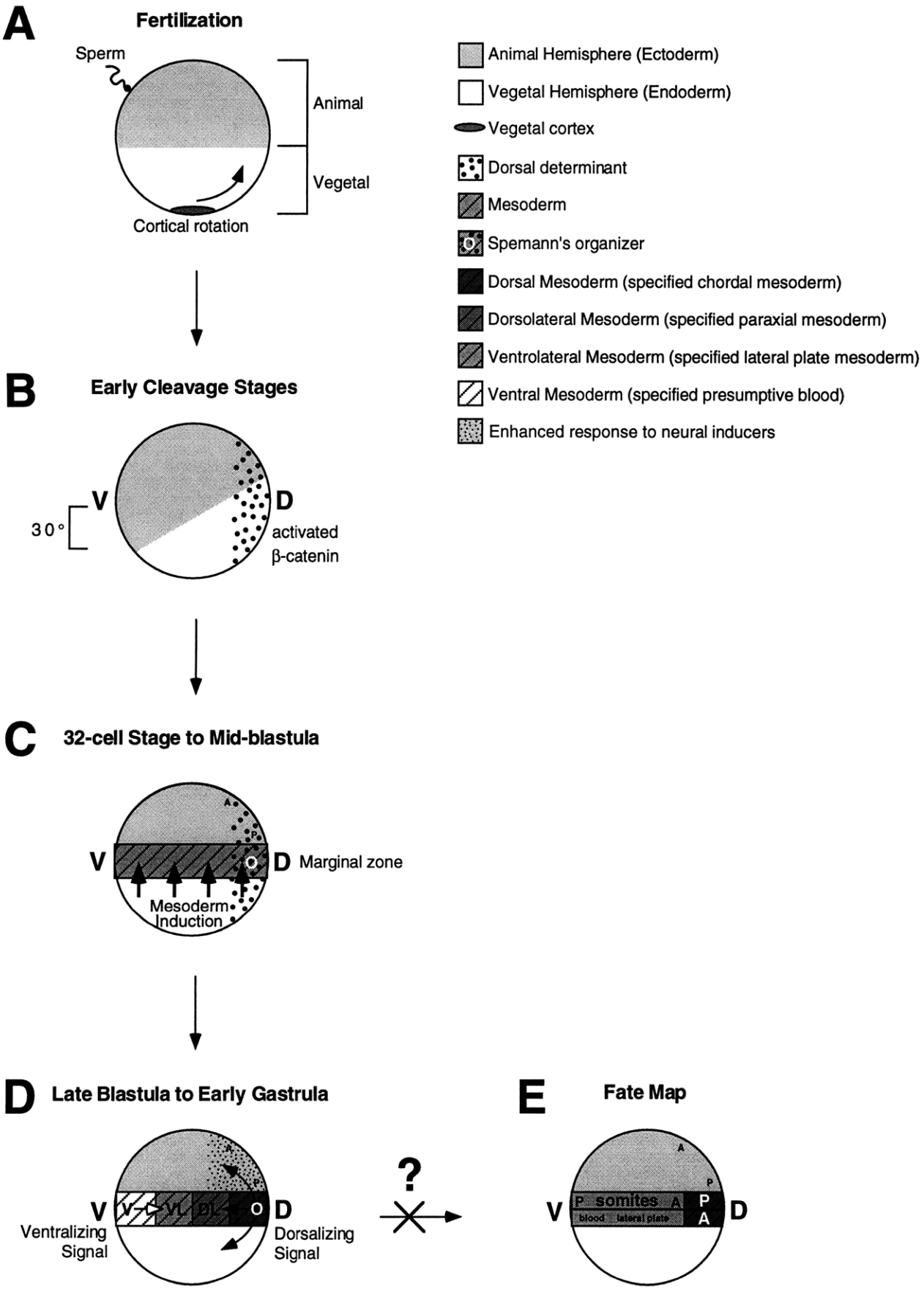

A
Fertilization
Sperm
Animal
Vegetal
Cortical rotation
B
Early Cleavage Stages
V
D
30°
activated
β-catenin
C
32-cell Stage to Mid-blastula
A
V
D
Marginal zone
Mesoderm
Induction
D
Late Blastula to Early Gastrula
V
D
Ventralizing
Signal
Dorsalizing
Signal
?
E
Fate Map
A
P
V
somites
blood
lateral plate
P
A
D
Animal Hemisphere (Ectoderm)
Vegetal Hemisphere (Endoderm)
Vegetal cortex
Dorsal determinant
Mesoderm
Spemann's organizer
Dorsal Mesoderm (specified chordal mesoderm)
Dorsolateral Mesoderm (specified paraxial mesoderm)
Ventrolateral Mesoderm (specified lateral plate mesoderm)
Ventral Mesoderm (specified presumptive blood)
Enhanced response to neural inducers

Figure 1.3 Pattern in the organizer.
As a result of early patterning events, the organizer region exhibits restricted expression of secreted factors (A) and transcription factors (B) within the mesoderm (darkest grey) and endoderm (white). Expression of these factors defines posterior ("P") trunk inducing (stippled) and anterior ("A") head inducing (hatched) domains of the organizer. **(A)** Expression of secreted factors within the early gastrula organizer. Posterior: *eFGF* (Isaacs, et al., 1992), *noggin* (Smith and Harland, 1992), and *chordin* (Sasai, et al., 1994). Anterior: *Xnr3* (Darras, et al., 1997; Smith, et al., 1995), *noggin*, and *chordin*. Endoderm: *Xnr3*, *cerberus* (Bouwmeester, et al., 1996), and *dickkopf* (Glinka, et al., 1998). **(B)** Expression of transcription factors within the early gastrula organizer. Posterior: *Xnot2* (von Dassow, et al., 1993; Zoltewicz and Gerhart, 1997) and *Xbra* (Smith, et al., 1991; Zoltewicz and Gerhart, 1997). Anterior: *Xlim-1* (Taira, et al., 1992), *goosecoid* (Cho, et al., 1991; Zoltewicz and Gerhart, 1997), *siamois* (Lemaire, et al., 1995), and *otx2* (Blitz and Cho, 1995; Pannese, et al., 1995). Endoderm: *Xlim-1*, *siamois*, *otx2*.

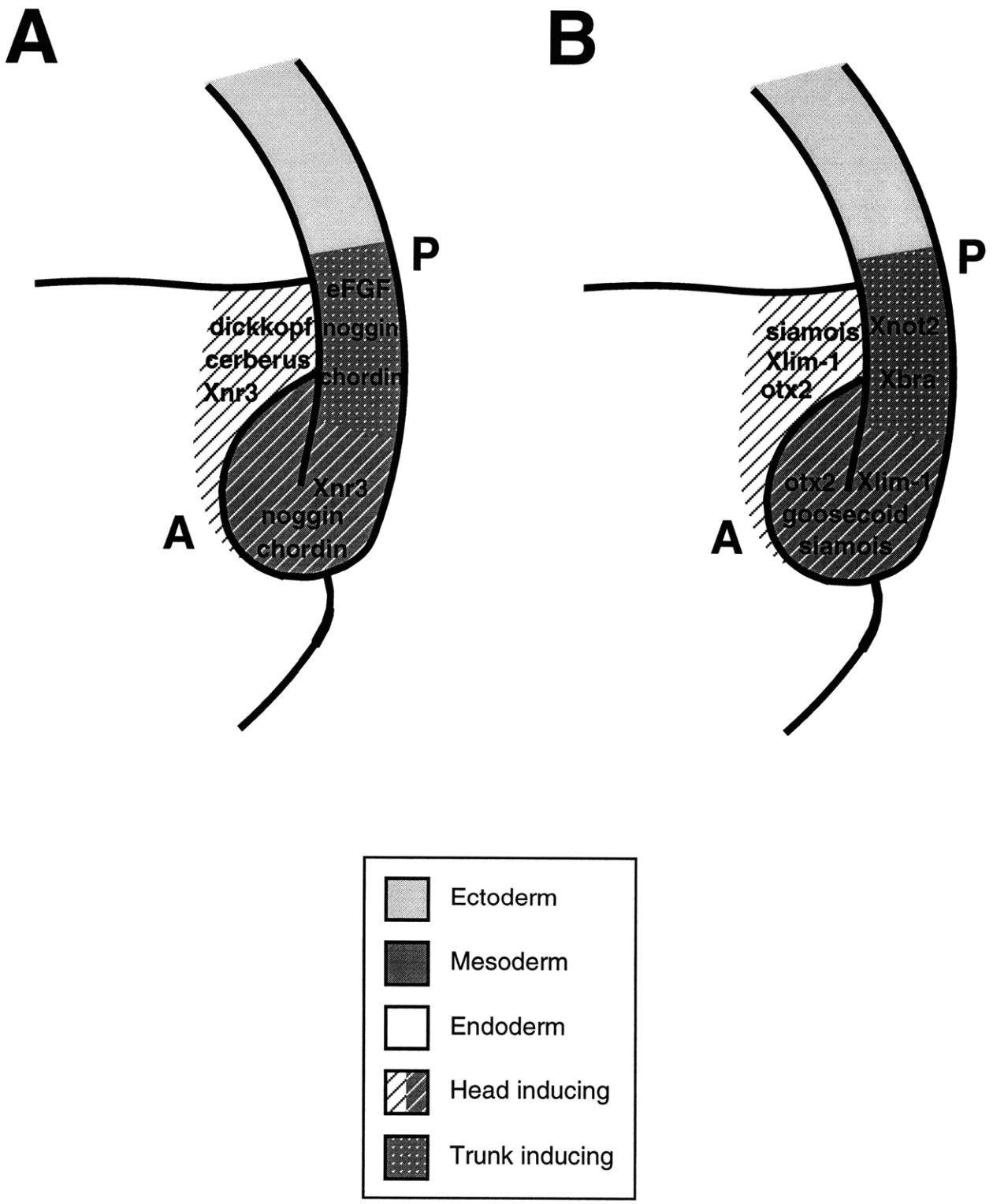
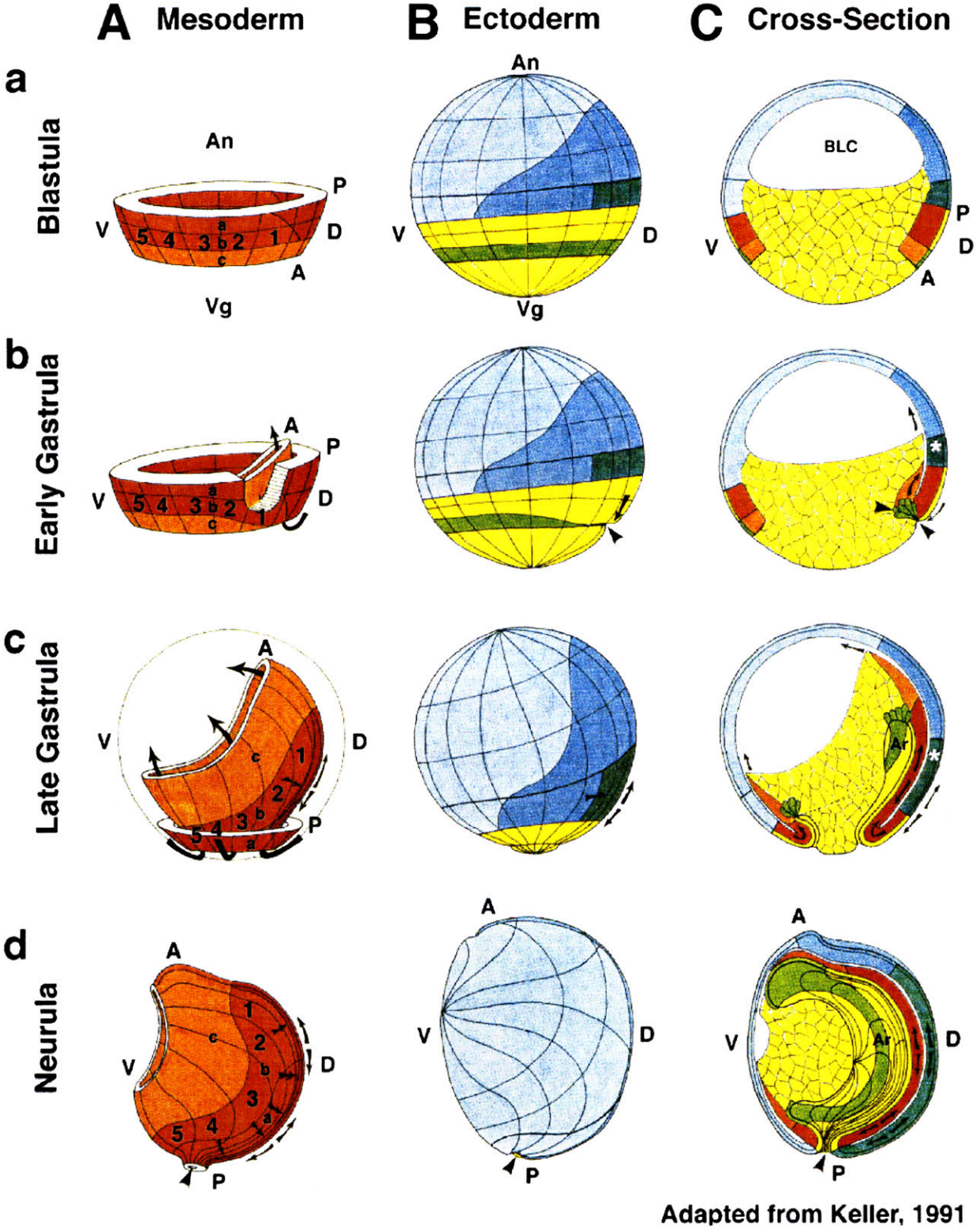

A
P
eFGF
dickkopf
noggin
cerberus
chordin
Xnr3
Xnr3
noggin
chordin
A
B
P
siamois
Xnot2
Xlim-1
otx2
Xbra
otx2 / Xlim-1
goosecoid
siamois
A
Ectoderm
Mesoderm
Endoderm
Head inducing
Trunk inducing

Figure 1.4 Gastrulation movements.

Adapted from Keller, 1991. Cell movements during gastrulation in the mesoderm (A), ectoderm (B), and in cross section (C) at blastula (a), early gastrula (b), late gastrula (c), and neurula stages (d). **(A)** At blastula stage, the dorsal ("D") mesoderm is organized along an inverted version of the future anterior ("A")-posterior ("P") axis. The remainder of the marginal zone has an apparent dorsal-ventral ("V") organization (1-5), and an animal ("An")-vegetal ("Vg") axis (a, b ,c). Involution of the mesoderm at the blastopore lip and convergent-extension movements toward the dorsal side (arrows) convert this D/V arrangement into the A/P axis of the paraxial and lateral plate mesoderm. **(B)** The future neurectoderm maps to a low, laterally extending region at early gastrula. Convergent-extension movements, most significantly in the future spinal cord, move this tissue to the dorsal side where it forms the neural plate. **(C)** In cross-section, it is possible to see the vertical contact between the mesoderm and the overlying ectoderm. Due to the gradual involution of the mesoderm, the posterior ectoderm first comes in contact with anterior mesoderm (white asterisk) at early gastrula stages. Later during gastrulation, this tissue is underlain and respecified by posterior mesoderm. BLC, blastocoel; BC, bottle cells; Ar, archenteron.

Epidermis, light blue; neurectoderm, blue; future spinal cord, dark green; notochord, red; somitic mesoderm, red-orange; leading edge mesoderm (includes prechordal plate dorsally and lateral plate mesoderm ventrally), orange; endoderm, yellow; bottle cells (pharyngeal endoderm), bright green.



Adapted from Keller, 1991

Figure 1.5 Patterns of gene expression in late gastrula dorsal ectoderm.
Pattern in the neurectoderm is evident in the restricted expression of transcription factors during gastrulation. *otx2* (grey) is expressed exclusively in the anterior ectoderm (Blitz and Cho, 1995; Pannese, et al., 1995), while *opl/Zic-1* (stippled) is present slightly more posteriorly in the anterior and lateral edges of the neural plate (Kuo et al, in press; Mizuseki, et al., 1998). *fkh5* expression (hatching) spans the gap between posterior and anterior gene expression (Gamse et al., in preparation). In the posterior neurectoderm, *HoxD1* (Epstein, et al., 1997; Kolm, et al., 1994) and *gbx-2* (von Bubnoff, et al., 1996) are restricted to posterior-lateral domains , while *Xcad-2* (Epstein, et al., 1997) is expressed a similar yet more posterior domain. "A", anterior; "P", posterior.

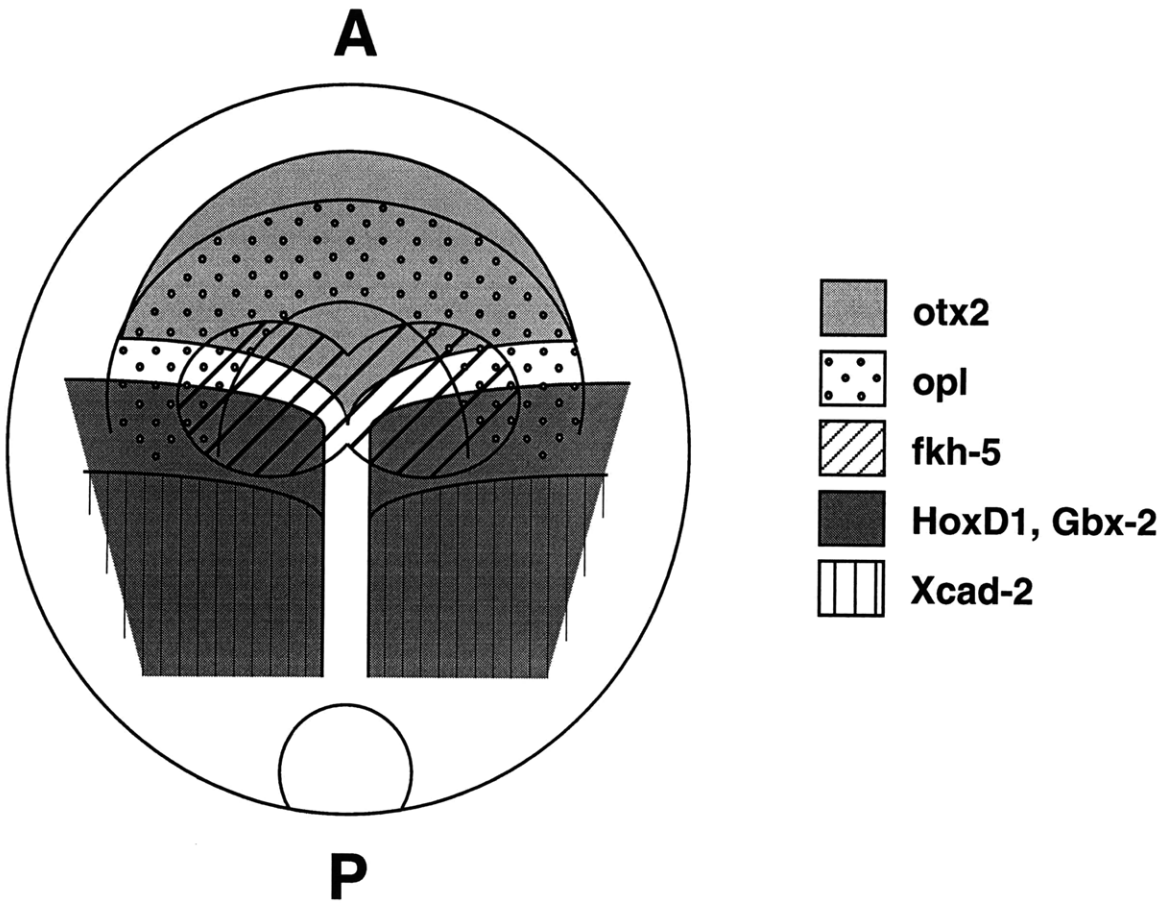
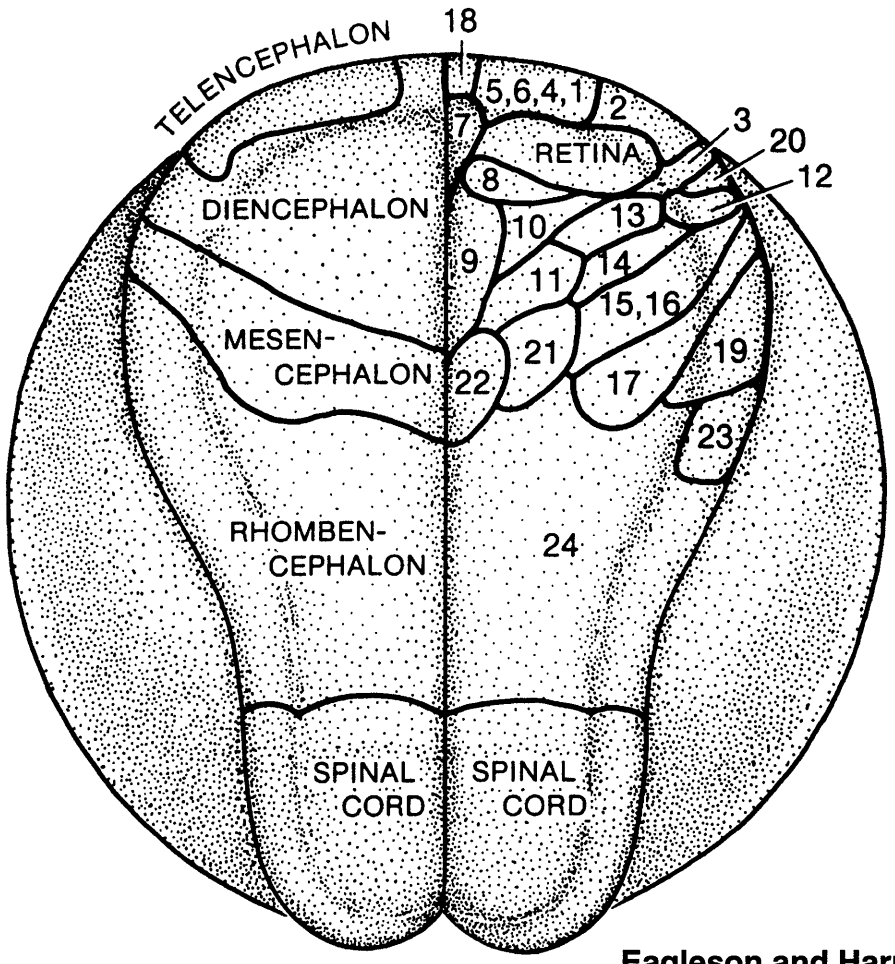

A
P
otx2
opl
fkh-5
HoxD1, Gbx-2
Xcad-2

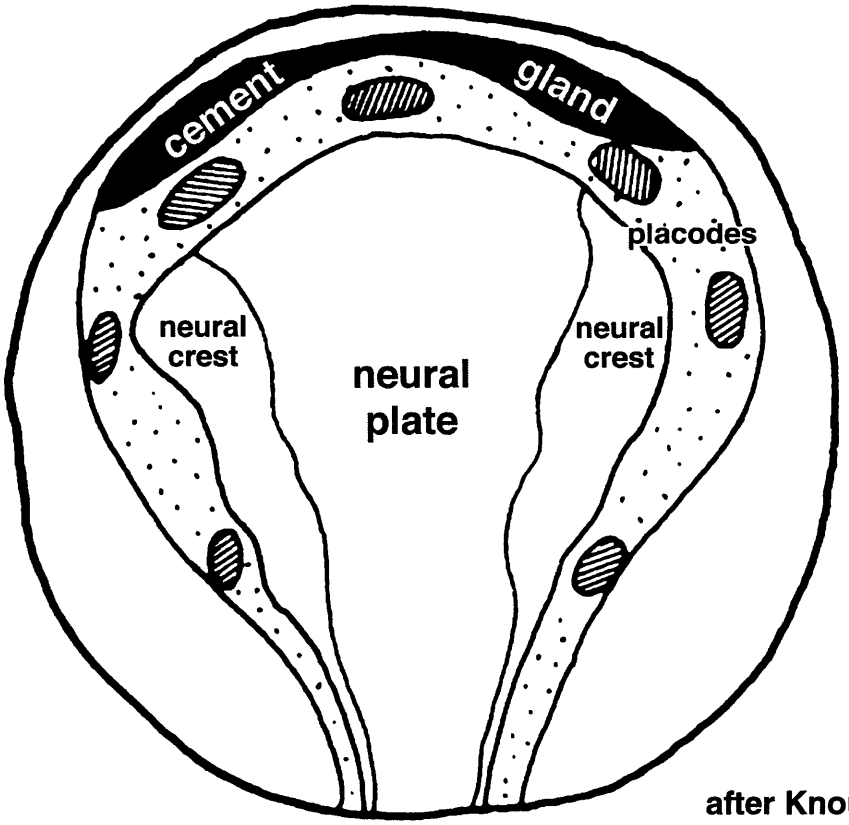
Figure 1.6 Fate maps of the neurula stage neurectoderm.
(A) The mid-neurula stage brain fate map of Eagleson and Harris, 1990. Cells in the neural plate were labeled with Hoechst and DiI at stage 15 (mid-neurula) and were followed until stage 47 (swimming tadpole). The descendants of cells in different regions of the neural plate contribute to particular structures in the adult brain (indicated by numbered regions on the right; key not shown) which map to the major subdivisions of the brain (indicated on the left): telencephalon and diencephalon (forebrain), mesencephalon (midbrain), rhombencephalon (hindbrain), and spinal cord. **(B)** A dorsoanterior view of a neurula embryo showing the location of the placodal region and cement gland in *Rana pipiens* (from Knouff, 1935). The disposition of structures is similar in *Xenopus*. The placodal region corresponds to the thickened area surrounding the neural plate in (A). Note that the cement gland forms at the anterior end of the neural plate.

A



Eagleson and Harris, 1990

B



after Knouff, 1935

Chapter 2. Identification of *otx2* target genes and restrictions in ectodermal competence during *Xenopus* cement gland formation.

(published as Gammill and Sive, 1997)

2.1 Introduction

The *Xenopus* cement gland is a mucus-secreting organ that forms from the anterior-most ectoderm at the dorsoventral border, equivalent to the ectoderm overlying the chin of mammals. The cement gland primordium becomes apparent at early neurula when the marker genes *XCG* and *XAG* are first expressed (Sive, et al., 1989; Sive, et al., 1996). The unique position and early differentiation of the cement gland make it useful for analyzing how an organ becomes precisely positioned along the anteroposterior axis (Sive and Bradley, 1996). Several tissue interactions during gastrula stages induce or repress cement gland formation (reviewed in Sive and Bradley, 1996). Positive signals arise from dorsal mesoderm, neural plate, and dorsoanterior endoderm (Bradley, et al., 1996; Drysdale and Elinson, 1993; Yamada, 1938). At least two inhibitory signals from posterior chordamesoderm and ventral ectoderm restrict cement gland development anteriorly and control cement gland size (Bradley, et al., 1996; Sive, et al., 1989).

Cement gland determination must result from localized activation of transcription factors that regulate its subsequent differentiation. One candidate regulatory gene is the homeobox gene *otx2*. *otx2* is a vertebrate homologue of *Drosophila orthodenticle* (*otd*), an anterior gap gene that specifies segment identity in the head (Cohen and Jurgens, 1990; Finkelstein and Perrimon, 1990), *otx2* is clearly a key regulator of positional identity in vertebrates, since mice lacking *otx2* exhibit defects in gastrulation, deficits in axial mesoderm, and deletions of the forebrain and midbrain (Acampora, et al., 1995; Ang, et al., 1996; Matsuo, et al., 1995). However, very little is known about the mechanism by which *otx2* acts. It is not clear whether *otx2* acts primarily in the dorsal ectoderm or in the underlying mesoderm, neither have the downstream target genes of *otx2* been identified.

Xenopus otx2 has been isolated by several groups (Blitz and Cho, 1995; Pannese, et al., 1995) We isolated *otx2* by subtractive cloning, in a search for early positional markers along the anteroposterior ectodermal axis (Kuo et al., in press). *Xenopus otx2* is initially expressed at early gastrula in the dorsal mesoderm and weakly in the dorsal ectoderm, while by late gastrula, *otx2* RNA is expressed in a broad anterodorsal domain including the presumptive forebrain and cement gland primordium (Blitz and Cho, 1995; Gammill and Sive, 1997; Pannese, et al., 1995). Retinoic acid, which inhibits cement gland formation (Durston, et al., 1989; Sive, et al., 1990), also inhibits *otx2* expression (Ang, et al.,

1994; Pannese, et al., 1995; Simeone, et al., 1995) further suggesting a connection between cement gland formation and *otx2*.

Two recent studies have shown that *otx2* RNA injected into fertilized *Xenopus* eggs leads to ectopic mesoderm, neural tissue, and cement gland formation (Blitz and Cho, 1995; Pannese, et al., 1995). This interesting result is consistent with the idea that endogenous *otx2* is involved in normal cement gland determination. However, in these studies, it was equally plausible that ectopic cement gland formed as a secondary effect of the neural tissue and mesoderm induced by *otx2*. Additionally, embryos injected with *otx2* RNA often show severe gastrulation defects, clouding interpretation of the mechanism by which ectopic tissues might have formed (Blitz and Cho, 1995; Pannese, et al., 1995; Gammill and Sive, unpublished). In particular, the results from these studies were ambiguous because it was not possible to separate early effects of misexpressed *otx2* from those that occurred later during gastrulation when cement gland is normally induced (Bradley, et al., 1996).

In this study we ask whether *otx2* activity is spatially or temporally regulated in embryonic ectoderm and whether *otx2* directly activates expression of cement gland marker genes. In order to address these issues, we controlled the timing of *otx2* activity using a hormone-inducible fusion protein (*otx2*-GR; Kolm, et al., 1995b). With this technique, the ligand-binding domain of a steroid hormone receptor, such as the glucocorticoid receptor, is fused in frame to a heterologous protein (Kolm and Sive, 1995b; Mattioni, et al., 1994). The fusion protein is inactive in the absence of ligand, most likely due to association of the hsp90 protein and conformation of the ligand binding domain, and is post-translationally activated by addition of hormone (Tsai, et al., 1994).

Our data uncover complex spatial and temporal modulations in the responsiveness of the ectoderm to *otx2*. We show that *otx2*-GR directly activates expression of a cement gland-specific gene and activates an *otx2* autoregulatory loop. Finally, we document that lack of *otx2* expression is responsible for some of the inhibitory effects of retinoic acid on cement gland formation.

2.2 Results

2.2A An *otx2*-glucocorticoid receptor fusion protein activates cement gland markers in embryos and animal caps.

In order to assay the activity of *otx2* specifically during gastrulation, we constructed a glucocorticoid-inducible *otx2* by inserting the ligand-binding

domain of the glucocorticoid receptor (GR) into the *otx2* coding region (*otx2*-GR, see Methods). We first asked whether the fusion protein elicited hormone-dependent ectopic cement gland formation in whole embryos (Fig. 2.1A). Embryos were injected with *otx2*-GR RNA mixed with *lacZ* RNA as a lineage tracer, and injections were targeted to the prospective ectoderm. Dexamethasone (dex) was added at early gastrula (stage 10.5), and cement gland formation was assayed at hatching (stage 32) by in situ hybridization for expression of the cement gland-specific gene *XCG*. Without dex, only endogenous *XCG* RNA was observed (Fig. 2.1B, panel a, white open arrowhead). However, after dex treatment, we observed extensive ectopic *XCG* expression in ventrolateral ectoderm, which normally forms epidermis (b, black arrowheads; Table 2.1). *XCG* was never expressed in the neural tube and was induced less efficiently in dorsal epidermis (b, white arrowheads; Table 2.1). Similar results were obtained when expression of another cement gland marker, *XAG*, was examined (data not shown). These data indicated that *otx2*-GR activity was regulated by dex and that the ability of *otx2*-GR to activate cement gland marker expression was not altered by the glucocorticoid receptor domain. Further, when dex was added at late blastula, gastrulation defects were observed (not shown) similar to the effects of injecting native *otx2* RNA (Pannese, et al., 1995).

We next asked whether *otx2*-GR induced cement gland formation in isolated ectoderm (animal caps) and when it could do so. Animal caps removed from *otx2*-GR injected embryos were incubated without or with dex at various stages until control embryos reached hatching (stage 32; defined as hatching stage "equivalent"; Fig. 2.1A). *XCG* expression was not detected in untreated caps (Fig. 2.1C, panel a; Table 2.2). In contrast, dex treatment at mid-gastrula (stage 11.5; b) or early neurula equivalent (stage 14; c) activated *XCG* to high levels over most of the explants. Ectopic *XCG* RNA was expressed in both the inner and outer ectodermal layers of animal caps (not shown). Co-injection of *otx2*-GR and *lacZ* RNA demonstrated that regions which did not express *XCG* after dex treatment at stage 11.5 equivalent did not inherit injected RNA (data not shown). When caps were treated at tailbud equivalent (stage 20; d), however, only small patches of *XCG* were expressed.

In order to determine whether decreased activation of *XCG* expression at tailbud was due to insufficient *otx2*-GR protein, we examined RNA and protein levels using a myc-tagged *otx2*-GR (MT-*otx2*-GR; see Methods). The activity of MT-*otx2*-GR was indistinguishable from the original *otx2*-GR fusion (data not

shown). Animal caps dissected from MT-*otx2*-GR injected embryos were harvested at various stage equivalents and examined by Northern (Fig. 2.1D) or Western analysis (Fig. 2.1E). Although the injected RNA was degraded and present at low levels by late gastrula equivalent (Fig. 2.1D, lanes 1-4), MT-*otx2*-GR protein was present at similar levels at all stages examined, including tailbud equivalent (Fig. 2.1E, lanes 1-4).

These data showed that *otx2*-glucocorticoid receptor fusion activity was tightly regulated by dexamethasone. The *otx2*-GR fusion protein was stable through tailbud and could efficiently activate ectopic cement gland formation in embryos and animal caps as late as mid-neurula.

2.2B Sensitivity to retinoic acid reveals a correlation between *otx2* and *XCG* expression.

In order to assess whether endogenous *otx2* expression was prerequisite for *XCG* expression, we exploited the ability of retinoic acid (RA) to inhibit cement gland formation (Sive, et al., 1990) and endogenous *otx2* expression (Ang, et al., 1994; Pannese, et al., 1995; Simeone, et al., 1995). Since sensitivity to retinoic acid is transient (Durston et al., 1989; Sive et al., 1990), we first asked whether loss of sensitivity to RA by the cement gland correlated with endogenous *otx2* expression. Embryos were treated with RA beginning at gastrula stages and gene expression was assayed at mid-neurula (stage 15; Fig. 2.2). Untreated embryos expressed both *XCG* (panel a, black open arrowhead) and *otx2* (e, white open arrowhead) in the cement gland primordium. When RA was added at early gastrula (stage 10.5), neither *XCG* (b) nor ectodermal *otx2* (f) was expressed. Weak *otx2* expression persisted in the mesoderm (Pannese, et al., 1995). Following RA treatment beginning at mid-gastrula (stage 11.5), both *XCG* (c; black open arrowheads) and *otx2* (g; white open arrowheads) were expressed weakly in the cement gland anlage. When RA was added at late gastrula (stage 12.5), *XCG* (d) and *otx2* (h) expression in the cement gland primordium was equivalent to untreated embryos, although *otx2* expression in the forebrain had not been restored. These data demonstrated that cement gland development and *otx2* expression were maximally sensitive to RA before mid-gastrula. As embryos lost sensitivity to RA, there was a strict temporal correlation between cement gland formation and *otx2* expression in the cement gland anlage.

2.2C *otx2*-GR can overcome the inhibitory effect of retinoic acid to activate *XCG* expression in whole embryos.

The correlation between *otx2* and *XCG* expression after RA treatment suggested that inhibition of cement gland formation by RA might be due to lack of *otx2* expression. This allowed us to test the sufficiency of *otx2*-GR for cement gland determination by asking whether *otx2*-GR could activate *XCG* expression in the presence of RA during the period when endogenous cement gland formation was inhibited by RA. *otx2*-GR injected embryos were incubated in RA, dex, or both for five hours from late blastula (stage 9) to early/mid-gastrula (stage 11), or from early/mid-gastrula to early neurula (stage 15; Fig. 2.3A). Embryos were harvested immediately after treatment and *XCG* expression was analyzed by in situ hybridization. Fig. 2.3B shows representative embryos from three experiments collated in Table 2.3.

Embryos treated with dex from stage 9 to 11, before the onset of endogenous *XCG* expression (Fig. 2.3Ba), were unresponsive to *otx2*-GR and did not express ectopic *XCG* RNA (b). In contrast, dex treatment from stage 11 to 15 (when endogenous *XCG* is expressed; c), led to ectopic *XCG* expression in the ventrolateral ectoderm of 71% of embryos examined (d, black arrowheads; Table 2.3). RA treatment from stage 11 to 15 completely prevented endogenous *XCG* expression (e; Table 2.3), however, 72% of embryos treated with RA and dex expressed *XCG* RNA (f; Table 2.3). In several cases, RA-insensitive *XCG* was expressed in what would have been the cement gland primordium. The absence of endogenous *XCG* RNA in RA-treated embryos (e; Table 2.3) and the observation that treatment of uninjected embryos with RA and dex did not alter the timing of RA sensitivity (not shown) led us to conclude that this was ectopic *XCG* expression. A similar level of MT-*otx2*-GR protein was present after stage 9 to 11 treatment (Fig. 2.3C, lanes 1-4), when embryos did not respond to *otx2*-GR, as after stage 11 to 15 treatment (lanes 5-8), when ectopic *XCG* was activated by *otx2*-GR. Neither RA nor dex altered MT-*otx2*-GR protein levels. dex treatment resulted in up to five separate higher mobility MT-*otx2*-GR bands (lanes 3, 4, 7 and 8). The reason for this shift in mobility has not been determined.

These results showed that *otx2*-GR was sufficient to activate ectopic *XCG* expression in whole embryos in the presence of RA at a time when endogenous cement gland formation was inhibited. Surprisingly, in both untreated and RA-treated embryos, *otx2*-GR was unable to activate *XCG* expression until after early/mid-gastrula (stage 11).

2.2D *otx2*-GR can activate *XCG*, *XAG* and endogenous *otx2* in isolated ectoderm only after early/mid-gastrula.

The inability of whole embryos to respond to *otx2*-GR and activate *XCG* before stage 11 showed that *otx2*-GR activity was temporally restricted. We therefore asked more precisely when isolated ectoderm (animal caps) became responsive to *otx2*-GR. We assayed expression of both cement gland markers and the endogenous *otx2* gene, to determine whether *otx2* expression was controlled by an autoregulatory loop. In order to distinguish endogenous from injected *otx2*, we deleted *otx2* 5′ and 3′UTR from *otx2*-GR to construct *otx2*/3′Δ-GR. Animal caps were isolated from *otx2*/3'Δ-GR-injected embryos and aged in saline for various lengths of time before dex was added (Fig. 2.4A). Gene expression was later monitored by a relative quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay (see Methods) using primers specific to *otx2* 3′UTR, *XCG*, and *XAG*.

Caps isolated at mid-blastula (stage 8) and incubated for five hours in dex until early gastrula (stage 10¼) did not show activation of *XCG*, *XAG*, or *otx2* (Fig. 2.4B, lanes 1,2), even when low background levels were visualized by long exposures of the autoradiogram (not shown). When caps were isolated at late blastula (stage 9) and incubated in dex for five hours until early/mid gastrula (stage 11), a slight activation of *XCG* was observed, while neither *XAG* nor endogenous *otx2* were significantly activated (lanes 3, 4). In contrast, when stage 9 caps were aged in saline, then treated with dex for five hours from stage 11 to mid-neurula (stage 15) equivalent, we observed strong activation of all three genes (lanes 5, 6). Relative to *EF-1α*, the induction of *XCG* was 6-fold greater after dex treatment between stages 11 and 15 than after dex treatment between stages 9 and 11. Since the background level of *XCG* was so low, it was not possible to quantitate the absolute level of induction. *XAG* was induced 5-fold over background, and endogenous *otx2* was induced 8-fold over background. dex treatment of caps aged to tailbud (stage 20) equivalent also led to significant activation of *XCG*, *XAG*, and *otx2* (lanes 7, 8), but to lower levels than prolonged treatment initiated at stage 11 (lane 9), in accord with the data shown in Fig. 2.1C. The inability of caps to respond to *otx2*-GR before early/mid-gastrula was not due to insufficient *otx2*-GR protein, since Western analysis showed equivalent levels of MT-*otx2*-GR protein at all stages (Fig. 2.1E, Fig. 2.3C).

These results showed that isolated ectoderm could not respond to *otx2*-GR until early/mid-gastrula. Further, once the ability to respond to *otx2*-GR was

acquired, it was retained through tailbud stages. Our data also uncovered an *otx2* autoregulatory loop.

2.2E Analysis of *XCG*, *XAG* and endogenous *otx2* activation in animal caps in the absence of protein synthesis and in the presence of retinoic acid.

We next asked whether *XCG*, *XAG*, or endogenous *otx2* were direct targets of *otx2*-GR, and could therefore be activated by *otx2*-GR in the absence of protein synthesis. Embryos were injected with *otx2*/3'Δ-GR RNA and animal caps removed at late blastula (stage 9). Caps were aged to early/mid gastrula equivalent (stage 11) when *otx2*/3'Δ-GR was activated by dex for 5 hours with or without inhibition of protein synthesis by cycloheximide (CHX; Fig. 2.5A, Methods). In addition, we asked whether *otx2*-GR could overcome the inhibitory effects of retinoic acid (RA) in animal caps (Sive et al., 1990) with simultaneous RA treatment given with or without dex from stage 11 to stage 15 equivalent, or from stage 11 to stage 30 equivalent (Fig. 2.5A). After harvest, expression of *XCG*, *XAG*, and endogenous *otx2* was assayed by RT-PCR.

As shown previously, *XCG*, *XAG*, and endogenous *otx2* were activated by dex treatment from stage 11 to 15 (Fig. 2.5B; lanes 1, 2). Although CHX alone did not alter background *XCG* RNA levels (lane 3), caps pre-treated with CHX for 30 minutes and incubated in CHX plus dex for 5 hours from stage 11 until stage 15 equivalent activated significant *XCG* expression (lane 4). The relative level of *XCG* RNA after dex plus CHX treatment was similar to that seen after dex treatment alone, with 70% of *XCG* expression independent of protein synthesis when normalized to *EF-1α*. In contrast, *XAG* was not activated by dex in the presence of CHX (lane 4). Similar results were obtained after two and three hour treatments with CHX and dex (not shown). A third situation was observed with endogenous *otx2*, which was expressed 28-fold above background after treatment with CHX alone (lane 3), compared to 4-fold above background after treatment with dex alone. Incubation in CHX plus dex did not lead to accumulation of endogenous *otx2* RNA to levels higher than seen after CHX alone (lanes 3, 4). CHX treatment of uninjected caps resulted in a similar accumulation of *otx2* RNA (not shown). CHX treatment did not alter MT-*otx2*-GR protein levels, either in the absence (Fig. 2.5C; lanes 1, 2) or presence (lanes 3, 4) of dex. As previously observed, dex treatment caused a mobility shift of the MT-*otx2*-GR protein.

We also asked whether *otx2*/3'Δ-GR was sufficient to activate *XCG*, *XAG*, and endogenous *otx2* expression in RA-treated animal caps. RA plus dex

treatment of *otx2*/3'Δ-GR expressing caps between stages 11 and 15 equivalent led to high *XCG* RNA expression, while *XAG* expression was also greater (6-fold) after treatment with dex plus RA than after RA alone (lanes 5, 6). Endogenous *otx2* was not, however, activated in the presence of RA (lanes 5, 6). Although these results showed that *XCG* and *XAG* could be activated by *otx2*/3'Δ-GR in the presence of RA, longer incubations of caps in dex plus RA from stage 11 to late tailbud (stage 30) equivalent prevented further accumulation of *XCG* and *XAG* RNAs (lanes 7-10). This effect was not general, since *EF-1α* and the cytokeratin *XK81* (Jamrich, et al., 1987) were expressed at normal levels after prolonged RA treatment (Sive et al., 1990; not shown).

These results demonstrated that *otx2*-GR could activate *XCG* RNA accumulation in the absence of protein synthesis, identifying *XCG* as a direct target of *otx2*. Activation of *XAG* was apparently dependent on protein synthesis, although we could not reach a firm conclusion since the response of this gene to *otx2*-GR was weak. Accumulation of endogenous *otx2* RNA was strongly activated by CHX alone. As in whole embryos, *otx2*/3'Δ-GR could activate *XCG* expression in the presence of RA, but accumulation was not sustained after prolonged RA treatment. In addition, RA blocked the endogenous *otx2* autoregulatory loop.

2.3 Discussion

By controlling the timing of *otx2* activity with a dexamethasone-inducible fusion protein (*otx2*-GR), we have shown that *otx2* directly regulates at least part of the *Xenopus* cement gland determination program. Our data make four points concerning the mechanism by which *otx2* acts. First, we have shown that regionally restricted co-factors modulate *otx2* activity, since *otx2*-GR can activate cement gland gene expression only in ventrolateral ectoderm. Second, we have determined that temporal competence of the ectoderm to respond to *otx2*-GR is restricted, becoming maximal only at mid-gastrula when naive ectoderm is losing the ability to respond to inducers secreted by mesoderm. Third, we have identified the cement gland differentiation markers *XCG*, *XAG*, and the endogenous *otx2* gene as targets of *otx2*-GR, with *XCG* likely to be a direct target. Fourth, we have shown that part of the inhibitory effect of retinoids on cement gland formation is likely to be due to the absence of *otx2* expression.

2.3A *otx2* activity is position dependent.

Ectopic *XCG* expression was activated by *otx2*-GR exclusively in ventrolateral ectoderm, although *otx2*-GR protein was present at similar levels dorsally and ventrally (not shown). We never observed activation of cement gland markers in the neural plate. Ectopic *XCG* RNA was expressed much less frequently in the epidermis over the neural tube and somites than in ventral epidermis, implying that the epidermis covering different regions of the embryo is not equivalent. The region responsive to *otx2*-GR included the cement gland primordium, which was often enlarged or skewed by ectopic *XCG* expression. After inhibition of the endogenous cement gland by retinoids, *otx2*-GR sometimes induced *XCG* in what would have been the cement gland anlage. Ventral ectoderm is a potent inhibitor of endogenous cement gland formation (Bradley and Sive, 1996), and the ability of *otx2* to activate cement gland formation ventrally indicates that *otx2* function can override inhibitory factors. Consistent results were obtained by misexpressing native *otx2* RNA, which activated cement gland markers more efficiently when injected into ventral blastomeres than when expressed dorsally (Pannese, et al., 1995). These data define an area of the ectoderm permissive for cement gland formation, and we propose that the normal restriction of *XCG* and *XAG* to the cement gland reflects the overlap of endogenous *otx2* expression with this permissive region (Fig. 2.6).

What prevents *otx2* from inducing cement gland dorsally in the neurectoderm? One possibility is a ventrally restricted co-factor required for cement gland differentiation. A second alternative is a dorsal-specific factor that synergizes with *otx2* to activate neural differentiation and passively inhibit cement gland formation. Consistent with this possibility, *otx2*-GR greatly expands the domain of *N-CAM* expression in the neural plate (not shown). Additionally, a dorsal-specific factor could be an active repressor of cement gland formation. Since *otx2*-GR activated cement gland markers in both anterior and posterior ventrolateral ectoderm, and did not activate these markers in either anterior or posterior neurectoderm, factors that restrict *otx2* activity must be expressed along the entire anteroposterior axis.

2.3B Responsiveness to *otx2* is temporally limited.

otx2-GR could not activate expression of either *XCG* or endogenous *otx2* before mid-gastrula, while these genes retained responsiveness at tailbud stages. Activity of endogenous *otx2* is also likely to be restricted. Although *otx2* RNA is

present in the dorsal ectoderm from early gastrula (Blitz and Cho, 1995; Pannese, et al., 1995). *XAG* and *XCG* are not expressed in the cement gland primordium until the end of gastrulation (Sive and Bradley, 1996). The restriction in responsiveness to *otx2* may reflect a general strategy to control timing of differentiation while axial patterning takes place. During gastrulation, posterior ectoderm is transiently specified to anterior fates (Eyal-Giladi, 1954; Sive, et al., 1989) and several genes that are eventually anterior-specific, including *otx2* (Blitz and Cho, 1995; Pannese et al., 1995; Kuo et al., in press), are initially expressed in posterior ectoderm. Preventing the ectoderm from responding to regulatory factors prematurely would avoid inappropriate differentiation before pattern has been established.

The period of competence to respond to *otx2* contrasts sharply with the earlier competence of ectoderm to respond to dorsal mesoderm (Kintner, et al., 1991; Nieuwkoop, 1958; Sharpe and Gurdon, 1990; Sive, et al., 1989). While early gastrula stage animal caps are efficiently induced by dorsal mesoderm to express cement gland markers, responsiveness is lost if caps are cultured to mid-gastrula equivalent before conjugation with dorsal mesoderm (Sive, et al., 1989). Thus, sequential changes in ectodermal competence take place, such that early gastrula ectoderm responds to secreted signals by expressing *otx2* and other transcription factors (Ekker, et al., 1995; Lamb, et al., 1993; Sasai, et al., 1994), while only older ectoderm is able to respond to the induced genes. The responsiveness of the ectoderm to downstream transcription factors is unlinked to prior induction by the mesoderm, since animal caps autonomously acquire sensitivity to *otx2*. Interestingly, competence to be induced to neural versus lens fates is also sequentially and autonomously regulated in animal caps (Servetnick, et al., 1991), however, we do not know whether the mechanism that regulates this phenomenon is related to the one described here.

This is one of the first demonstrations in a vertebrate that competence to respond to a transcription factor is temporally regulated during development (analogous observations have been made for the *Xbra* gene using a hormone-inducible protein; Tada et al., 1997). Conditional gene expression has been difficult to effect in vertebrates (Spencer, 1996), but has been widely used in invertebrate embryos to define periods during which transcription factors can act. For example, *Drosophila otd* is able to specify ocelli only when expressed over a narrow, two hour window (Royet and Finkelstein, 1995). However, in this and other invertebrate examples, "classical" induction assays are difficult to perform,

preventing comparisons between the timing of responsiveness to secreted inducers and downstream regulators.

2.3C Targets of *otx2*.

Several lines of evidence suggest that *XCG* is a direct target of *otx2*. *otx2* and *XCG* are always co-expressed in the cement gland anlage. Induction of *XCG* by *otx2*-GR is rapid, with significant RNA accumulation one hour after dex addition (not shown). A significant component of *XCG* activation by *otx2*-GR is independent of protein synthesis, supporting the idea that *otx2* binds to the *XCG* promoter to activate its expression. *XCG* is the major cement gland-specific RNA and encodes a mucin (Sive and Bradley, 1996) that is likely to be part of the cement gland "glue." *XCG* is therefore a "differentiation gene," suggesting that *otx2* may directly activate other terminal differentiation genes elsewhere in its expression domain. *otx2* is able to initiate the entire cement gland differentiation program, since elongated cells characteristic of the mature cement gland form after *otx2*-GR activation (not shown; Blitz and Cho, 1995; Pannese et al., 1995). It is not clear, however, how many aspects of this program can be directly controlled by *otx2*. Another early cement gland marker, *XAG*, does not appear to be directly activated by *otx2*, although the weak responsiveness of this gene made results unclear.

We also demonstrated that an autoregulatory loop controls *otx2* expression. It was not possible to address whether the endogenous *otx2* gene was a direct target of *otx2*-GR since treatment with cycloheximide led to high levels of *otx2* expression, probably by stabilizing the *otx2* RNA which is transcribed in animal caps. The results suggest that *otx2* RNA is unstable, a property that would make it possible to rapidly alter the levels and pattern of *otx2* expression (reviewed in Surdej et al., 1994). It has been suggested that *Drosophila otd* RNA is unstable, since it rapidly responds to maternal control genes (Surdej, et al., 1994), but this point has not been explicitly addressed. Activated *otx2*-GR did not synergize with cycloheximide to lead to higher levels of endogenous *otx2* RNA than seen with cycloheximide alone. This lack of synergy may be because *otx2*-GR activates transcription of the endogenous *otx2* gene, but requires synthesis of factors induced by *otx2*. Alternatively, *otx2*-GR may act by stabilizing endogenous *otx2* RNA, but this effect may not be obvious in the presence of cycloheximide when *otx2* RNA stability may already be maximal.

otx2 can be induced by conjugation with dorsal mesoderm (Ang et al., 1994) and by several mesendodermally derived factors including noggin, chordin, and hedgehog proteins (Lamb et al., 1993; Ekker et al., 1995; Sasai et al., 1995). Since the *otx2* autoregulatory loop cannot be activated in early gastrula animal caps, our data suggest that at least until mid-gastrula, ectodermal *otx2* expression is maintained by continued induction from the underlying mesendoderm. After this, as inhibitory factors are lost, or as co-activators are synthesized, autoregulation is initiated (Fig. 2.6). Consistent results have been observed using ectoderm induced in embryos, where dorsal ectoderm isolated at mid-gastrula maintains expression of *otx2* in the absence of inducing mesendoderm (Ang et al., 1994; Kolm and Sive, unpublished).

2.3D Teratogenic effects of retinoids and inhibition of *otx2* expression.

Retinoic acid severely truncates the anteroposterior axis in vertebrates, including deletion of the cement gland in *Xenopus* (Conlon, 1995; Durston, et al., 1989; Sive, et al., 1990). The ability of *otx2*-GR to activate *XCG* expression in the presence of retinoids was not only another test for the sufficiency of *otx2* to activate cement gland determination, but also suggested that failure to express *otx2* (Ang et al., 1994; Pannese et al., 1995; Simeone et al., 1995) is partly responsible for suppression of cement gland formation by retinoids. Curiously, *otx2*-GR was unable to sustain *XCG* RNA accumulation in the presence of retinoic acid. One reason for this could be that endogenous *otx2* is required for maximal accumulation of *XCG*, perhaps after *otx2*-GR protein is degraded. It is equally plausible, however, that prolonged treatment with retinoic acid activates inhibitory factors that suppress *XCG* expression, even in the presence of active *otx2*, or that retinoids inhibit factors required in addition to *otx2* for maximal *XCG* expression.

How is endogenous *otx2* inhibited by retinoic acid? One possibility is that *otx2* expression never begins, either because mesodermal signaling is defective (Cho, et al., 1991; Ruiz i Altaba and Jessell, 1991a; Sive and Cheng, 1991) or because retinoid-induced inhibitors in the ectoderm block accessibility of the *otx2* promoter to activators (Simeone et al., 1995). Such inhibitors may explain why endogenous *otx2* expression never establishes (Fig. 2.5B) or maintains (not shown; Ang et al., 1994) an autoregulatory loop in the presence of retinoids.

2.3E *otx2* and cement gland induction: a model.

These results can be collected into the model shown in Fig. 2.6. Early during gastrulation, *otx2* RNA begins to accumulate in the dorsal ectoderm, after induction by factors secreted from the dorsal mesoderm (Lamb et al., 1993; Ang et al., 1994; Ekker et al., 1995; Sasai et al., 1995). At this time, the ectoderm is unable to respond to *otx2* to activate cement gland marker expression or the *otx2* autoregulatory loop due to the presence of an inhibitor or the absence of a co-activator. Later during gastrulation, the inhibitor is lost or the co-activator synthesized, allowing the ectoderm to respond to *otx2* and initiate *otx2* autoregulation. *XCG* expression is activated at this time, by binding of *otx2* to the *XCG* promoter, and possibly through activating additional factors. The ventrolateral ectoderm may express a factor that synergizes with *otx2* to allow cement gland formation ventrally, or a dorsal factor may inhibit cement gland formation in the neural plate. During the period permissive for cement gland formation, the cement gland develops in the region where *otx2* expression and the permissive area overlap.

This study has begun to uncover the mechanisms by which *otx2* controls regional identity in the embryonic ectoderm. Future challenges lie in determining the molecular basis for modulating *otx2* activity, in addressing what mechanisms control *otx2* activity in the neural plate and in identifying other targets of this gene, an area where *otx2*-GR constructs may again be very helpful.

2.4 Acknowledgments

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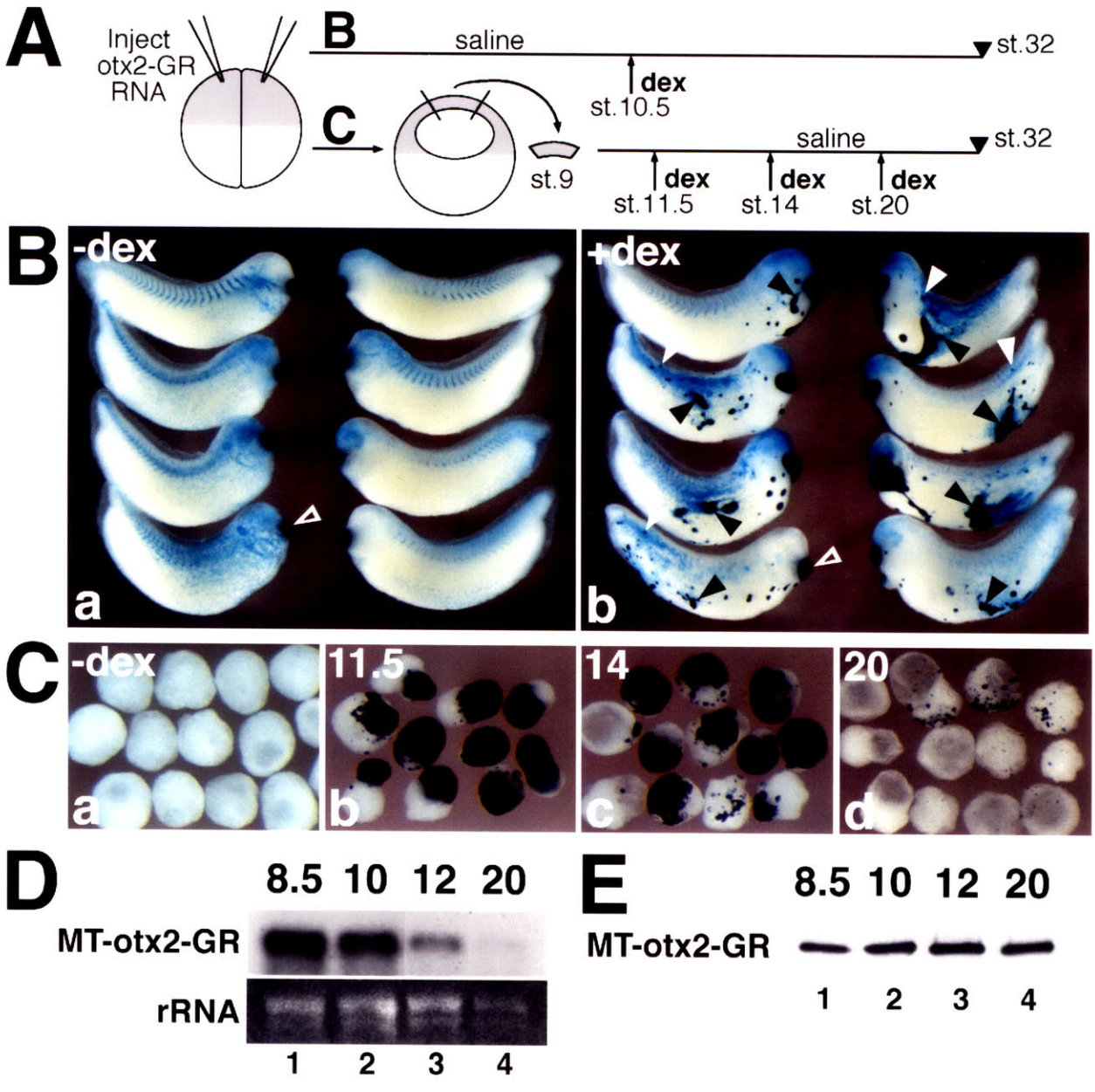
Figure 2.1 An *otx2*-glucocorticoid receptor fusion activates hormone-dependent ectopic *XCG* expression in embryos and explants.
(A) Albino embryos were injected with *otx2*-GR RNA at the two-cell stage. Injected embryos or blastula (stage 8 or 9) animal caps were incubated alone or with dexamethasone (dex) until embryos reached hatching (stage 32). Arrows indicate the stage treatment was initiated, triangles indicate the stage of harvest. **(B)** Ectopic cement gland induction in whole embryos. Embryos were injected in one blastomere with 100 pg *otx2*-GR RNA mixed with 60 pg *lacZ* RNA. Embryos were incubated without (a) or with (b) dex at early gastrula (stage 10.5) until stage 32, when they were stained for *lacZ* (light blue) and for *XCG* expression by in situ hybridization (black). Endogenous *XCG*, white open arrowhead; ventrolateral ectopic *XCG*, black arrowheads; ectopic *XCG* in dorsal epidermis, white arrowheads. Lateral view, anterior to the center. **(C)** Ectopic cement gland induction in animal caps. Embryos were injected in both blastomeres with 50 pg *otx2*-GR RNA. Animal caps dissected at stage 9 were transferred to dex when control embryos reached the stages indicated. *XCG* expression was analyzed by in situ hybridization when control embryos reached stage 32. (a) untreated; (b) +dex st. 11.5; (c) +dex st. 14; (d) +dex st. 20. **(D)** *otx2*-GR RNA levels in animal caps. Embryos were injected in both blastomeres with 50 pg MT-*otx2*-GR RNA. Animal caps were dissected at stage 8 and pools of ten caps were analyzed by Northern blot for *otx2* expression when control embryos reached stage 8.5 (lane 1), stage 10 (lane 2), stage 12 (lane 3), or stage 20 (lane 4). Ethidium bromide-stained 28S rRNA is a loading control. **(E)** *otx2*-GR protein levels in animal caps. Explants prepared as in (D) were harvested in groups of ten when sibling embryos reached stage 8.5 (lane 1), stage 10 (lane 2), stage 12 (lane 3), or stage 20 (lane 4). Two cap equivalents of protein were analyzed by Western blot analysis using an anti-myc antibody. Ponceau S staining indicated that all lanes were loaded equally (data not shown).


A
Inject
otx2-GR
RNA
B
saline
st.32
dex
st.10.5
C
st.9
saline
st.32
dex
st.11.5
dex
st.14
dex
st.20
B
-dex
+dex
a
b
C
-dex
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20
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b
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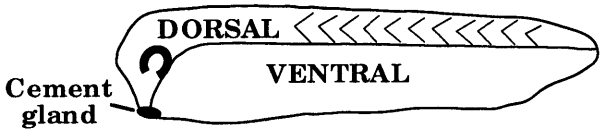
d
D
8.5 10 12 20
MT-otx2-GR
rRNA
1 2 3 4
E
8.5 10 12 20
MT-otx2-GR
1 2 3 4

location of *lacZ*[a]	number of embryos[c]	*XCG* expression (%)[d]
dorsal	154	34 (22)[e]
ventral	137	123 (90)
neural[b]	25	0 (0)



Schematic of stage 32 embryo, with "dorsal and "ventral" divisions used for scoring purposes.

Table 2.1 Frequency of dorsal vs. ventral ectopic *XCG* expression in *otx*2-GR injected embryos.

Embryos were prepared as described in Fig. 2.1A. 160 embryos were scored in five independent experiments. *XCG* expression was scored by in situ hybridization when embryos reached stage 32 (see diagram above).

[a] Location of *lacZ* staining. Staining was scored as "dorsal" when in or over the head, spinal cord, or somites, "ventral" when on the belly (see diagram of stage 32 embryo above).

[b] "neural" staining was scored by coarse sectioning of selected embryos representative of those which stained for *lacZ* in the neural tube.

[c] Of the 160 embryos examined, the number of embryos with *lacZ* staining in the region scored. When *lacZ* staining was present in more than one region, the embryo was scored once for each category.

[d] Of the embryos with *lacZ* staining in the region scored, the number and frequency of embryos with ectopic *XCG* expression overlapping *lacZ* staining.

[e] Only small points of "dorsal" ectopic *XCG* expression were observed in the dorsal epidermis.

dex treatment[a]	strong *XCG* (%)[b]	patchy *XCG* (%)[c]	number of explants[d]	number of experiments[e]
–	0 (0)	0 (0)	37	3
st. 11.5	26 (84)	4 (13)	31	3
st. 14/15	22 (71)	7 (23)	31	3
st. 20	0 (0)	17 (77)	22	2

Table 2.2 Frequency of hormone-dependent *XCG* induction by *otx2*-GR in animal caps.

Explants were prepared as described in Fig. 2.1A. *XCG* expression was scored by in situ hybridization when control embryos reached stage 32 equivalent.

[a] Stage equivalent when treatment with dexamethasone (dex) was initiated. All caps were cultured until stage 32 equivalent.

[b] Number and frequency of animal caps with large areas of *XCG* expression.

[c] Number and frequency of animal caps with patchy *XCG* expression.

[d] The total number of animal caps examined.

[e] The number of independent experiments.

Figure 2.2 *otx2* expression correlates with insensitivity of the cement gland to retinoic acid.

Albino embryos were left untreated (a, e) or incubated in retinoic acid (RA) beginning at stage 10.5 (b, f), stage 11.5 (c, g), or stage 12.5 (d, h) and until harvest at stage 15 for analysis by in situ hybridization. **Panels a-d:** *XCG* expression. Black open arrowheads indicate *XCG* expression in the cement gland anlage. Anterior view, dorsal up. **Panels e-h:** *otx2* expression. White open arrowheads indicate *otx2* expression in the cement gland anlage. Anterior view, dorsal up.

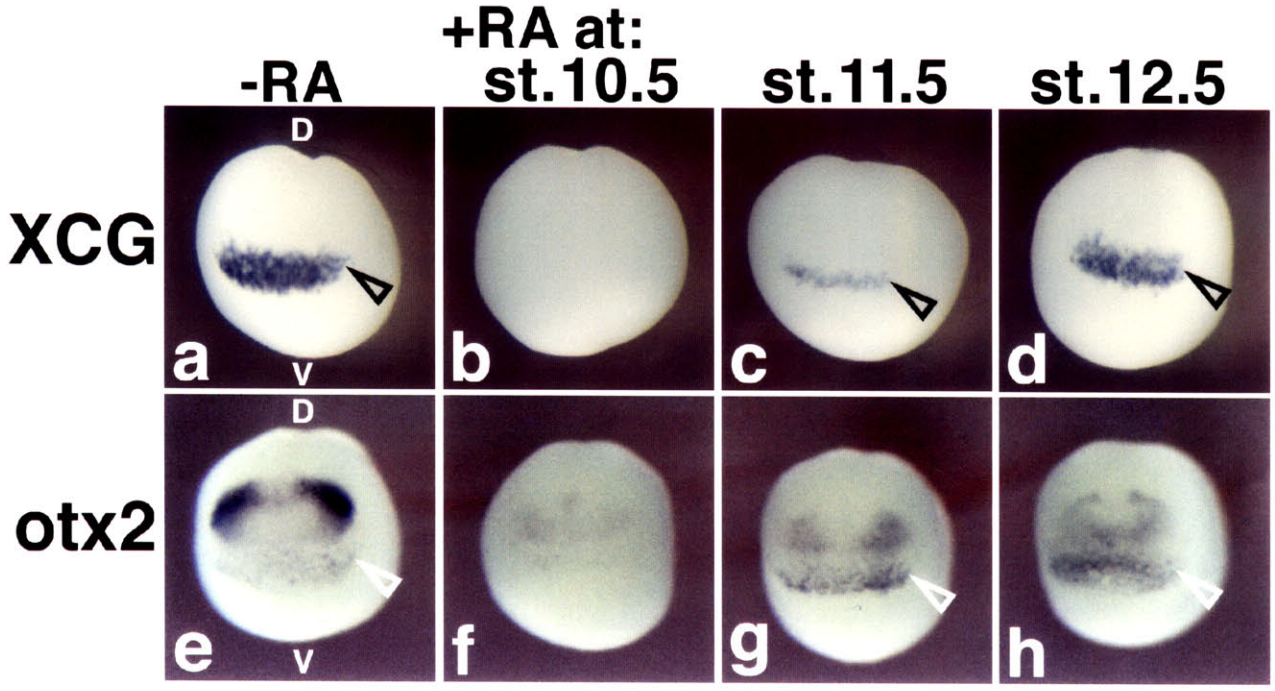
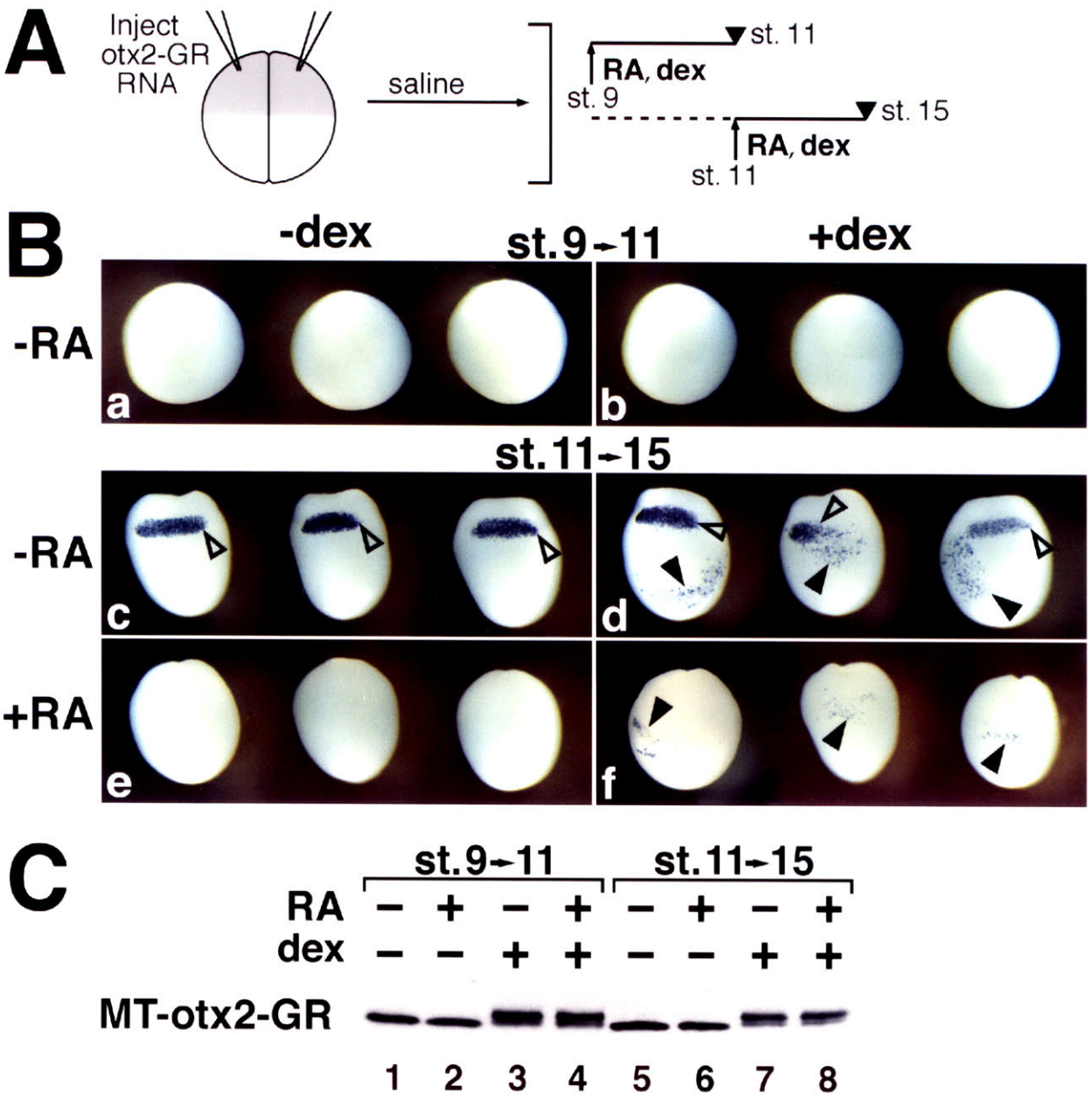

-RA
+RA at:
st.10.5
st.11.5
st.12.5
XCG
otx2
D
V
a
b
c
d
e
f
g
h

Figure 2.3 *otx2*-GR can activate retinoic acid-insensitive *XCG* expression in whole embryos.

(A) Albino embryos were injected with *otx2*-GR RNA at the two-cell stage. Injected embryos were incubated alone or in dexamethasone (dex) and/or retinoic acid (RA) for 5 hours beginning at blastula (stage 9) or mid-gastrula (stage 11). Arrows indicate the stage treatment was initiated, triangles indicate the stage of harvest. **(B)** *XCG* induction in whole embryos. Embryos injected in one blastomere with 50 pg *otx2*-GR RNA were left untreated or incubated in dex and/or RA from stage 9 to 11 (a, b) or stage 11 to 15 (c-f). *XCG* expression was analyzed by in situ hybridization. Open arrowheads indicate endogenous *XCG*, solid arrowheads mark ectopic *XCG*. Anteroventral view. (a) untreated; (b) +dex st. 9; (c) untreated; (d) +dex st. 11; (e) +RA st. 11; (f) +RA+dex st. 11. **(C)** Western blot analysis of whole embryos. Embryos injected with 50 pg MT-*otx2*-GR RNA in both blastomeres and prepared as in (B) were lysed in pools of five for Western blot analysis with an anti-myc antibody. One embryo equivalent of protein was analyzed per lane. Ponceau S staining indicated that all lanes were loaded equally (data not shown). Lane 1, untreated; lane 2, +RA st. 9; lane 3, +dex st. 9; lane 4, +RA+dex st. 9; lane 5, untreated; lane 6, +RA st. 11; lane 7, +dex st. 11; lane 8, +RA+dex st. 11.


A
Inject
otx2-GR
RNA
saline
st. 11
RA, dex
st. 9
st. 15
RA, dex
st. 11
B
-dex
st.9→11
+dex
-RA
a
b
st.11→15
-RA
c
d
+RA
e
f
C
st.9→11
st.11→15
RA
dex
MT-otx2-GR
1 2 3 4 5 6 7 8

treatment period[a]	treatment[b] dex	RA	endogenous *XCG* (%)[c]	ectopic *XCG* (%)[d]	number of embryos[e]	number of experiments[f]
st. 9-11	–	–	0 (0)	0 (0)	39	3
	–	+	0 (0)	0 (0)	33	3
	+	–	0 (0)	0 (0)	36	3
	+	+	0 (0)	0 (0)	36	3
st. 11-15	–	–	58 (100)	0 (0)	58	3
	–	+	0 (0)	0 (0)	47	2
	+	–	55 (100)	39 (71)	55	3
	+	+	0 (0)	33 (72)	46	2

Table 2.3 Frequency of *XCG* expression in RA-treated *otx2*-GR injected embryos. Embryos were prepared as described in Fig. 2.3A. *XCG* expression was scored by in situ hybridization, with harvest at either stage 11 or stage 15 as indicated.

[a] Treatments were for five hours from stage 9 to 11 or from stage 11 to 15.

[b] Embryos were incubated without (–) or with (+) dexamethasone (dex) and retinoic acid (RA) as indicated.

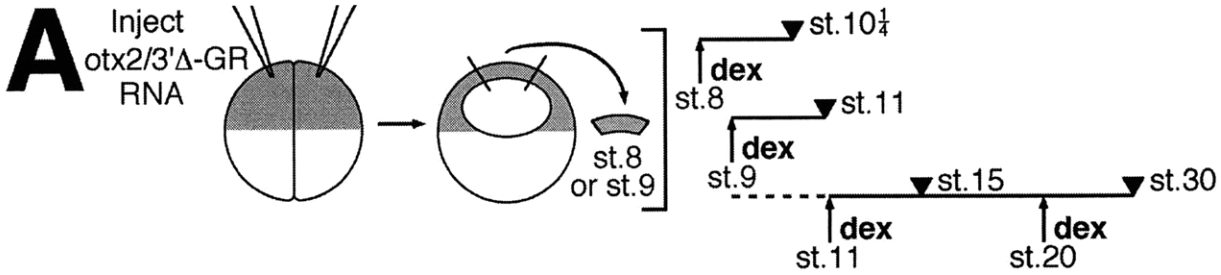
[c] Number and frequency of embryos with *XCG* expression in the cement gland anlage. Expression was scored as endogenous by its position at the extreme anterior of the embryo.

[d] Number and frequency of embryos with ectopic *XCG* expression in the ventrolateral ectoderm. Expression was scored as ectopic by the shape and location of the region expressing *XCG*.

[e] The total number of injected embryos examined.

[f] The number of independent experiments.

Figure 2.4 *otx2*-GR cannot activate cement gland marker expression or an *otx2* autoregulatory loop until early/mid-gastrula.
(A) Wildtype embryos were injected with *otx2*/3'Δ-GR RNA at the two-cell stage. Blastula (stage 8 or 9) animal caps were isolated from injected embryos and incubated without or with dexamethasone (dex) for five hours beginning at stage 8 or stage 9, for five hours or 20 hours beginning at early/mid-gastrula (stage 11), or for 12 hours beginning at tailbud (stage 20). Arrows indicate the stage treatment was initiated, triangles indicate the stage of harvest. **(B)** *XCG*, *XAG*, and *otx2* expression in animal caps. Embryos were injected in both blastomeres with 50 pg *otx2*/3'Δ-GR RNA. Animal caps were dissected at stage 8 or 9, and pools of ten explants were left untreated or were incubated in dex for 5 hours immediately after dissection (lanes 1-4), or for 5 hours (lanes 5, 6) or 20 hours (lanes 7, 9) beginning when control embryos reached stage 11, or for 12 hours (lane 8) when controls embryos reached stage 20. *XCG*, *XAG*, and *otx2* expression were analyzed by RT-PCR using *EF-1α* as a control. Since cement gland markers are expressed at very high levels in the mature cement gland compared to their initial expression levels, samples were assayed at high number of PCR cycles (lanes 1-6) or low number of PCR cycles (lanes 7-9) in order to remain in the linear range of amplification at the stage assayed. Early expression is not detectable at low cycle numbers. Identical results were obtained in two independent experiments. Lane 1, untreated; lane 2, +dex st. 8; lane 3, untreated; lane 4, +dex st. 9; lane 5, untreated; lane 6, +dex st. 11; lane 7, untreated; lane 8, +dex st. 20; lane 9, +dex st. 11.


A
Inject
otx2/3'Δ-GR
RNA
st.8
or st.9
st.8
dex
st.10¼
st.9
dex
st.11
st.11
dex
st.15
st.20
dex
st.30

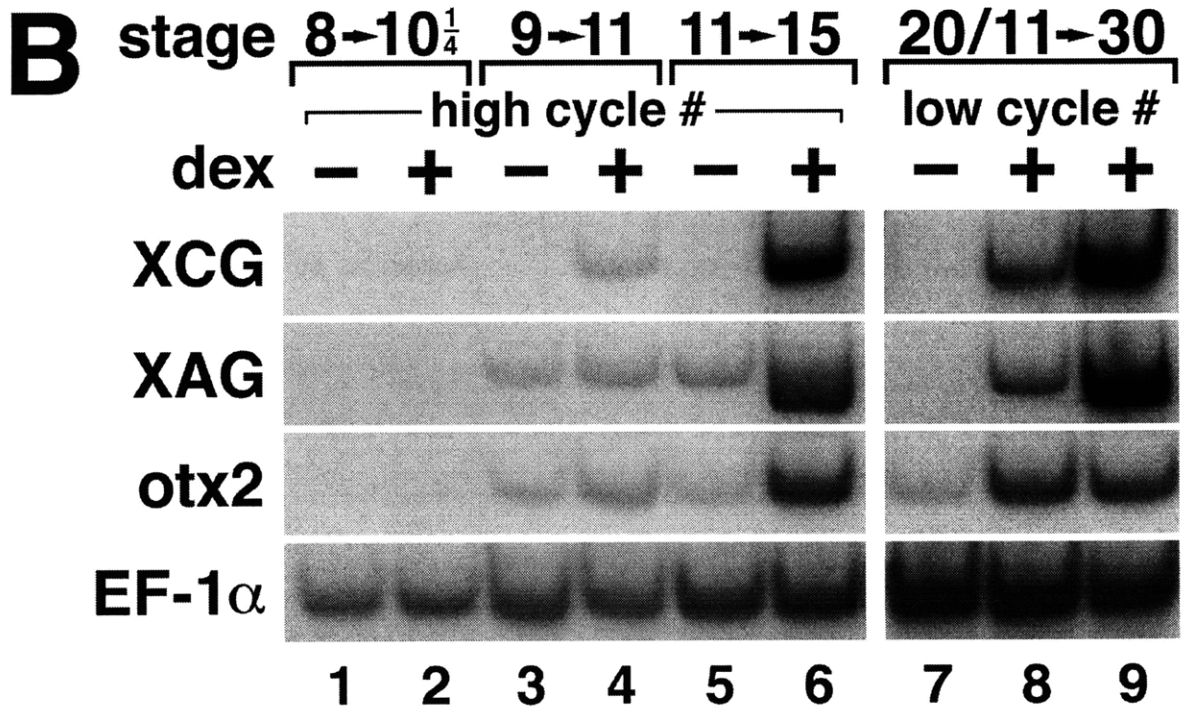

B
stage
8→10¼
9→11
11→15
20/11→30
high cycle #
low cycle #
dex
– + – + – + – + +
XCG
XAG
otx2
EF-1α
1 2 3 4 5 6 7 8 9

Figure 2.5 *otx2* regulation of target gene expression.
(A) Embryos were injected with *otx2*/3'Δ-GR RNA at the two-cell stage. Blastula (stage 9) animal caps were isolated and incubated in dexamethasone (dex) and/or cycloheximide (CHX) or retinoic acid (RA) for 5 hours or for 20 hours beginning at early/mid-gastrula (stage 11). Arrows indicate the stage treatment was initiated, triangles indicate the stage of harvest. **(B)** RT-PCR analysis. Embryos were injected in both blastomeres with 50 pg *otx2*/3'Δ-GR RNA. Animal caps were dissected at stage 9 and left untreated or incubated in dex without or with CHX or RA for 5 hours (lanes 1-6) or 20 hours (lanes 7-10) beginning when control embryos reached stage 11. *XCG*, *XAG*, and *otx2* expression were analyzed by RT-PCR using *EF-1α* as a control. Since cement gland markers are expressed at very high levels in the mature cement gland compared to their initial expression levels, samples were assayed at high number of PCR cycles (lanes 1-6) or low number of PCR cycles (lanes 7-10) to remain in the linear range of amplification at the stage assayed. Early expression is not detectable at low cycle numbers. Comparable results were obtained with cycloheximide in eight independent experiments and by in situ hybridization (three independent experiments), and with RA in four independent experiments. Lane 1, untreated; lane 2, +dex; lane 3, +CHX; lane 4, +CHX+dex; lane 5, +RA; lane 6, +RA+dex; lane 7, untreated; lane 8, +dex; lane 9, +RA; lane 10, +RA+dex. **(C)** Western blot analysis. Embryos were injected in both blastomeres with 50 pg MT-*otx2*-GR RNA and animal caps dissected at stage 9. When control embryos reached late gastrula (stage 12.5), caps were treated without or with dex and/or CHX for 3 hours, a period of time encompassed by treatments described in (B), and were lysed in groups of ten for Western blot analysis with an anti-myc antibody. Two cap equivalents of protein were analyzed per lane. Ponceau S staining indicated that all lanes were loaded equally (data not shown). Lane 1, untreated; lane 2, +CHX; lane 3, +dex; lane 4, +CHX+dex .

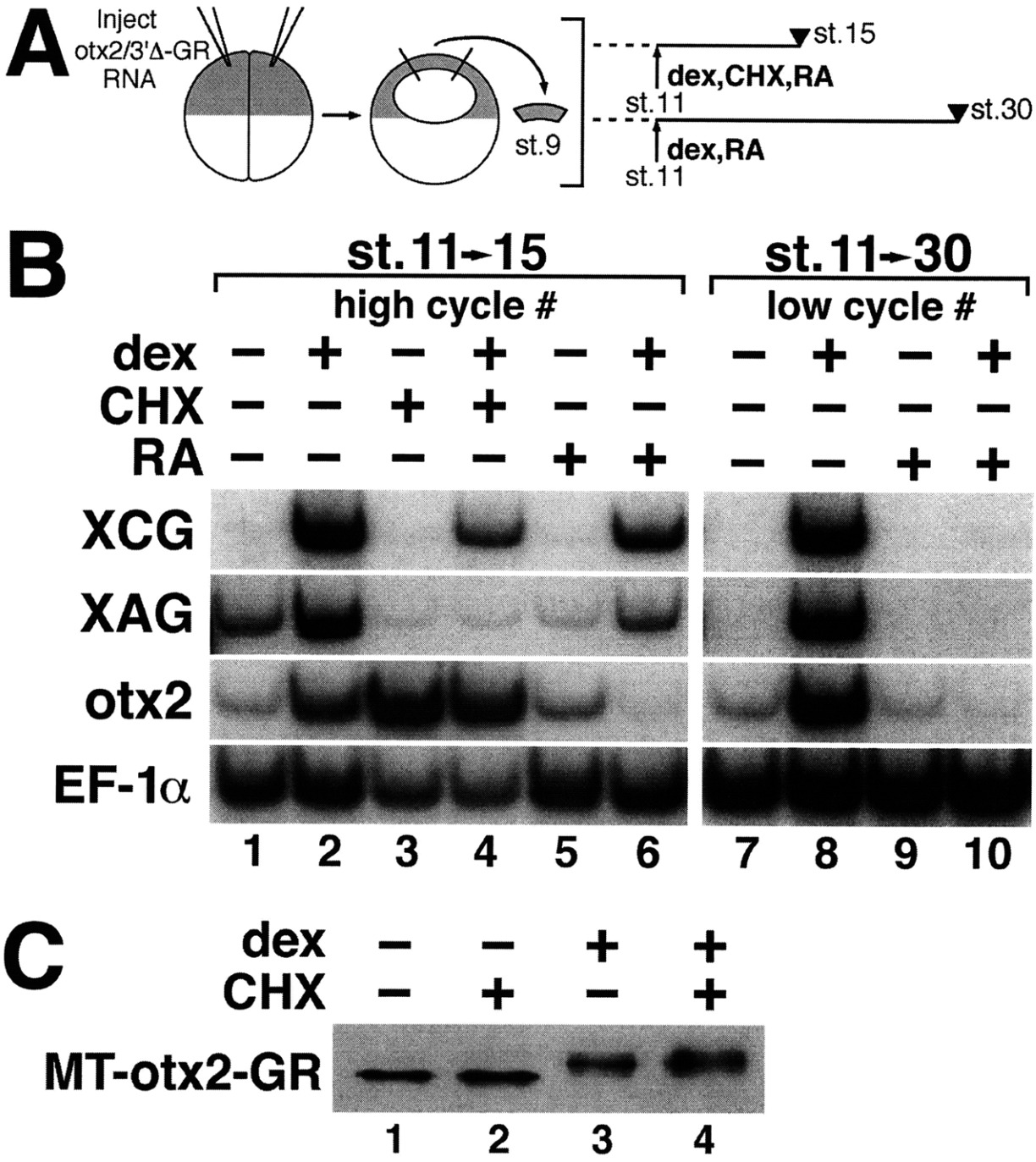
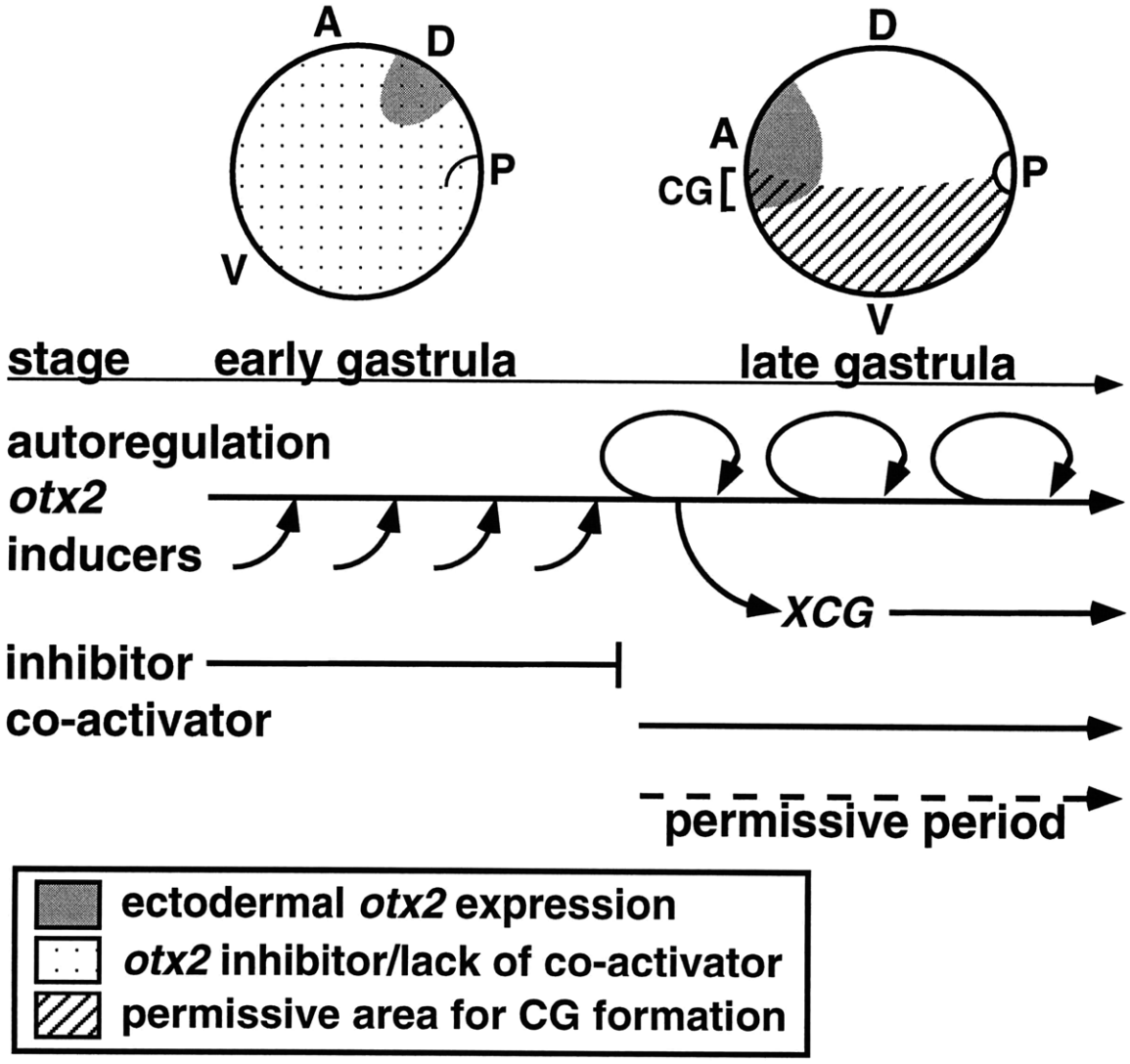

A
Inject
otx2/3'Δ-GR
RNA
st.9
st.15
dex,CHX,RA
st.11
st.30
dex,RA
st.11
B
st.11→15
high cycle #
st.11→30
low cycle #
dex
CHX
RA
XCG
XAG
otx2
EF-1α
1 2 3 4 5 6 7 8 9 10
C
dex
CHX
MT-otx2-GR
1 2 3 4

Figure 2.6 *otx2* and a model for cement gland induction.
Interaction of signals that regulate cement gland (CG) formation. See text for details. *otx2* is expressed in the ectoderm beginning at early gastrula (*otx2* line, grey shading) as a result of induction from the underlying mesendoderm (inducers). *otx2* is unable to autoregulate or activate *XCG* expression at this time due to the presence of an inhibitor (⊣) or the absence of a co-activator (→; stippling on embryo schematic). At the end of gastrulation, *otx2* expression defines an anterior domain (grey shading). Due to absence of the inhibitor or synthesis of the co-activator, *otx2* activates *XCG* expression and an autoregulatory loop. *otx2* can activate cement gland differentiation only where its expression domain overlaps with the ventrolateral area permissive for cement gland formation (stripes on embryo schematic).


A
D
P
V
D
A
CG
P
V
stage
early gastrula
late gastrula
autoregulation
otx2
inducers
XCG
inhibitor
co-activator
permissive period
ectodermal otx2 expression
otx2 inhibitor/lack of co-activator
permissive area for CG formation

Chapter 3. *otx2* and BMP4 signaling cooperate during cement gland formation.

3.1 Introduction

The anterior ectoderm of a *Xenopus* embryo gives rise to anterior neural tissue as well as an additional, non-neural derivative, the cement gland. A small mucus-secreting organ, the cement gland forms at the dorsoventral boundary and anterior-most extent of the ectoderm. In addition to occupying a unique location in the embryo, secreted factors and tissue interactions that induce anterior neural determination also induce cement gland (reviewed in Sive and Bradley, 1996). The coincidence of cement gland and anterior neural fates provides an attractive opportunity to consider how distinct anterior positional identities are defined in the ectoderm.

Anterior neural and cement gland lineages are linked by their common expression of the homeobox gene *otx2* (Blitz and Cho, 1995; Pannese, et al., 1995). The vertebrate homologue of the *Drosophila* anterior gap gene *orthodenticle* (*otd*), *otx2* expression begins at the onset of gastrulation and delineates an anterior domain in the mesendoderm and ectoderm of the gastrula embryo (Blitz and Cho, 1995; Pannese, et al., 1995). *otx2* is essential for the formation of anterior fates, as mice lacking *otx2* function do not form head structures rostral to rhombomere 3 (Acampora, et al., 1995; Ang, et al., 1996; Matsuo, et al., 1995). Meanwhile, *otx2* overexpression in *Xenopus* embryos induces ectopic mesoderm, neural tissue, and cement gland (Blitz and Cho, 1995; Pannese, et al., 1995). Although the requirement of *otx2* specifically in the anterior ectoderm is not clear (Rhinn, et al., 1998), *otx2* directly regulates *Xenopus* cement gland formation, indicating that *otx2* instructs anterior ectodermal identity (Gammill and Sive, 1997). However, the factors that determine that ability of *otx2* to induce anterior neural or cement gland fates within its broad expression domain are not known.

Recent work in *Xenopus* has indicated that the activities of the bone morphogenetic proteins (BMPs) are of central importance during patterning of the embryo. BMPs induce ventral mesoderm and the ventral derivative of the ectoderm, epidermis (Dale, et al., 1992; Jones, et al., 1992; Wilson and Hemmati-Brivanlou, 1995). The demonstration that the neural inducers noggin (Zimmerman, et al., 1996) and chordin (Piccolo, et al., 1996) act by antagonizing BMP activity has lead to a model in which neural tissue forms in the absence of ventral signaling (see Hemmati-Brivanlou and Melton, 1997). In the anterior ectoderm, diffusion of noggin and chordin from a source in the mesoderm may give rise to a gradient of BMP4 activity that produces anterior dorsoventral

pattern. This gradient can be reproduced in animal caps, where no BMP4 activity induces anterior neural tissue, intermediate BMP4 signaling activates cement gland formation, and high BMP4 activity induces epidermis (Knecht and Harland, 1997; Wilson, et al., 1997). Although a gradient of Smad1, which relays BMP4 signaling to the nucleus, has a similar effect, the mechanism through which BMP signaling generates pattern is not known (Wilson, et al., 1997). Smad1 binds DNA with Smad4 (Lagna, et al., 1996; Zhang, et al., 1997), a sequence-specific transcriptional activator (Zawel, et al., 1998), and the level of Smad1 activity could determine target specificity (see Wilson, et al., 1997). Alternatively, by analogy to activin-responsive Smad2, which interacts with the winged-helix transcription factor FAST-1 to activate gene expression (Chen, et al., 1996), Smad1 could interact with transcription factors such as *otx2* to instruct anterior ectodermal fates, however this possibility has not been explored.

In our previous work, overexpression of an inducible *otx2* was used to define the ventrolateral ectoderm as an area permissive for cement gland formation. We postulated that overlap of *otx2* and this ventral permissive region positioned cement gland formation exclusively at the anterior extent of *otx2* expression. Here, we begin to explore the molecular basis for this modulation of *otx2* activity as a means to address how the decision to induce cement gland versus anterior neural tissue is made. We first show that *otx2* can induce ectopic neural tissue and cement gland in the correct dorsoventral pattern. By examining *otx2* and *noggin* injected animal caps, we demonstrate that the induction of cement gland correlates with *otx2* and *BMP4* coexpression. We also show that *otx2* and *BMP4* RNA normally coexist in the cement gland, and that coinjection of *otx2* and *BMP4* promotes cement gland formation. We propose that interaction between otx2 and an anterior gradient of BMP4 signaling selects between cement gland or neural induction.

3.2 Results

3.2A *otx2* induces neural tissue and cement gland in the correct pattern.

Since *otx2* activates cement gland formation only in the ventrolateral ectoderm (Gammill and Sive, 1997), we wondered whether the ability of *otx2* to induce neural tissue (Blitz and Cho, 1995; Pannese et al., 1995) would also be spatially restricted in the embryo. To minimize the effects of *otx2* activity in the mesoderm, which would secondarily affect the overlying ectoderm, an inducible form of *otx2* that includes the glucocorticoid receptor ligand binding domain

(*otx2*-GR) was targeted to the ectoderm (Gammill and Sive, 1997). This construct allowed *otx2* activity to be regulated by the addition of the hormone dexamethasone (dex) during gastrula stages when competence for mesoderm induction was low (Jones, et al., 1987). *otx2*-GR RNA was injected into one of two blastomeres at the two-cell stage, and dex was added at mid-gastrula stage 11.5. At tailbud stage 20 or hatching stage 28, embryos were analyzed by in situ hybridization for induction of *NCAM* (a general neural marker) and *XCG* (a cement gland marker) expression (Fig. 3.1A).

At both stages, embryos incubated in the absence of dex expressed normally restricted *NCAM* and *XCG* RNA (Fig. 3.1B, a and b). Following hormone treatment, however, *NCAM* was ectopically expressed throughout the ectoderm, both dorsally and ventrally (c-f, white arrowheads). As previously reported, *XCG* expression (black arrowheads) was induced only in the ventrolateral ectoderm (Gammill and Sive, 1997) and was typically observed ventral to ectopic *NCAM* expression within the *lacZ* expressing domain. This pattern of ectopic expression was similar to the normal dorsoventral disposition of *NCAM* and *XCG* expression in the embryo. Exceptions to this trend were generally determined by the shape of the region expressing *otx2*-GR. Since *XCG* was usually induced at the edge of the *lacZ* domain, when *otx2*-GR expression had a sharp vertical border or spread ventrally to the uninjected side, *XCG* could be observed next to or effectively dorsal to *NCAM* expression.

These data showed that *otx2*-GR can induce neural tissue throughout the dorsal and ventral ectoderm. Ectopic *NCAM* expression was generally induced dorsal to ectopic *XCG*.

3.2B Neural tissue and cement gland are induced by different levels of *otx2*.

Since cement gland and neural tissue were not induced evenly within the *otx2*-GR injected region, we wondered whether the pattern of *NCAM* and *XCG* ectopic expression was related to the amount of *otx2*-GR RNA inherited by an injected cell. To address this question, embryos were injected with different concentrations of unmodified *otx2* RNA ranging from 10 pg to 400 pg. Animal caps were dissected at mid-blastula stage 9, and at mid-neurula stage 15, expression of *XCG*, *XAG* (a cement gland marker), *NCAM*, *BMP4* (a ventral inducer), and *XVent-1* (a ventral marker) was assayed by RT-PCR (Fig. 3.2A).

Injection of 400 pg of *otx2*, the highest level tested, induced low levels of cement gland markers *XCG* and *XAG*, while maximal levels of *NCAM* were

induced (Fig. 3.2B, lane 1). Ventral markers *BMP4* and *XVent-1* were suppressed in these caps. Expression of *NCAM* remained high even in animal caps from embryos injected with 100 pg *otx2* (lane 3), then rapidly declined at lower doses (lanes 4-6). As *NCAM* induction decreased, cement gland marker expression increased dramatically to maximum levels at 50 pg of injected *otx2* (lanes 2-4). This pattern of cement gland marker expression was accompanied by increased levels of ventral markers *BMP4* and *XVent-1*. Cement gland induction drops precipitously in caps injected with 20 pg and 10 pg of injected *otx2*, which begin to resemble uninjected caps in their spectrum of gene expression (lanes 5-7).

These results demonstrated that neural tissue and cement gland were induced at different levels of *otx2* RNA. The ability of *otx2* to induce cement gland paralleled expression of the ventral inducer *BMP4*.

3.2C Cement gland induction correlates with *BMP4* and *otx2* expression.

Since noggin has been shown to induce *otx2* and may be one of the endogenous signals regulating *otx2* expression (Lamb, et al., 1993), it was possible that different doses of noggin might lead to variable levels of *otx2* expression. To address this possibility, the effect of noggin concentration was examined by analyzing expression of *otx2* and its targets in animal caps. Embryos were injected with a range of *noggin* RNA doses at the two cell stage, and animal caps were cut at mid-blastula stage 9. At tailbud stage 20, the explants were analyzed by RT-PCR for expression of *XCG*, *XAG*, *NCAM*, and *BMP4* (Fig. 3.3A).

The dose response to noggin was similar to the pattern of gene expression observed after injection of *otx2* RNA. High levels of noggin suppressed *BMP4* expression and did not induce cement gland, while strong expression of *NCAM* and *otx2* was induced (Fig. 3.3B, lanes 1, 2). Decreasing the dose of noggin lead to a decline in *NCAM* expression, while cement gland induction increased to a maximal level at 0.5 pg of *noggin* (lanes 3, 4). The induction of cement gland rather than neural tissue was accompanied by equally strong expression of *otx2* and very little suppression of *BMP4* RNA. As induction of *otx2* decreased in caps injected with 0.25 and 0.1 pg of *noggin*, *XCG* expression was also lost (lanes 5 and 6) and gene expression levels resembled uninjected caps (lane 7).

These data showed that *otx2* was expressed at similar levels when neural tissue or cement gland were induced by noggin. Cement gland was induced by doses of noggin which simultaneously activated *otx2* and allowed high *BMP4* expression.

3.2D *BMP4* and *otx2* are normally coexpressed in the cement gland.

Since the ability of *otx2* to induce cement gland seemed to correlate with *BMP4* expression, it was important to determine whether *otx2* and *BMP4* RNA normally overlapped in the embryo. *otx2* and *BMP4* expression were compared at mid-gastrula (stage 11), early neurula (stage 13), and mid-neurula (stage 15) by single and double whole mount in situ hybridization (Fig. 3.4).

Strikingly, *otx2* and *BMP4* RNA were coexpressed at all stages in an anterior region that completely included the cement gland primordium. At stage 11, *otx2* was evenly expressed in a triangular shaped anterior region (a). The anterior edge of the *otx2* domain, the cement gland primordium, was coincident with the band of maximal *BMP4* expression (b, white arrowhead; c). *BMP4* staining was also observed ventral, and to some extent dorsal, to the region of high level expression in the cement gland (c). By stage 13, *otx2* was still strongly expressed in the cement gland primordium (d), and continued to overlap with maximal expression of *BMP4* (e, white arrowhead; f), which was now excluded from the neural plate (f). *otx2* expression at stage 15 was complex, however, *otx2* RNA was still apparent in the cement gland (g), overlapping with expression of *BMP4* (h, white arrowhead). *BMP4* was most highly expressed in the ventral cement gland at stage 15 (i), a reflection of the pattern that exists within the cement gland (Bradley et al., 1996). These results demonstrated that *otx2* and *BMP4* normally coexist in the cement gland at the time of cement gland formation.

3.2E Coinjection of *BMP4* and *otx2* preferentially induces cement gland.

To test directly whether otx2 and BMP4 might act in concert to induce cement gland, the ability of BMP4 to promote cement gland formation at *otx2* levels that do not favor cement gland induction was examined. Since the inducible *otx2* is more potent that unmodified *otx2* RNA (data not shown), embryos were injected in both blastomeres with 100 pg *otx2*-GR and/or 1 ng *BMP4* RNA. Animal caps were isolated at late blastula stage 9 and incubated with or without dex until mid-neurula stage 15 (Fig. 3.5A). Gene expression in the caps was determined by RT-PCR.

XCG and *NCAM* RNA were not detectable in uninjected, *BMP4* injected, or *otx2*-GR injected animal caps in the absence of dex (Fig. 3.5B, lanes 1, 2, 3). However, *XVent-1* was expressed at high levels in these caps (lanes 1, 2, 3). *otx2*-GR injected caps treated with dex exhibited induction of *XCG* and *NCAM* and

inhibition of *XVent-1* expression (lane 4). However, animal caps from embryos injected with both *otx2*-GR and *BMP4* and treated with dex expressed *XCG* at two-fold higher levels than after injection of *otx2*-GR alone (lane 5). These caps expressed both *NCAM* and *XVent-1*, but at levels 2 to 2.5-fold lower than those observed after injection of *otx2*-GR or *BMP4* alone (compare lanes 4 and 5 or lanes 2 and 5). These data showed that forced expression of *otx2* and *BMP4* favors cement gland formation.

3.3 Discussion

In order to understand how the cement gland and neural inducing capacity of *otx2* is modulated, we have examined the signals that restrict *otx2* cement gland inducing activity. First, we have demonstrated that, although cement gland induction by *otx2* was limited to the ventrolateral ectoderm, induction of neural tissue by *otx2* was not regionally restricted and was induced in the correct pattern with cement gland in the ventrolateral ectoderm. Second, we explored this difference and found that the ability of *otx2* to induce cement gland correlated with expression and activity of the ventral inducer BMP4. Third, *otx2* and *BMP4* normally coexist in the cement gland and coexpression of *otx2* and *BMP4* favored cement gland formation over ventral and neural fates. These results show that ventrolateral permissiveness for cement gland formation is molecularly defined by BMP4 expression and indicate that dorsal and ventral signals converge during cement gland induction. Together, these data suggest that the overlap of the BMP4 gradient with *otx2* expression positions the formation of cement gland and neural tissue.

3.3A Induction of neural tissue by *otx2* is not regionally restricted.

Although *otx2* overexpression activated ectopic cement gland formation only in the ventrolateral ectoderm, ectopic neural tissue was induced in both dorsal and ventral ectoderm (Fig. 3.1). Dorsal ectopic *NCAM* expression was typically manifested as an enlargement of the neural tube, whereas ventral expression was often punctate, but did occasionally appear as a solid field (see Fig. 3.1f, for example). This result demonstrates that *otx2* activity is sufficient to induce neural tissue throughout the ectoderm and that *otx2* is active in the neural plate, despite its inability to induce cement gland there. This indicates that it is the cement gland inducing capacity of *otx2* that is modulated dorsally versus ventrally in the ectoderm. This could be achieved either by the absence of a co-

factor or the presence of an inhibitor in the dorsal ectoderm (Gammill and Sive, 1997).

How does *otx2* induce neural tissue ventrally? There is extensive evidence to suggest that the inhibition of BMP4 signaling is sufficient for neural induction. The neural inducers noggin and chordin act by binding to BMP4 (Piccolo, et al., 1996; Zimmerman, et al., 1996), autoneuralization seems to be achieved at least in part by the dilution of BMP4 in dissociated cells (Wilson and Hemmati-Brivanlou, 1995), and dominant negative BMP4 cleavage mutants neuralize animal caps (Hawley, et al., 1995). Since *otx2* is able to inhibit *BMP4* expression, and *BMP4* inhibition is inversely proportional to the level of *NCAM* expression (Fig. 3.2B), neural induction by *otx2* is presumably achieved by downregulating BMP4 levels. Whether this is relevant to in vivo neural induction is not clear. Although noggin and chordin bind BMP4, the inhibition of *BMP4* transcription is not a proximal response to these signals (Harland and Gerhart, 1997). To date, it is not known how *BMP4* RNA is cleared from the dorsal side during neural induction, but it is possible that *otx2* is involved in this process.

3.3B *BMP4* correlates with cement gland formation.

Different concentrations of *otx2* lead to the expression of distinct markers in the ectoderm (Fig. 3.2). As discussed above, high doses of *otx2* result in efficient induction of neural tissue, as well as repression of ventral markers including *BMP4*. Low doses, however, activate massive expression of cement gland markers, with minimal or no inhibition of *BMP4* expression. This difference may explain why *otx2* overexpression activates the correct dorsal-ventral pattern of neural tissue and cement gland in the ventrolateral ectoderm of whole embryos. It is even possible that *otx2* is normally present in a gradient in the embryo. In *Drosophila*, a gradient of otd protein is required during patterning of the eye-antennal disk (Royet and Finkelstein, 1995). In addition, the level of *otx1* and *otx2* activity is important for the proper pattern of morphogenesis in the brain, but not in any strict anterior to posterior progression (Acampora, et al., 1997).

More importantly, however, the dose response to *otx2* provides insight on how *otx2* activity may be modulated. The correlation between *BMP4* and cement gland formation suggests that the presence or absence of BMP4 determines whether *otx2* will induce cement gland or neural tissue, respectively. This prediction was tested in animal caps by coexpressing *otx2* and *BMP4*, shifting the

balance of BMP4 levels to favor cement gland formation over neural induction (Fig. 3.5). A relationship between BMP4 and *otx2* is further supported by data from animal caps expressing different levels of noggin (Fig. 3.3). noggin activates *otx2* expression in an all or none fashion, suggesting that it probably does not create a gradient of *otx2* in the embryo. However, the simultaneous expression of *otx2* and *BMP4* at intermediate noggin doses closely predicts activation of *XCG* expression, again suggesting that BMP4 modulates the ability of *otx2* to induce cement gland.

These observations are especially interesting given the recent demonstration that a BMP4 gradient recapitulates the dorsoventral pattern of the anterior ectoderm (Knecht and Harland, 1997; Wilson, et al., 1997). Using noggin-treated or dissociated cells, these studies showed that high levels of BMP4 activity induce epidermis, intermediate BMP4 levels activate cement gland, while no BMP4 signaling leads to neural induction. However, *otx2* expression is efficiently induced by noggin treatment (Fig. 3.3; Lamb, et al., 1993) and animal cap dissociation (Sagerström et al, submitted), thus both methods used to define the BMP4 activity gradient lead to different levels of BMP4 with or without *otx2* expression (see Fig. 3.3). Therefore, the correlation between BMP4 and *otx2* activity established here suggests that it is not simply the gradient of BMP4 activity that leads to correct dorsal-ventral patterning, but the interaction of transcription factors that regulate regional differentiation with different levels of BMP4 signaling within the gradient. At the transcriptional level, this could mean that Smad1, which mediates intercellular BMP4 signaling (Graff, et al., 1996), interacts with otx2 to induce cement gland in a manner similar to the interaction between the winged helix transcription factor FAST-1 and Smad2 (Chen, et al., 1996). Smad4, which binds active Smad1 (Lagna, et al., 1996), was recently shown to be a sequence specific transcription factor (Zawel, et al., 1998), so otx2 and Smad1/Smad4 together could activate the expression of different target genes (cement gland) than either otx2 (neural) or Smad1/Smad4 (epidermis) alone. In this manner the BMP4 gradient and *otx2* expression could combine to correctly position structures within the anterior ectoderm. The ability of otx2 and Smad1 to physically interact is currently being addressed.

Interestingly, a Smad4 mouse mutant was recently described (Sirad, et al., 1998). These mice do not form mesoderm or visceral endoderm and exhibit gastrulation defects. These defects are rescued in aggregation chimeras with wildtype visceral endoderm, however, chimeric mice have anterior truncations,

indicating that Smad4 is also necessary for ectodermal patterning. The authors note that Smad4 mutants resemble BMP4 and BMP4 receptor mutant embryos (Mishina, et al., 1995; Winnier, et al., 1995). Although Smad4 is a cofactor for both Smad1/BMP4 and Smad2/activin-TGFβ signaling (Lagna, et al., 1996; Zhang, et al., 1997), the Smad2 loss of function phenotype appears to be due to a requirement for Smad2 in extraembryonic tissues (Waldrip, et al., 1998). Although the ability of wildtype endoderm to rescue the Smad2 mutants has not been addressed, and although the Smad4 ectodermal phenotype could be due to a requirement for signaling by other TGF-βs, these findings are consistent with BMP4 playing a role in anterior ectodermal pattern formation. As in *Xenopus*, several BMPs are expressed in the anterior-most neurectoderm of mouse embryos (Furuta, et al., 1997).

If BMP4 is involved in cement gland formation, why had it previously been identified as a cement gland inhibitor (reviewed in Sive and Bradley, 1995)? Overexpression of BMP4 ablates cement gland and other head structures (Fainsod, et al., 1994; Schmidt, et al., 1995), while dominant negative BMP4 cleavage mutants (Hawley, et al., 1995) and dominant negative BMP receptors (Frisch and Wright, 1998; Graff, et al., 1994; Suzuki, et al., 1994) lead to cement gland formation. Since BMP4 ventralizes dorsal mesoderm (Dale, et al., 1992; Jones, et al., 1992), the ability of BMP4 to inhibit cement gland formation in whole embryos could be a secondary effect of ventralized mesoderm, which would not induce cement gland. In addition, cement gland is induced when BMP4 is inhibited, for example after treatment with noggin or chordin, but only at intermediate levels of BMP4 signaling (Knecht and Harland, 1997; Wilson, et al., 1997). Thus the cement gland is exquisitely sensitive to BMP4 activity, simultaneously requiring inhibition of BMPs for anterior neural/cement gland induction and a degree of BMP4 signaling to achieve cement gland formation. For this reason, a high level of BMP4 is a cement gland inhibitor, while dominant negative constructs must inhibit BMP4 sufficiently to induce neural tissue, but never entirely abolish BMP4 signaling since cement gland is induced.

There are several indications that the relationship between *otx2* and BMP4 is likely to be relevant to normal development. First, *BMP4* and *otx2* RNA coexist in the cement gland during late gastrula and early neurula stages when cement gland induction takes place (Fig. 3.4). Second, the BMP-specific receptor *BMPRII* is expressed in the cement gland primordium, so this region should be competent to respond to BMP4, although it is not clear how early this expression

begins (Frisch and Wright, 1998). Third, BMP4 is expressed in a ventral domain that correlates with the region of the embryo previously described as "permissive" for cement gland formation (Gammill and Sive, 1997), suggesting that BMP4 is a ventrally restricted factor that limits cement gland formation.

3.3C Why not wnts?

Ventrolateral ectopic cement gland formation has also been observed following overexpression of the wnt pathway inhibitory signaling intermediate, *GSK-3*, suggesting that regulation of wnt signaling may be involved in cement gland induction (Itoh, et al., 1995; Pierce, et al., 1996). However, GSK-3 probably does not interact with or modulate *otx2* activity since it is unlikely that GSK-3 directly regulates expression of the cement gland markers. Although *GSK-3* overexpression results in ectopic cement gland formation, *otx2* expression is simultaneously induced in this tissue (Itoh, et al., 1995). This suggests that ectopic *XCG* is activated as consequence of ectopic *otx2* expression rather than as a direct effect of injected *GSK-3*. Furthermore, *otx2* can induce ectopic cement gland throughout the anteroposterior extent of the ventrolateral ectoderm (Gammill and Sive, 1997; Pannese, et al., 1995), while *GSK-3* leads to cement gland formation only in the anterior ventral ectoderm (Pierce and Kimelman, 1996). wnt signaling is more likely involved in modulating the competence of the ectoderm to respond to anterior inducing signals. In support of this, GSK-3 expressing caps are more sensitive to noggin and form cement glands at much lower noggin doses (Pierce and Kimelman, 1996). In addition, the endogenous, anteriorly expressed wnt inhibitor frz-b has no activity on its own in uninduced ectoderm, but augments head formation when expressed in whole embryos (Leyns, et al., 1997; Wang, et al., 1997). Indeed, head induction has been shown to accompany inhibition of wnt signaling, although the actual mechanism responsible for this effect is not clear (Glinka, et al., 1997). Regulation of wnt signaling at gastrula stages is likely to be very complex due to expression of Notch, which by analogy to *Drosophila*, may interact with wnt signaling pathway components to regulate neural patterning and competence (Axelrod, et al., 1996; Couso, et al., 1994).

3.3D The convergence of dorsal and ventral signals.

If *otx2* and *BMP4* cooperate to induce cement gland, how do they come to be expressed in the same place? It has previously been demonstrated that BMP4

inhibits *otx2* expression (Schmidt, et al., 1995), and we have shown that *otx2* can inhibit *BMP4* (Fig. 3.2). It is possible that *otx2* and *BMP4* are expressed in the same cells, but at levels where they do not efficiently inhibit each other. Importantly, *otx2* does not inhibit expression of the BMP4 signal transducer Smad1 (data now shown).

One puzzling aspect of a cooperative role for *otx2* and BMP4, however, is the regulation of their expression. Inhibition of BMP4 signaling by the neural inducers/BMP4 antagonists noggin and chordin induces *otx2* expression and may be involved in establishing the BMP4 activity gradient discussed earlier. Nevertheless, BMP4 antagonists are unlikely to be the only signals involved in this process, since noggin concentrations that induce cement gland inhibit BMP4 transcription (Fig. 3.3B). In contrast, BMP4 expression in the embryo is maximal in the cement gland primordium (Fig. 3.4). This problem is compounded by data suggesting that a BMP4 activity gradient is established by diffusion of noggin or chordin to a BMP4 source, since BMP4 does not appear to have long range effects (Dosch, et al., 1997; Jones, et al., 1998). This indicates that, while a BMP4 activity gradient may be the end result, there must be signals that actively maintain or induce *BMP4* expression in the anterior ectoderm to achieve such a gradient. This is contrary to the passive view that *BMP4* is simply expressed where it is not inhibited.

Where would such a *BMP4* inducing signal arise? Based on the tissue interactions that regulate cement gland induction (reviewed in Sive and Bradley, 1995), there are two possible sources. First, a signal could be produced ventral to the cement gland, in the ventral ectoderm or ventral mesoderm, both of which repress cement gland formation. The ventral ectoderm is in close proximity to maximal *BMP4* expression throughout gastrulation. However, *BMP4* RNA levels run in a reverse gradient from what would be expected if ventral ectoderm were a source of an inducer. The ventral mesoderm is similarly limited, plus it lies at some distance from the cement gland primordium throughout gastrulation.

A second possible source for a *BMP4*-maintaining signal is the head endoderm, which lies directly underneath the cement gland primordium and highest levels of *BMP4* expression. Unlike ventral tissues, the head endoderm has been identified as a tissue that induces cement gland (Bradley, et al., 1996). Intriguingly, the endoderm is also a source of anterior inducers in chick (Foley, et al., 1997) and mouse (Thomas and Beddington, 1996; Varlet, et al., 1997).

However, head endoderm can only act on a neuralized ectodermal substrate, suggesting that head induction requires prior action of BMP antagonists (Bradley, et al., 1996; Foley, et al., 1997; Sive, et al., 1989). Furthermore, induction of the neurectoderm by the anterior endoderm in mouse also appears to take place at the time neural induction would be expected to occur, despite prior contact of these tissues (Hermesz, et al., 1996; Thomas and Beddington, 1996). In contrast, two secreted factors that are expressed in the head endoderm, cerberus (Bouwmeester, et al., 1996) and dickkopf (Glinka, et al., 1998), are sufficient to activate *otx2* and cement gland marker expression in uninduced animal caps. Why doesn't the head endoderm induce cement gland in animal caps if it is a source of factors that induce cement gland on their own? It is possible that the ratio of BMP4 to cerberus or dickkopf determines when the head inducers are active, and neither is expressed at high enough levels in the head endoderm to induce anterior markers in the absence of a BMP4-inhibiting activity. In support of this idea, cerberus activity is blocked by coexpression of BMP4 in animal caps (Bouwmeester, et al., 1996). This could also suggest that the head endoderm secretes a signal that maintains or induces BMP4. Therefore, only in the presence of BMP4 antagonists, or in ectoderm which has been cleared of BMP4 activity, would cerberus and dickkopf activity be revealed and cement gland induced.

3.3E A revised model for cement gland formation.

These data make several points regarding existing models for cement gland and neural induction, illustrated in Figure 3.6. During gastrulation, noggin and chordin diffuse from the mesoderm (Dosch, et al., 1997; Jones and Smith, 1998) and create a dorsal to ventral gradient of BMP4 protein in the ectoderm (Fig 3.6A; (Knecht and Harland, 1997; Wilson, et al., 1997). This clearing of BMP4 protein activates *otx2* (Lamb, et al., 1993; Sasai, et al., 1995) and allows cerberus and dickkopf, anterior signals from the underlying endoderm, to strongly activate and reinforce *otx2* expression in the anterior-most ectoderm as well (Bouwmeester, et al., 1996; Glinka, et al., 1998). The complete inhibition of BMP4 signaling plus *otx2* expression leads to the formation of neural tissue, while the coincidence of BMP4 protein/activated Smad1 and *otx2* results in cement gland induction.

How is *BMP4* expression regulated to achieve this effect? As neural induction occurs, noggin and chordin inhibit BMP4 protein (Piccolo, et al., 1996; Zimmerman, et al., 1996), leading to the induction of genes such as *otx2* in the dorsal ectoderm (Fig. 3.6B; Lamb, et al., 1993; Sasai, et al., 1995). *BMP4*

transcription is also inhibited dorsally, perhaps by activation of these factors in the ectoderm. Although a gradient of BMP4 protein is the end result, *BMP4* RNA is expressed at maximal levels in the region of the cement gland primordium. Since dorsal signaling inhibits *BMP4* expression, a signal from the ventral ectoderm or underlying anterior endoderm must maintain *BMP4* transcription in order to achieve this expression profile. Since BMP4 does not diffuse efficiently (Dosch, et al., 1997; Jones and Smith, 1998) and noggin is expressed in the anterior edge of the neural plate (Knecht and Harland, 1997), maximal production of BMP4 protein just ventral to noggin expression could help ensure a steep gradient of BMP4 activity (Fig. 3.6C).

3.4 Acknowledgments

Many thanks to Edoardo Boncinelli, Chris Kintner, Christof Niehrs, Paul Krieg, Richard Harland, and Jerry Thomsen for the kind gifts of plasmids. Special thanks to Vladimir Apekin for frog care, and to members of the Sive lab for many useful discussions.

Note: The work described in this chapter is being prepared as a manuscript, Gammill and Sive.

Figure 3.1 *otx2* induces patterned *NCAM* and *XCG* expression.
(A) One cell of a two-cell stage albino embryo was injected with 100 pg *otx2*-GR RNA mixed with 60 pg *lacZ* RNA as a lineage tracer. At mid-gastrula (stage 11.5), embryos were incubated without or with dexamethasone (dex) until tailbud (stage 20) or hatching (stage 28). **(B)** Injected embryos were stained for *lacZ* (blue) and processed for *NCAM* (purple, white arrowheads) and *XCG* (orange, black arrowheads) expression by in situ hybridization. Lateral view, anterior to the left, dorsal up. (a) -dex, harvest st.20; (b) -dex, harvest st.28; (c, e) +dex, harvest st.20; (d, f) +dex, harvest st.28.

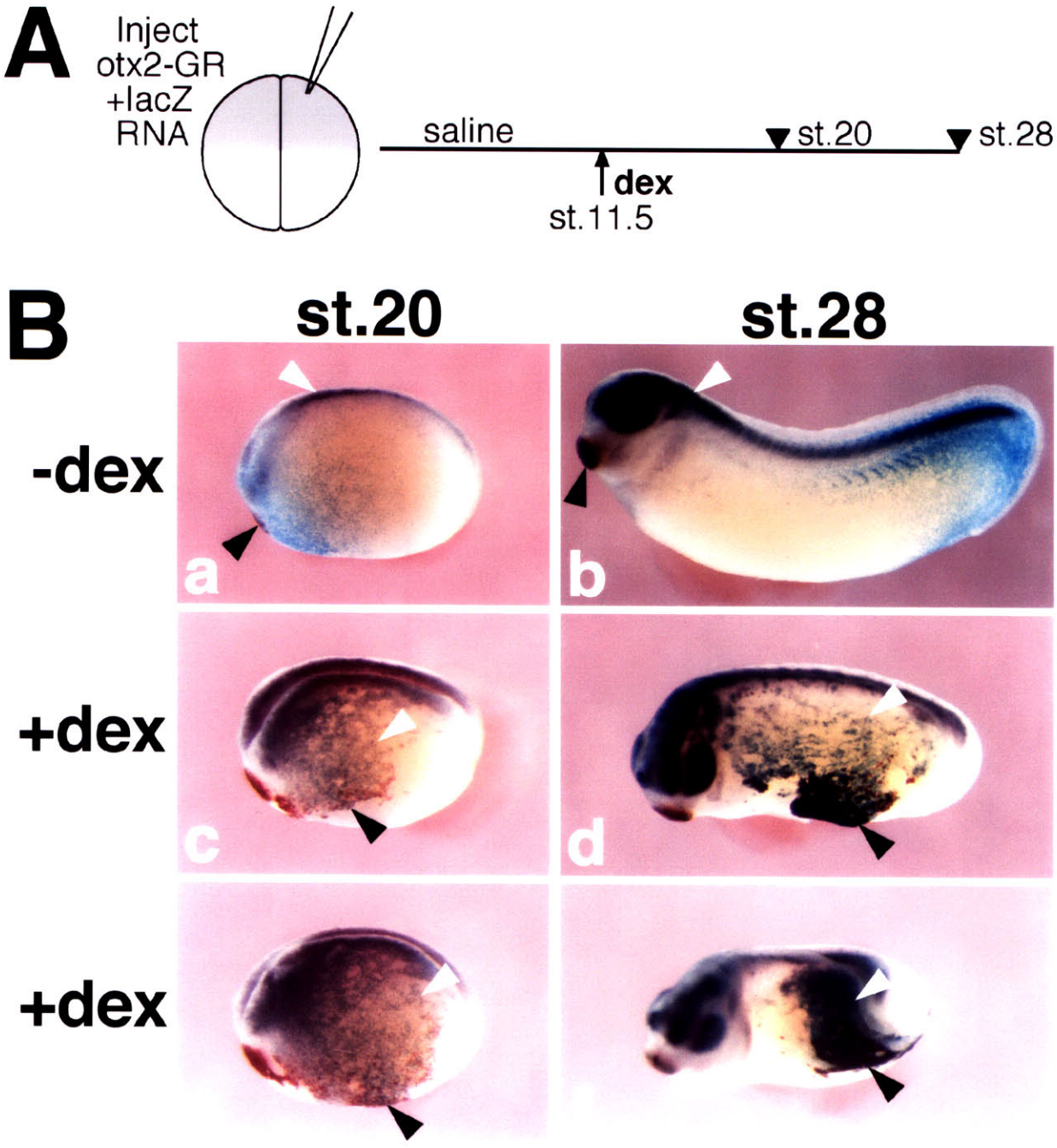

A
Inject
otx2-GR
+lacZ
RNA
saline
st.20
st.28
dex
st.11.5
B
st.20
st.28
-dex
+dex
+dex
a
b
c
d

Figure 3.2 *otx2* induces *NCAM* and *XCG* at different concentrations.
(A) Both blastomeres of a two-cell stage embryo were injected with different doses of *otx2* RNA. Animal caps were dissected at mid-blastula stage 9 and aged until mid-neurula stage 15. **(B)** Animal caps isolated from embryos injected with 10 pg to 400 pg *otx2* RNA as indicated were assayed by RT-PCR for expression of *XCG*, *XAG*, *NCAM*, *BMP4*, or *XVent-1* using *EF-1α* as a loading control. Lane 1, 400 pg *otx2*; lane 2, 200 pg *otx2*; lane 3, 100 pg *otx2*; lane 4, 50 pg *otx2*; lane 5, 20 pg *otx2*; lane 6, 10 pg *otx2*; lane 7, uninjected animal caps; lane 8, stage 15 whole embryo.

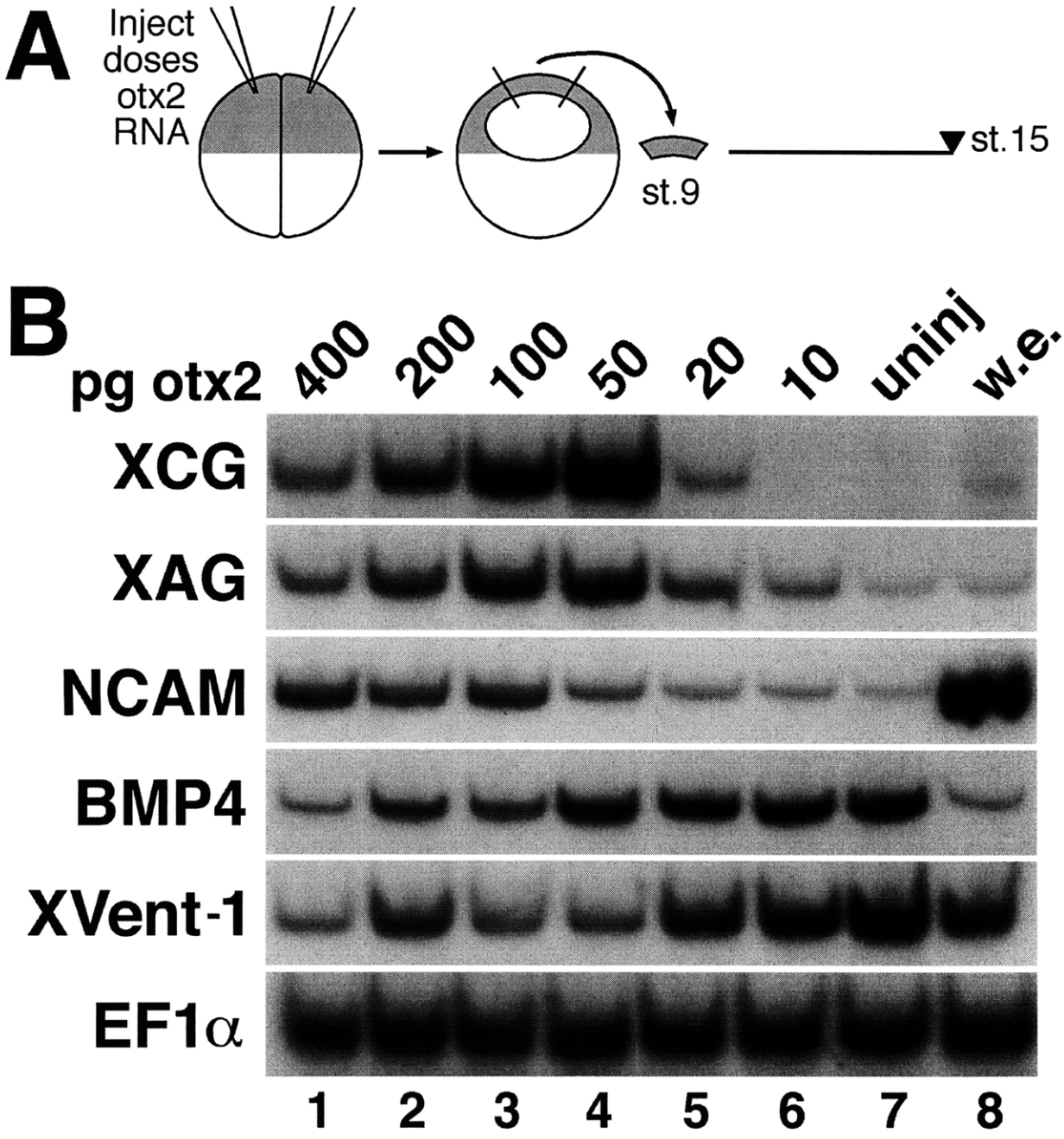

A
Inject
doses
otx2
RNA
st.9
st.15
B
pg otx2
400
200
100
50
20
10
uninj
w.e.
XCG
XAG
NCAM
BMP4
XVent-1
EF1α
1
2
3
4
5
6
7
8

Figure 3.3 Noggin induced cement gland formation correlates with *otx2* and *BMP4* coexpression.

(A) Both blastomeres of a two-cell stage embryo were injected with different concentrations of *noggin* RNA. Animal caps were dissected at mid-blastula stage 9 and aged until tailbud stage 20. **(B)** Animal caps isolated from embryos injected with 0.1 pg to 5 pg of *noggin* RNA as indicated were assayed by RT-PCR for expression of *XCG*, *NCAM*, *otx2*, or *BMP4* using *EF-1α* as a loading control. Lane 1, 5 pg *noggin*; lane 2, 2.5 pg *noggin*; lane 3, 1 pg *noggin*; lane 4, 0.5 pg *noggin*; lane 5, 0.25 pg *noggin*; lane 6, 0.1 pg *noggin*; lane 7, uninjected animal caps; lane 9, stage 20 whole embryo.

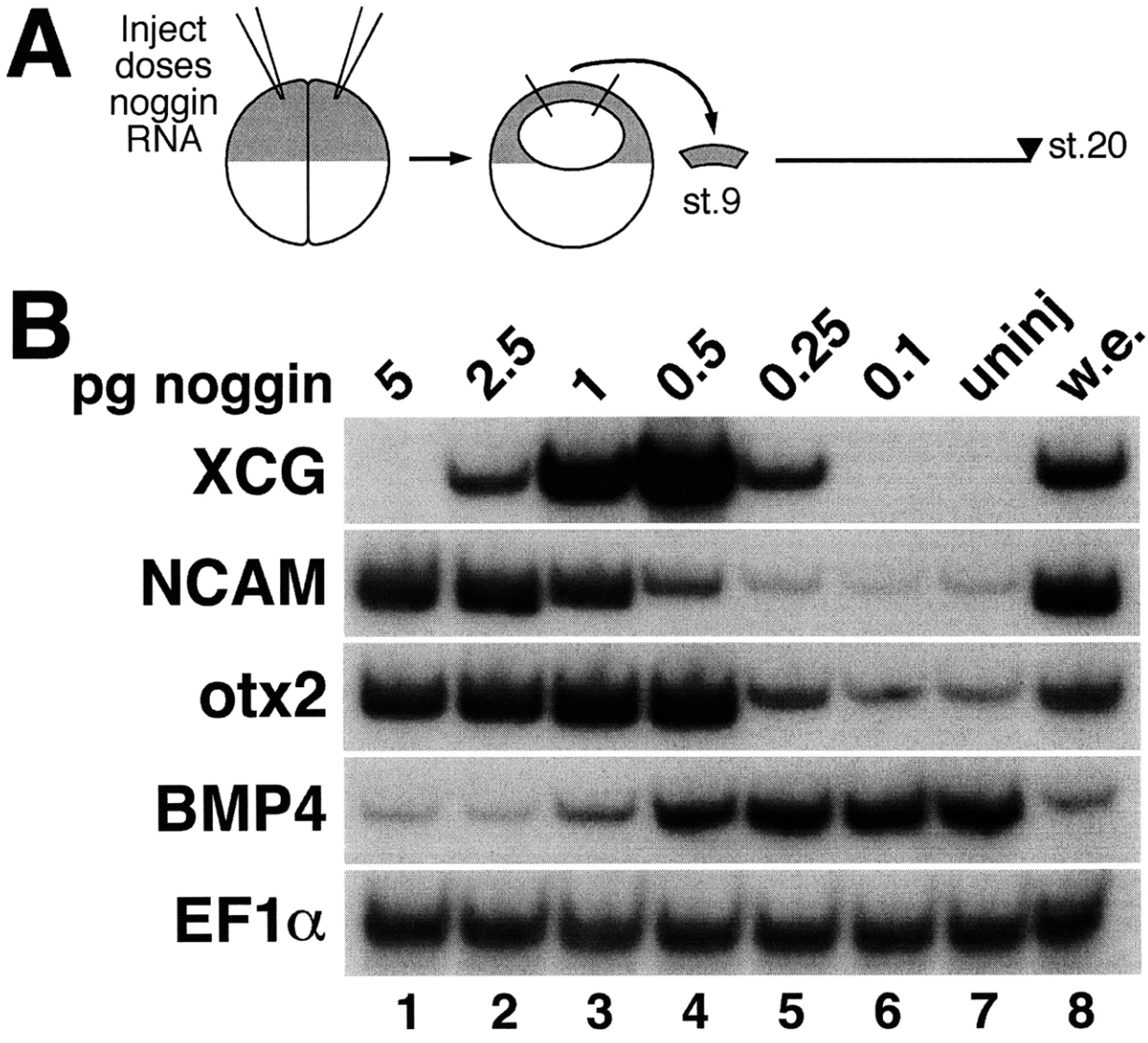

A
Inject
doses
noggin
RNA
st.9
st.20
B
pg noggin
5
2.5
1
0.5
0.25
0.1
uninj
w.e.
XCG
NCAM
otx2
BMP4
EF1α
1
2
3
4
5
6
7
8

Figure 3.4 *otx2* and *BMP4* expression overlaps in whole embryos.
Albino embryos at mid-gastrula (stage 11; a, b, c), early neurula (stage 13; d, e, f), or mid-neurula (stage 15; g, h, i) were analyzed by whole mount in situ hybridization for expression of *otx2* only (a, d, g), *otx2* (orange) and *BMP4* (purple; b, e, h), or *BMP4* only (c, f, i). White arrowheads point to the cement gland primordium, where *otx2* and *BMP4* expression overlap.

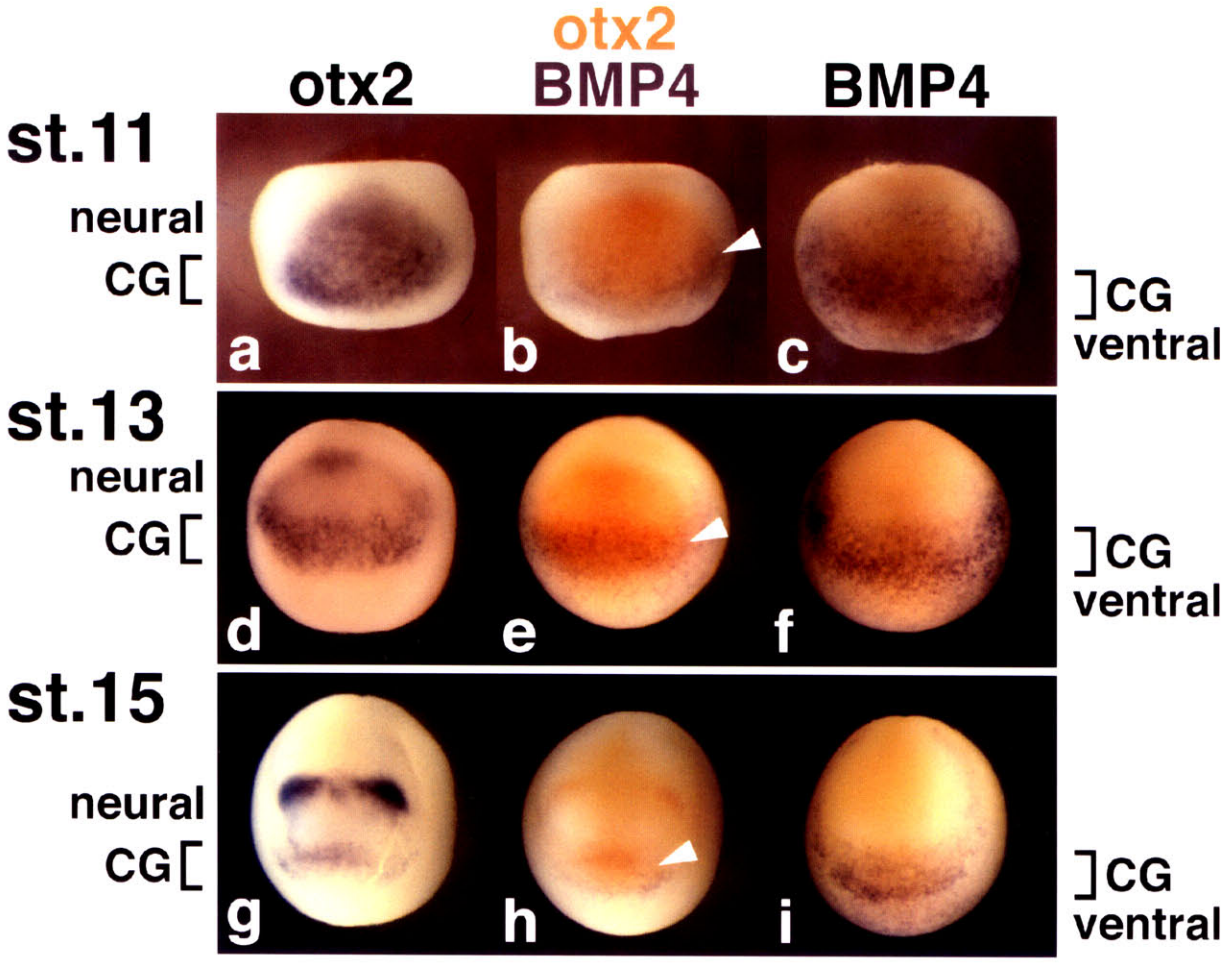

otx2
otx2
BMP4
BMP4
st.11
neural
CG[
]CG
ventral
a
b
c
st.13
neural
CG[
]CG
ventral
d
e
f
st.15
neural
CG[
]CG
ventral
g
h
i

Figure 3.5 *otx2* and *BMP4* coinjection induces cement gland.
(A) Both blastomeres of a two-cell stage embryo were injected with *otx2*-GR and/or *BMP4* RNA. Animal caps were dissected at mid-blastula stage 9, treated as indicated without or with dexamethasone (dex), and aged until mid-neurula stage 15. **(B)** Animal caps from embryos injected with 100 pg of *otx2*-GR and/or 1 ng BMP4 RNA were assayed by RT-PCR for expression of *XCG*, *NCAM*, or *XVent-1* using *EF-1α* as a loading control. Lane 1, uninjected, -dex; lane 2, *BMP4* injected, -dex; lane 3, *otx2*-GR injected, -dex; lane 4, *otx2*-GR injected, +dex; lane 5, *BMP4* and *otx2*-GR injected, +dex.

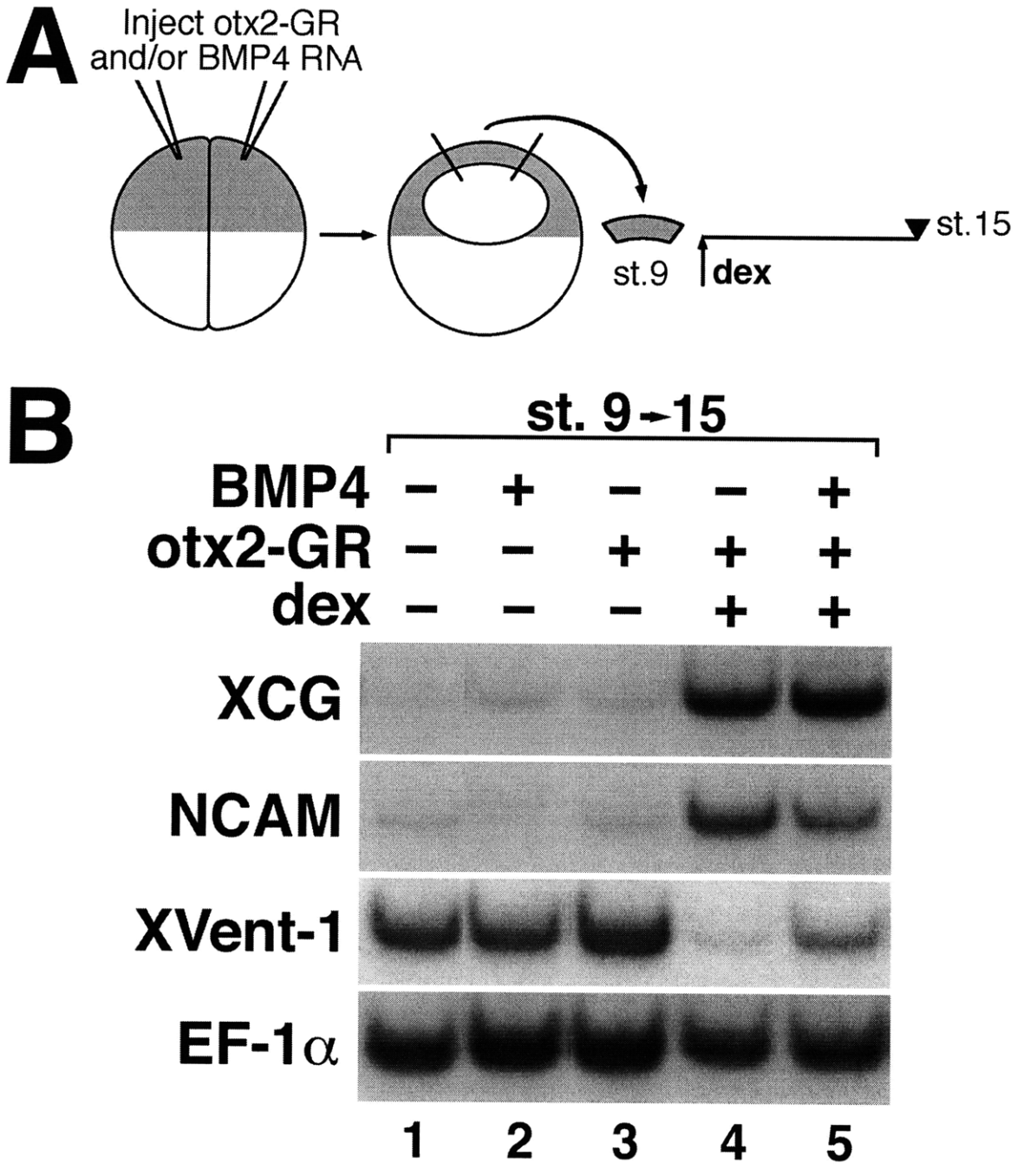
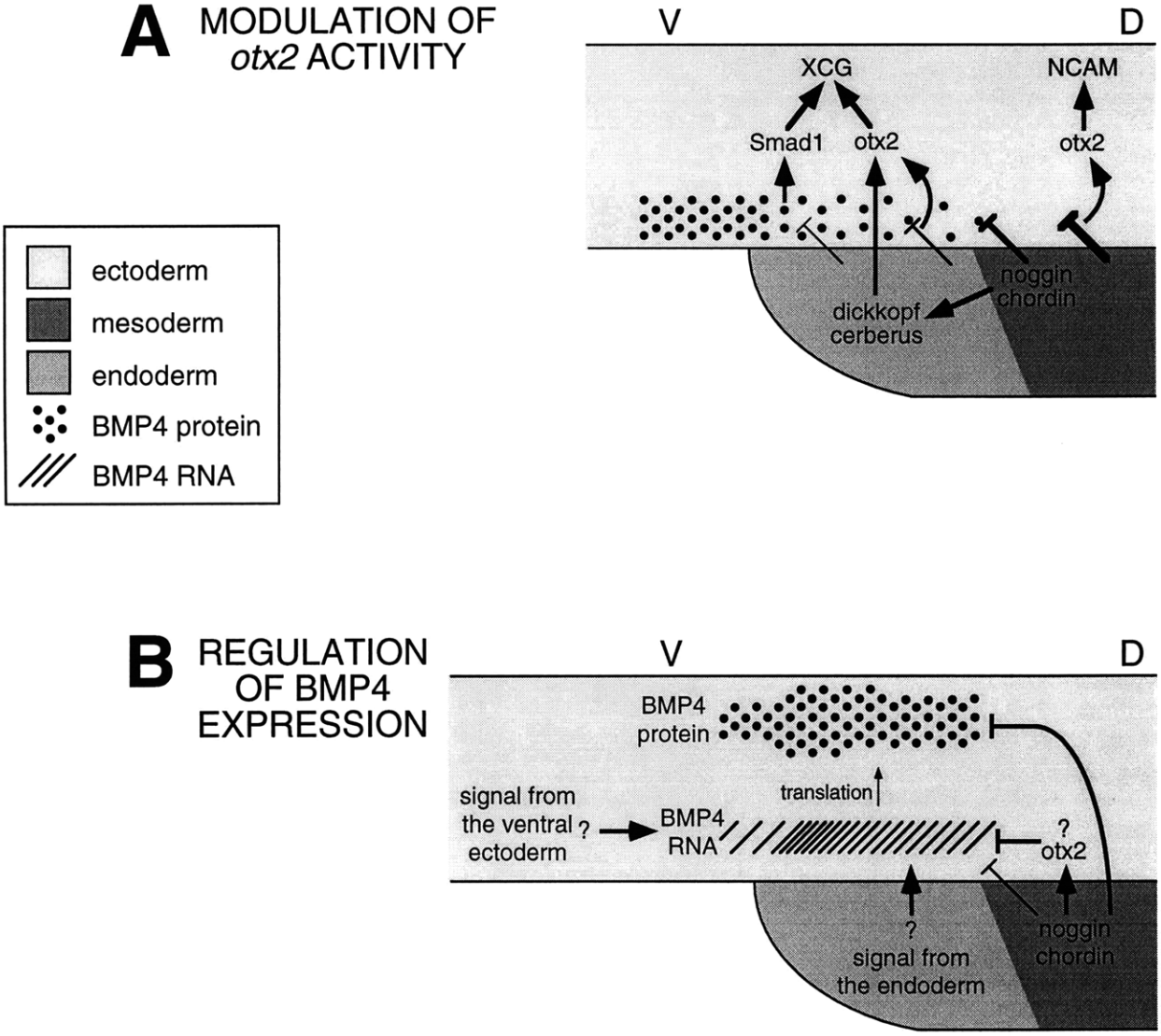

A
Inject otx2-GR
and/or BMP4 RNA
st.9
dex
st.15
B
st. 9→15
BMP4 – + – – +
otx2-GR – – + + +
dex – – – + +
XCG
NCAM
XVent-1
EF-1α
1 2 3 4 5

Figure 3.6 Cement gland formation and the BMP4 gradient.
(A) Modulation of *otx2* activity. A close-up of the anterior limit of dorsal mesendoderm involution is shown at late gastrula stage. Noggin and chordin (⋀) diffuse from the mesoderm (dark grey) to the ectoderm (light grey) where they inhibit BMP4 protein (•) and create a gradient of BMP4 activity. The dorsalizing effect of noggin and chordin activates cerberus and dickkopf expression in the endoderm (medium grey) and *otx2* in the ectoderm. cerberus and dickkopf reinforce *otx2* expression in the anterior-most ectoderm. In the absence of BMP4 protein, *otx2* induces neural tissue (*NCAM*), while in the presence of BMP4 and active Smad1, *otx2* induces cement gland (*XCG*). D is dorsal, V is ventral. **(B)** Regulation of BMP4 expression. Noggin and chordin inhibit BMP4 protein, but also prevent *BMP4* transcription (///), perhaps by activating expression of factors such as *otx2* in the ectoderm. *BMP4* expression is maximal in the cement gland primordium and must be maintained by active signaling, perhaps from the anterior endoderm or ventral ectoderm, in order to counteract inhibition during neural induction. **(C)** Predicted BMP4 gradient. Although BMP4 expression is highest in the cement gland primordium, diffusion of noggin creates a steep gradient of BMP4 protein.


A
MODULATION OF
otx2 ACTIVITY
V
D
XCG
NCAM
Smad1
otx2
otx2
noggin
chordin
dickkopf
cerberus
ectoderm
mesoderm
endoderm
BMP4 protein
BMP4 RNA
B
REGULATION
OF BMP4
EXPRESSION
V
D
BMP4
protein
translation
signal from
the ventral
ectoderm
?
BMP4
RNA
?
otx2
?
signal from
the endoderm
noggin
chordin
C
THE PREDICTED
BMP4 GRADIENT
noggin
chordin
BMP4 protein

Chapter 4. *otx2* maintains anterior specification and is required for cement gland formation.

4.1 Introduction

During gastrulation in *Xenopus*, the dorsal mesoderm involutes at the blastopore lip and induces the overlying ectoderm to give rise to neural tissue. Anteroposterior patterning of the neurectoderm takes place during this process in two steps, as the ectoderm is first "activated" to form neural tissue throughout the dorsal ectoderm and then "transformed" in posterior regions by a posteriorization signal (reviewed in Kolm and Sive, 1997). Since posterior specification is derived from anterior, posterior fates are said to be dominant to anterior (reviewed in Slack, et al., 1992). The anterior neurectoderm is induced by signals from both the axial mesoderm (Ang, et al., 1994; Ang, et al., 1993; Sive, et al., 1989) and the anterior head endoderm (Bradley, et al., 1996; Rhinn, et al., 1998; Thomas and Beddington, 1996; Varlet, et al., 1997). However, it is not clear how the responding ectoderm interprets these inductions to give rise to complex pattern within the anterior neural plate.

Throughout gastrulation, expression of one gene, *otx2*, faithfully delineates anterior position in the embryo. A homeobox gene, *otx2* is expressed in a broad, anterior domain that includes all presumptive anterior ectodermal derivatives (midbrain, forebrain, and associated non-neural cement gland), as well as the underlying mesendoderm (Blitz and Cho, 1995; Pannese, et al., 1995). Like its *Drosophila* homologue, the anterior gap gene *orthodenticle* (*otd*), *otx2* is clearly an essential early regulator of anterior development, since mice deficient for *otx2* lack all rostral head structures (Acampora, et al., 1995; Ang, et al., 1996; Matsuo, et al., 1995). Generation of mice chimeric for $otx2^{-/-}$ cells has demonstrated that wildtype visceral endoderm is sufficient to rescue formation of deleted neural tissue in *otx2* mutants (Rhinn, et al., 1998). However, the rescued neurectoderm in the chimeras was not appropriately patterned, indicating an additional requirement for *otx2* in the remaining mutant tissue: in the ectoderm and/or in the underlying mesendoderm, which induces *otx2* in the anterior neurectoderm (Ang, et al., 1994). Except in the midbrain/hindbrain boundary, where *otx2* is required specifically in the ectoderm for expression of *en-2* (Rhinn, et al., 1998), the necessity of *otx2* in each of these tissues has not been distinguished, and the requirement of *otx2* in the ectoderm during anterior pattern formation remains unclear.

We have examined the role of *otx2* during neurectodermal patterning and have begun to ask whether *otx2* is necessary in the ectoderm for establishment of pattern and subsequent differentiation of anterior structures such as the cement

gland. To address the ectodermal requirement of *otx2*, a dominant negative *otx2*-engrailed repressor fusion (*otx2*-En) was constructed by deleting the *otx2* transcriptional activation domain and replacing it with the engrailed repressor domain. As a result of this substitution, *otx2*-En is converted into a repressor and dominant negatively interferes with *otx2* activity, in theory by competitively binding to *otx2* promoter elements to repress rather than activate *otx2* targets (Conlon, et al., 1996).

We have previously demonstrated that *otx2* directly regulates formation of the anterior ectodermal adhesive organ, the cement gland (Gammill and Sive, 1997). Here, we show that anterior neural fates are also specified by *otx2* activity in whole embryos and in isolated ectoderm. Furthermore, *otx2* actively maintains anterior identities and represses posterior and ventral gene expression. Using the dominant negative *otx2*-En, we go on to demonstrate that *otx2* activity is required during cement gland formation. Further experiments are underway to specifically address the requirement of *otx2* in the ectoderm for determination of cement gland and anterior neural fates.

4.2 Results

4.2A Ectopic *otx2* expression modifies ectodermal patterning.

In order to consider the requirement of *otx2* during ectodermal pattern formation, it was first important to characterize the overall effect of *otx2* overexpression on pattern in the ectoderm and determine the nature of the neural tissue that was induced by ectopic *otx2* (Chapter 3). To this end, gene expression was examined by whole mount in situ hybridization in embryos injected with *otx2*-GR and *lacZ* RNA in one blastomere at the two-cell stage. The inducible *otx2* was used to limit *otx2* activity to late gastrula stages (Gammill and Sive, 1997). Embryos were left untreated or incubated in dexamethasone (dex) at mid-gastrula stage 11.5 to activate *otx2*-GR. Depending on the expression profile of the marker assayed, embryos were harvested at early neurula (stage 13 or 14), mid-neurula (stage 16), or hatching (stage 28 or 30). In uninjected embryos, *otx2* was expressed in a broad anterior domain at stage 13 (Fig. 4.1Aa) that resolved into a band of expression in the mid/hindbrain boundary and in the cement gland (not shown) at stage 16 (b). At hatching stages, *otx2* was still strongly expressed in the midbrain and more weakly in the cement gland, as well as in the eyes and in a patch in the forebrain (c).

4.2Ai General and anterior neural

The expression of the general neural markers *NCAM* (Fig. 4.1Ba, c; Kintner, et al., 1987) and *N-tubulin* (e; *N-tub*; Richter, et al., 1988) were normally restricted to the eye and neural tube of hatching stage embryos. Following activation of *otx2*-GR at mid-gastrula, *NCAM* expression was expanded dorsally, often to the extent that an eye was no longer discernible on the injected side (b, white arrowhead). Ectopic *NCAM* was also induced ventrally (d). A similar effect was observed when *N-tubulin* (f) and *nrp-1* (another general neural marker, Knecht, et al., 1995; data not shown) were examined. To determine the character of this ectopic neural tissue, anterior neural markers were assayed. *Lipocalin* (*cpl-1*) was normally expressed at the anterior edge of the neural plate in early neurula embryos (Fig. 4.1Ca) and in a complex pattern in the anterior brain at hatching stage (c; Knecht, et al., 1995). Activation of *otx2*-GR at mid-gastrula resulted in a posterior expansion of the *lipocalin* domain at both stages (b, black arrowhead; d, white arrowhead). In addition, ectopic *lipocalin* expression was much stronger than endogenous levels in hatching stage embryos (compare c and d), with staining concentrated at the edge of the region expressing *otx2*-GR (d). Ectopic *lipocalin* expression was observed in the ventrolateral ectoderm of *otx2*-GR injected embryos, but at very low levels (data not shown). Another anterior marker, *XBF*, was restricted to the telencephalon in untreated hatching embryos (e; Papalopulu and Kintner, 1996). When *otx2*-GR was activated at mid-gastrula, *XBF* was induced posteriorly in the brain and spinal cord (f, white arrowhead). Ectopic *XBF* expression was also excluded from the middle of the *otx2*-GR expressing domain (f), similar to the effect observed in embryos stained for *lipocalin* (d). *XBF* expression was not detectable in the ventrolateral ectoderm. These data showed that ectopic *otx2* activity induced anterior neural tissue in the posterior dorsal ectoderm.

4.2Aii Midbrain/hindbrain boundary

otx2 has been implicated in the formation of the mid/hindbrain boundary (Acampora, et al., 1997; Rhinn, et al., 1998; Suda, et al., 1996), so we examined markers of this region in *otx2*-GR injected embryos as well. *gbx-2*, which is required for establishment of the mid/hindbrain organizer region (Wassarman, et al., 1997), was expressed in a stripe corresponding to the anterior hindbrain and in the ventrolateral ectoderm of untreated early neurula embryos (Fig. 4.1Da; von Bubnoff, et al., 1996). When *otx2*-GR was activated at mid-gastrula, ectopic *gbx-2* was strongly induced throughout the posterior-dorsal ectoderm, except in cells

ectopically expressing *otx2*-GR and never anterior to the normal extent of *gbx-2* expression (b, white arrowhead). When *lacZ* staining overlapped endogenous *gbx-2* domain, expression in that region was ablated (b). In the ventrolateral ectoderm, *otx2*-GR ablated, but did not increase, *gbx-2* expression (data not shown). Similarly, at early neurula, *engrailed-2* (*en-2*) marked the mid/hindbrain boundary in embryos not treated with dex (c; Hemmati-Brivanlou, et al., 1991), while *otx2*-GR activity induced ectopic *en-2* expression along the edges of the *lacZ* expressing cells and ablated endogenous *en-2* expression within the *lacZ* domain (d, black arrowhead). Ectopic staining was never observed in anterior neural tissue, and was strongest in the future hindbrain. At hatching stages, the mid/hindbrain expression of *en-2* (e) was greatly expanded in embryos with active *otx2*-GR, and *en-2* expressing cells were even observed in the lateral ectoderm (f, white arrowhead). These results demonstrated mid/hindbrain boundary markers were induced at edges of *otx2* expression and were detected even in the ventrolateral ectoderm.

4.2Aiii Posterior neural and ventral ectoderm

Since anterior neural gene expression was expanded posteriorly and ventrally by ectopic *otx2* activity, we wondered what effect this had on expression of posterior and ventral markers that do not normally overlap with *otx2*. *Krox20* was expressed in two stripes in the hindbrain of mid-neurula embryos incubated in the absence of dex (Fig. 4.1Ea; Bradley, et al., 1993). Following activation of *otx2*-GR at mid-gastrula stages, this expression was ablated (b). A more posterior marker, *HoxB9*, was expressed in the future spinal cord of mid-neurula untreated embryos (c; Sharpe, et al., 1987). Activation of ectopic *otx2*-GR completely abolished *HoxB9* expression as well (d). The suppression of posterior fates was even more striking when *N-tubulin* was examined. In *Xenopus*, neuronal differentiation, marked by *N-tubulin* expression, occurs more quickly in the posterior than in the anterior neural plate (Papalopulu and Kintner, 1996). Thus, at mid-neurula stages, *N-tubulin* was expressed in three longitudinal stripes exclusively in the posterior dorsal ectoderm of untreated embryos (e). When *otx2*-GR was activated at mid-gastrula, however, this expression was ablated (f).

To determine whether *otx2* can inhibit ventral gene expression in whole embryos as it does in animal caps (Fig. 3.2B), ventral markers were also examined. *GATA-2* was expressed throughout the ventrolateral ectoderm in early neurula embryos cultured in the absence of dex (Fig. 4.1Fa; Kelley, et al., 1994). Activation of *otx2*-GR by dex treatment at mid-gastrula abolished *GATA-2* expression in *lacZ*

expressing cells (b). Likewise, *XVent-1* was normally expressed in a posterior ventral domain at early neurula (c; Gawantka, et al., 1995) that was inhibited by *otx2*-GR activity (d). *Vox/XVent-2* was also expressed in a ventral domain in early neurula untreated embryos (e; Onichtchouk, et al., 1996) that was ablated in the presence of active *otx2*-GR at mid-gastrula (f). These data showed that *otx2* inhibited posterior neural and ventral gene expression.

4.2Aiv Mesoderm

To address whether *otx2* was affecting ectodermal gene expression by altering patterning in the mesoderm, mesodermal markers were assayed in *otx2*-GR injected embryos. *goosecoid* was expressed in the prechordal plate in early neurula embryos that developed in the absence of dex (Fig. 4.1Ga; Cho, et al., 1991). Surprisingly, when *otx2*-GR was activated by dex treatment at mid-gastrula, *goosecoid* was induced posteriorly in what appeared to be the notochord (b; black arrowhead). Ectopic goosecoid was not detected in the ectoderm. In contrast, *brachyury*, which was expressed in the notochord and in a ring around the blastopore at early neurula (c; Smith, et al., 1991), was ablated by active *otx2*-GR (d). The ability of *otx2* to modify mesodermal patterning during gastrula stages was further demonstrated by examining *muscle actin* (*m-actin*), which was normally expressed in somites of hatching stage embryos (e; Gurdon, et al., 1985). This expression was abolished by activation of *otx2*-GR at mid-gastrula stage (f). These results showed that *otx2* was able to promote anterior and inhibit posterior mesoderm as late as mid-gastrula stage.

Taken together, the expression data shown in Fig. 4.1 demonstrated that *otx2* activated anterior neural and anterior mesodermal fates while suppressing posterior neural, ventral ectodermal, and posterior mesodermal fates. *otx2* most effectively activated markers of the mid/hindbrain boundary. These markers were induced at the edge of ectopic *otx2* expression domains in the dorsal and ventral ectoderm.

4.2B *otx2* induces anterior and represses ventral fates in isolated ectoderm.

The changes in ectodermal patterning that were observed in whole embryos could have been the direct result of the activity of *otx2* in the ectoderm, or a secondary effect of the anteriorized underlying mesoderm. In order to isolate the ectoderm from the effects of the mesoderm, we examined gene expression in ectodermal explants ectopically expressing *otx2*-GR. Embryos were injected with *otx2*-GR RNA at the 8-cell stage, targeting either the dorsal or ventral blastomeres

(Fig. 4.2A). At stage 11.5, posterior dorsal or ventral ectoderm was dissected and incubated without or with dex for 3.5 hours until early neurula, when gene expression was assayed by RT-PCR (A).

The effects observed in isolated ectoderm were very similar to those described in Fig. 4.1, although in many cases a different panel of markers was examined because of the stage of harvest. Dorsal ectoderm expressed low levels of the general neural markers *nrp-1* and *neurogenin-1* (*ngnr-1*; Ma, et al., 1996) in the absence of dex (Fig. 4.2B, lane 1), and these levels increased in the presence of active *otx2*-GR (lane 2). However, the cement gland marker *XCG* was not normally expressed in this piece of dorsal ectoderm (lane 1) and was not induced following activation of *otx2*-GR (lane 2). This is consistent with the ventrolateral restriction of cement gland formation observed in whole embryos (Fig. 2.1B, Gammill and Sive, 1997). When cultured without dex, dorsal ectoderm expressed moderate levels of anterior neural markers *opl* (Kuo et al., in press) and *Xanf-1* (Zaraisky, et al., 1992), although the mid/hindbrain marker *engrailed-2* (*en-2*) was not expressed (lane 1). Activation of *otx2*-GR increased *opl* and *Xanf-1* expression levels and dramatically induced *en-2* (lane 2). Since dorsal ectoderm closest to the blastopore lip was dissected, this explant unfortunately did not include the lateral tissue specified as hindbrain and spinal cord, as *Krox20* and *HoxB9* were not expressed in tissue cultured with or without dex (lanes 1 and 2). Although ectoderm close to the mesoderm was dissected, explants did not contain mesoderm as indicated by background expression of the prechordal plate marker *goosecoid* and the notochord marker *brachyury* (lane 1). Activation of *otx2*-GR led to a slight increase in *goosecoid* RNA levels (lane 2), however, this most likely represented ectodermal expression of *goosecoid* (Thisse, et al., 1994). Gene expression was not altered when uninjected dorsal ectoderm was incubated in dex (data not shown).

Similar results was obtained when ventral ectoderm expressing *otx2*-GR was examined. Very low (background) or no expression of neural, cement gland, and anterior neural markers were observed in ventral ectoderm cultured without dex (lane 4). However, when *otx2*-GR was activated, these markers were induced, although *opl* was only weakly induced (lane 5). Ventral ectodermal markers *XVent-1*, *Vox*, *Xhox3* (Ruiz i Altaba, 1990), and *BMP4* (Schmidt, et al., 1995) were normally expressed in the ventral ectoderm (lane 4). In the presence of active *otx2*-GR, ventral marker gene expression was inhibited (lane 5). Inhibition was probably incomplete due to uneven distribution of the *otx2*-GR RNA, since

equivalent targeted injections with *GFP* RNA indicated that injected RNA did not always spread evenly throughout the targeted region (data not shown). *XMad-1*, which transduces BMP4 signaling into the nucleus (Graff, et al., 1996), was expressed in untreated ventral ectoderm (lane 4) and was not affected by the presence of active *otx2*-GR (lane 5).

These results demonstrated that *otx2* was able to promote anterior and neural fates and repress ventral fates in isolated dorsal and ventral ectoderm.

4.2C *otx2* is a transcriptional activator.

In order to determine whether *otx2* activity was required to specify anterior and repress posterior and ventral fates in the ectoderm, we wanted to construct a dominant negative *otx2*. First, it was necessary to confirm that *otx2* was a transcriptional activator and to map the activation domain. Mouse *otx2* can activate gene expression through a bicoid target sequence (Simeone, et al., 1993), so it was anticipated that *Xenopus otx2* would positively regulate gene expression as well. The "activin responsive element" (DE) in the *goosecoid* promoter was chosen as a reporter since *goosecoid* appears to be a target of *otx2* (Fig 4.1Gb; Pannese, et al., 1995) and this element contains a paired-class binding site of the type recognized by *otx2* (Watabe, et al., 1995; Wilson, 1993). Coinjection of *otx2* RNA and a DNA luciferase reporter construct containing this element (Watabe, et al., 1995) gave a 2.5-fold increase in luciferase activity (10699 activity units) over injection of the reporter construct alone (4276 activity units), indicating that *otx2* was a transcriptional activator.

A series of deletions was constructed in MT-*otx2* to identify the *otx2* domain responsible for cement gland induction. Since the homeobox in *otx2* was located about 100 base pairs after the transcription start site, analysis was concentrated on the 3' end of the gene (Fig. 4.3A). Deletions were created by restriction endonuclease digestion and were tested by microinjecting RNA transcribed from each construct into embryos at the two-cell stage (Fig. 4.3B). Animal caps were isolated at late blastula stage 9 and assayed at mid-neurula stage 15 by RT-PCR for expression of the cement gland markers *XCG* and *XAG*. When full-length *otx2* was injected, *XCG* was induced 50-fold and *XAG* was induced 7.4-fold compared to expression in uninjected animal caps (Fig. 4.3C, lanes 1 and 2). Injection of constructs in which *otx2* was deleted in the carboxyl terminus, however, led to essentially background expression of *XCG* and *XAG* (1.4 to 3.3-fold difference in *XCG* expression; 0.5 to 1.8-fold difference in *XAG* expression;

compare lanes 1 and 3-6). An internal 61 amino acid deletion of *otx2* (NgoMI-BsmBI) retained cement gland inducing activity, activating *XCG* expression 29-fold and *XAG* expression 8.5-fold over uninjected levels (lane 6). It was possible that the deleted constructs did not induce cement gland because they were no longer translated or were not transported to the nucleus. This was addressed by Western analysis and immunocytochemical analysis of animal caps from embryos injected with deleted MT-*otx2*. Protein was expressed at equivalent levels from all constructs and was nuclear (data not shown). Together, these data showed that the domain of *otx2* required to induce cement gland resides in the 47 amino acids of the carboxyl terminus. In support of this conclusion, *otx2* constructs deleted for this region and replaced with the glucocorticoid receptor ligand binding domain, which contains an activation domain, retain the ability to induce cement gland (data not shown, see Appendix C and Gammill and Sive, 1997).

4.2D A dominant negative *otx2* inhibits cement gland formation.

A dominant inhibitory *otx2* was constructed by replacing the 3' region of *otx2*, including the activation domain, with the engrailed repressor domain (Fig. 4.4A; Conlon, et al., 1996; Han, et al., 1993). Although previously described engrailed fusions have used a large fragment of engrailed (see for example Conlon et al., 1996), *otx2* was fused to the minimal repressor domain, which mediates active repression (Han and Manley, 1993). This was done to avoid including a region of engrailed located immediately upstream of the minimal repressor, the highly conserved eh-1 repressor domain (Smith and Jaynes, 1996). This domain appears in diverse homeodomain proteins, including goosecoid (Smith and Jaynes, 1996). otx2 and goosecoid are both paired class homeodomain proteins that bind as a homo- or heterodimers to palindromic TAAT sites spaced by three nucleotides (P3C sites, Wilson, 1993). The goosecoid eh-1 domain was recently shown to confer repression by mediating dimerization of goosecoid with other P3C homeodomains (Mailhos, et al., 1998). Consequently, this region might have been expected cause otx2-En to interact with proteins that otx2 would not have recognized and have an activity similar to that of goosecoid.

The ability of *otx2*-En to interfere with cement gland formation was first examined in an animal cap assay. Embryos were injected at the two-cell stage with *otx2* RNA with or without 10-fold excess of *otx2*-En RNA, and animal caps were dissected at late blastula stage 9 (Fig. 4.4B). Explants were cultured to

hatching stage 30 and gene expression was assayed by RT-PCR. Injection of *otx2*-En alone did not affect expression of *XCG*, *XAG*, or the general neural marker *NCAM* (Fig. 4.4C, lane 1). Meanwhile, *otx2* induced massive expression of *XCG* and *XAG* and moderate levels of *NCAM* RNA (lane 2). This induction was completely blocked by coinjection of *otx2*-En (lane 3), indicating that the engrailed repressor fusion abolished *otx2* activity.

To assess the specificity of the dominant negative, the ability of *otx2*-En to interfere with the activity of another paired class homeodomain protein, siamois, was assayed. Embryos were injected at the two-cell stage with RNA for *siamois*, *otx2*-En, or both (Fig. 4.5A). Animal caps were isolated at late blastula stage 9 and cultured 4 hours until early gastrula stage 10.5 when gene expression was assayed by RT-PCR. Injection of *siamois* alone induced expression of the organizer genes *goosecoid, chordin*, and *noggin* (Fig. 4.5B, lane 1; Carnac, et al., 1996). Coinjection of *siamois* and *otx2*-En led to equivalent if not increased expression of the organizer transcripts (lane 2). The observed increase was most likely due to experimental variation and probably not significant, as in another experiment, coexpression of *siamois* and *otx2*-En produced a slight decrease in levels of these markers (data not shown). Injection of *otx2*-En alone did not affect organizer gene expression, resembling uninjected explants (lanes 3, 4).

otx2-En was next tested for the ability to inhibit endogenous cement gland formation. To further demonstrate that *otx2*-En specifically interfered with *otx2* activity, the ability of wildtype *otx2* to rescue dominant negative phenotypes was also assayed. Embryos were injected with *otx2*-En and *lacZ* RNA without or with *otx2* in equal amounts or in two-fold excess (Fig. 4.6A). Embryos were allowed to develop until hatching stage and scored for cement gland formation when lineage tracer overlapped with the region of the cement gland. Overexpression of *otx2*-En alone inhibited or reduced cement gland formation in 65% of the embryos assayed (Figs. 4.6Ba, 4.6Ca). Mixing an equal amount of wildtype *otx2* (Bb and Cb; 22% inhibited or reduced) or two-fold excess *otx2* (Bc and Cc; 28% inhibited or reduced) with the injected *otx2*-En restored cement gland formation to control levels (Bd and Cd; 24% inhibited or reduced).

These data showed that the dominant negative *otx2*-En inhibited cement gland formation. This effect was likely due to interference with *otx2* activity since *otx2*-En did not inhibit another paired class homeodomain, siamois, and the *otx2*-En phenotype was rescued by excess *otx2*.

4.3 Discussion

By overexpressing wildtype and dominant negative versions of *otx2*, we have characterized the effect of *otx2* activity in the neurectoderm and have begun to address the requirement of *otx2* for the determination of anterior fates. *otx2* was sufficient to induce anterior neural markers in posterior ectoderm, indicating that posterior gene expression is not dominant to anterior despite posterior dominance during neural induction. An edge of *otx2* expression may be important for establishing the mid/hindbrain boundary, as the boundary-specific marker, *engrailed-2*, and the anterior hindbrain marker, *gbx-2*, were induced when a new border of *otx2* expression is created. *otx2* was also able to repattern the mesoderm, promoting a prechordal fate. Characterization of a dominant negative *otx2* demonstrated that *otx2* activity was required for head and cement gland formation. Taken together, these data indicate that *otx2* plays an active role in specifying anterior neurectodermal fates through its activity in both the ectoderm and the mesoderm. Additional experiments will address whether this activity is necessary.

4.3A *otx2* activates and maintains anterior neural fates.

When *otx2* was overexpressed in whole embryos, anterior neural markers were induced and posterior and ventral markers were repressed (Fig. 4.1). This indicates that *otx2* expression maintains anterior identity in the neurectoderm by inhibiting posterior and ventral gene expression. Since posteriorly expressed genes *Xcad-2* (Epstein, et al., 1997) and *HoxD1* (Kolm, 1997) have likewise been shown to repress expression of *otx2*, anteroposterior patterning seems to involve a complex regulatory network of mutual anterior and posterior repression. These activities could be critical for sustaining regional specification as pattern is induced and elaborated during gastrulation. This hypothesis is supported by the anterior expansion of posterior gene expression in *otx2* mutant chimeras (Rhinn, et al., 1998) and the expansion of hindbrain at the expense of midbrain in mice lacking *otx1* and heterozygous for an *otx2* mutation (Acampora, et al., 1997).

The ability of *otx2* to inhibit posterior gene expression is interesting given the theory of posterior dominance during neural induction and patterning. This idea evolved from the activation-transformation model for neural induction, in which posterior neural tissue is derived from anterior (discussed in Slack and Tanahill, 1992). In support of posterior dominance, posteriorizing agents such as retinoic acid and FGF inhibit expression of anterior genes like *otx2* (Gammill and

Sive, 1997; Pannese, et al., 1995; Pownall, et al., 1996). However, the inhibition of posterior neural markers by *otx2* indicates that, while the posterior signal may be dominant to anterior, anterior and posterior gene expression are mutually inhibitory, as discussed above. Interestingly, even the dominance of posterior signals is limited, since the anterior ectoderm becomes insensitive to retinoic acid at late gastrula stages (Appendix A, Avantaggiato, et al., 1996; Ruiz i Altaba and Jessell, 1991a; Simeone, et al., 1995; Sive, et al., 1990). The change in sensitivity to retinoic acid may be due to changes in responsiveness to retinoic acid, for example retinoic acid receptor gene expression, but could also be realized by stabilization of posterior-repressive anterior gene expression, for example, initiation of the *otx2* autoregulatory loop at mid gastrula (Gammill and Sive, 1997).

Although patterning was altered in the neurectoderm, *otx2* was not very effective at inducing anterior neural markers in the ventral ectoderm of whole embryos, despite induction of general neural markers there (Fig. 4.1). Although the dearth of early, anterior-specific, strongly expressed neural markers make an absolute conclusion difficult, this result does suggest that neural induction and anterior pattern can to some extent be separated. Since most anterior neural gene expression was efficiently activated in isolated ventral ectoderm (Fig. 4.2), this indicates that a signal in the embryo, perhaps from the ventral mesoderm, overrides the patterning influences of *otx2*. Since *otx2* can inhibit BMP4 expression in whole embryos (data not shown) and is likely to induce neural tissue through this mechanism (see Chapter 3), it is probably not BMP4, but some other factor which inhibits the anterior neural patterning effect of *otx2* ventrally.

4.3B The posterior border of *otx2* expression is involved in establishing the mid/hindbrain boundary.

The mid/hindbrain boundary occurs at the interface of anterior and posterior domains of gene expression, and has been proposed to form in part as a result of repressive interactions between *otx2* anteriorly and *gbx-2* posteriorly (von Bubnoff, et al., 1996; Wassarman, et al., 1997). This model is supported by expansion of *otx2* expression in *gbx-2* mutant mice and expansion of mid/hindbrain markers in *otx2* mutant chimeras (Rhinn, et al., 1998; Wassarman, et al., 1997). Overexpression of *otx2* further supports this idea, as *gbx-2* was ablated when it fell within a region of ectopic *otx2* expression.

However, our data suggest that the relationship between *otx2* and *gbx-2* is complex, and that it is not simply *otx2* expression per se, but the boundary of *otx2* expression that is important for early mid/hindbrain patterning. Although *otx2* ablated *en-2* and *gbx-2* when it covered the mid/hindbrain region, both markers were induced in the neurectoderm at the edges of ectopic *otx2* expression. This surprising induction of *gbx-2* demonstrates that, although *otx2* and *gbx-2* interact repressively during mid/hindbrain organizer formation, *otx2* is involved in activating *gbx-2* expression. Furthermore, ectopic *otx2* RNA was able to induce *en-2* expression in the ventrolateral ectoderm of whole embryos at mid-neurula (data not shown) and hatching stages (Fig 4.1Df). These data indicate that *otx2* expression is sufficient to initiate at least some aspects of mid/hindbrain boundary formation. Interestingly, *otx2* has also recently been shown to be required in the ectoderm for *en-2* expression (Rhinn, et al., 1998). However, additional factors must regulate this activity of *otx2*, since ectopic *en-2* and *gbx-2* staining were most strongly induced in the region of the future hindbrain and never observed anterior to the region corresponding to the mid-hindbrain boundary (where *otx2* is already expressed). Taken together, these data suggest that the overlap of the posterior edge of *otx2* expression and factors expressed in the anterior hindbrain are involved in positioning the mid/hindbrain boundary. The posterior border of *otx2* expression has been described as a marker of mid-hindbrain boundary in chick (Millet, et al., 1996), and *wnt-1* is required to establish the *otx2* expression border (Bally-Cuif, et al., 1995), but no functional relationship has previously been suggested.

It is somewhat surprising that *otx2* can activate expression of the posterior gene *gbx-2*. This induction is non-cell autonomous, as *gbx-2* expression was ablated in *lacZ/otx2*-GR expressing cells and induced in neighboring cells. Furthermore, this induction clearly required additional factors, since *gbx-2* was not induced by *otx2* ventrally. It could be that, in the neural plate, *otx2* stimulates expression of mid/hindbrain organizer secreted factors, such as FGF8 or *wnt1*, and that these factors induce *gbx-2* expression in cells not expressing *otx2*. Since *otx2* and *gbx-2* expression domains abut, it has been previously suggested that *otx2* might establish the anterior border of *gbx-2* (von Bubnoff, et al., 1996).

4.3C *otx2* alters mesodermal patterning.

Although mesoderm induction and patterning occur during blastula stages (reviewed in Heasman, 1997), ectopic *otx2* activity at mid-gastrula stage was

sufficient to repattern the mesoderm, as indicated by inhibition of *brachyury* expression and induction of the prechordal plate marker *goosecoid* (Fig. 4.1). Although constitutive expression of *otx2* can induce ectopic mesoderm (Blitz and Cho, 1995; Pannese, et al., 1995), it is important to note that ectopic *goosecoid* was not observed except in the mesoderm, indicating that *otx2*-GR did not induce de novo mesoderm formation at this stage. The ability of *otx2* to repattern mesoderm at such a late stage is striking, indicating that mesodermal patterning is not fixed during gastrulation. However, this activity is consistent with the ability of *otx2* to convert tail organizer into head organizer (Andreazzoli, et al., 1997). Although it has not been determined whether *otx2* function is required in the prechordal plate of mouse embryos (Rhinn, et al., 1998), these observations suggest that *otx2* is involved in the establishment or maintenance of mesodermal pattern.

4.3D Is *otx2* required in the ectoderm for cement gland and anterior neural fates?

To address the requirement of *otx2* for the patterning events discussed above, a dominant negative *otx2*, *otx2*-En, was created. When *otx2*-En was overexpressed in embryos, head structures including the cement gland were ablated (Fig. 4.6), essentially phenocopying mice that lack *otx2* (Acampora, et al., 1995; Ang, et al., 1996; Matsuo, et al., 1995). These defects were rescued by coexpression of wildtype *otx2*, suggesting that the deletion phenotype was due to interference with *otx2* function. This conclusion is supported by the demonstration that *otx2*-En inhibits *otx2* activity (Fig. 4.4), but not activity of another paired class homeodomain, *siamois* (Fig. 4.5). This indicates that *otx2* is required for head formation in *Xenopus* as it is in mice.

To understand the role of *otx2* during neurectodermal patterning, however, it is critical to address the specific requirement of *otx2* in the neurectoderm. Mouse chimeras containing a mixture of *otx2* mutant and wildtype cells have indicated that *otx2* activity is required in the visceral endoderm for proper formation of the anterior neural plate (Rhinn, et al., 1998). Unfortunately, the presence of mutant cells in both the neurectoderm and prechordal plate of these chimeras preclude conclusions regarding the necessity of *otx2* specifically in the neurectoderm, although conjugates of *otx2* mutant ectoderm and wildtype mesoderm demonstrated that *otx2* is required in the ectoderm for induction of *en-2* (Rhinn, et al., 1998). However, this question can be much more broadly addressed in *Xenopus*, where constructing conjugates of

ectoderm expressing *otx2*-En and wildtype mesoderm will be much simpler. These experiments are currently underway.

4.3E Summary of *otx2* activity.

The patterning effects of *otx2* are summarized on a schematic of an embryo at early neurula in Fig. 4.7. *otx2* stimulates ectodermal and mesodermal gene expression that normally overlaps with that of *otx2*. This includes cement gland, general neural, anterior neural, and anterior mesodermal markers. Although the ability of *otx2* to induce cement gland is restricted to the ventrolateral ectoderm, general neural markers and, to some extent, anterior neural markers are induced throughout the ectoderm. Posterior and ventral markers, which do not fall within the *otx2* expression domain, are inhibited by *otx2*. These observations suggest that anterior specification is actively maintained by *otx2* activity in the ectoderm and mesoderm.

4.4 Acknowledgments

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Note: The experiments described in this chapter are in progress. Upon completion, they will be written for publication as Gammill and Sive.

Figure 4.1 *otx2* activates anterior and represses posterior and ventral gene expression in whole embryos.

Gene expression was assayed by whole mount in situ hybridization in uninjected (A) and *otx2*-GR injected embryos (B-G) incubated without ("-"; a, c, and e) or with ("+"; b, d, and f) dexamethasone (dex). Embryos are oriented with anterior at the top, except Fc,d, where posterior is at the top (ventral view). Dorsal view: Aa,b, Ba,b, Ca,b, Da-d, Ea-f, Ga-d. Dorsal to the right: Ac, Bc-f, Cc-f, De,f, Fa,b,e,f, Ge,f. **(A)** *otx2*; a, st. 13; b, st. 16; c, st. 30. **(B)** general neural; a, *NCAM*, st. 28 -dex; b, *NCAM*, st. 28 +dex; c, *NCAM*, st. 28 -dex; d, *NCAM*, st. 28, +dex; e, *N-tubulin* (*N-tub*), st. 30, -dex; *N-tubulin*, st. 30, +dex. **(C)** anterior neural; a, *lipocalin* (*cpl-1*), st. 14, -dex; b, *lipocalin*, st. 14, +dex; c, *lipocalin*, st. 30, -dex; d, *lipocalin*, st. 30, +dex; e, *XBF*, st. 32, -dex; f, *XBF*, st. 32, +dex. **(D)** mid/hindbrain boundary; a, *gbx-2*, st. 13, -dex; b, *gbx-2*, st. 13, +dex; c, *engrailed-2*, st. 16, -dex; d, *engrailed-2* (*en-2*), st. 16, +dex; e, *engrailed-2*, st. 32, -dex; f, *engrailed-2*, st. 32, +dex. **(E)** posterior neural; a, *Krox20*, st. 16, -dex; b, *Krox20*, st. 16, +dex; c, *HoxB9*, st. 16, -dex; d, *HoxB9*, st. 16, +dex; e, *N-tubulin*, st. 16, -dex; *N-tubulin*, st. 16, +dex. **(F)** ventral ectoderm; a, *GATA-2*, st. 13, -dex; b, *GATA-2*, st. 13, +dex; c, *XVent-1*, st. 13, -dex; d, *XVent-1*, st. 13, +dex; e, *Vox*, st. 13, -dex; f, *Vox*, st. 13, +dex. **(G)** mesoderm; a, *goosecoid* (*gsc*), st. 13, -dex; b, *goosecoid*, st. 13, +dex; c, *brachyury* (*Xbra*), st. 13, -dex; d, *brachyury*, st. 13, +dex; e, *muscle actin* (*m-actin*), st. 30, -dex; f, *muscle actin*, st. 30, +dex.

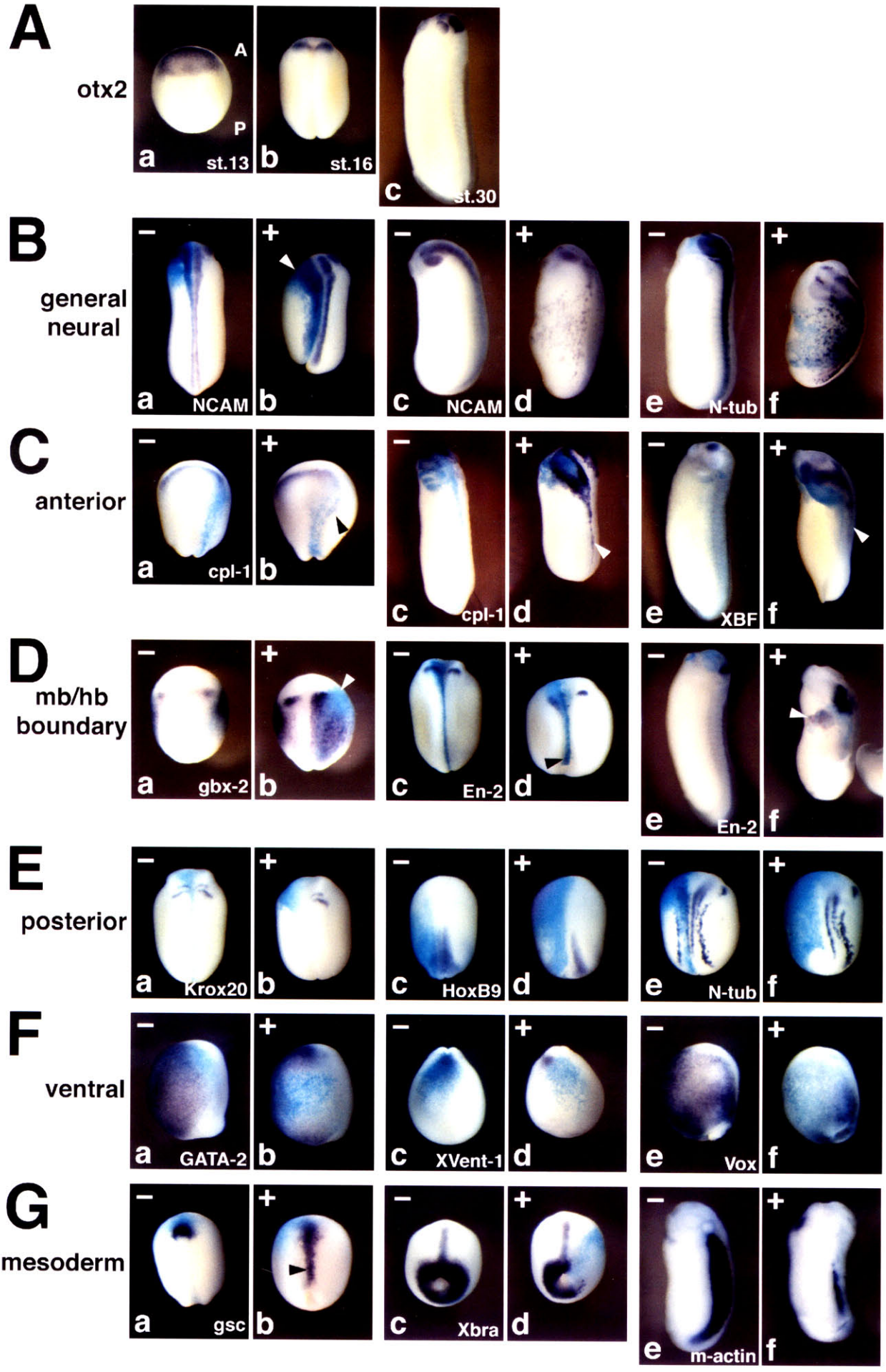

A
otx2
A
P
a st.13
b st.16
c st.30
B
general neural
a NCAM
b
c NCAM
d
e N-tub
f
C
anterior
a cpl-1
b
c cpl-1
d
e XBF
f
D
mb/hb boundary
a gbx-2
b
c En-2
d
e En-2
f
E
posterior
a Krox20
b
c HoxB9
d
e N-tub
f
F
ventral
a GATA-2
b
c XVent-1
d
e Vox
f
G
mesoderm
a gsc
b
c Xbra
d
e m-actin
f

Figure 4.2 *otx2* alters patterning in explants of dorsal and ventral ectoderm.
(A) Embryos were injected at the 8 cell stage in two dorsal or ventral blastomeres with 50 pg *otx2*-GR RNA (100 pg per embryo). At mid-gastrula stage 11.5, posterior dorsal or ventral ectoderm was dissected from appropriately injected embryos. Explants were cultured without or with dexamethasone (dex) for 3.5 hours until early neurula stage 13.5. **(B)** Explants of posterior dorsal ectoderm (dorsal) or ventral ectoderm (ventral) from embryos injected with 100 pg *otx2*-GR were incubated without (-) or with (+) dex and assayed by RT-PCR. Expression of general neural (*nrp-1*, *neurogenin* (*ngnr-1*)), cement gland (*XCG*), anterior neural (*opl*, *Xanf-1*, *engrailed-2* (*en-2*)), posterior neural (*Krox20*, *HoxB9*) ventral ectodermal (*XVent-1*, *Vox*, *Xhox3*, *BMP4*, *XMad-1*), and mesodermal (*goosecoid* (*gsc*), *brachyury*, (*Xbra*)) markers were examined by RT-PCR, using *EF-1α* expression as a loading control. Lane 1, posterior dorsal ectoderm, -dex; lane 2, posterior dorsal ectoderm, +dex; lane 3, stage 13.5 whole embryo; lane 4, ventral ectoderm, -dex; lane 5, ventral ectoderm, +dex.

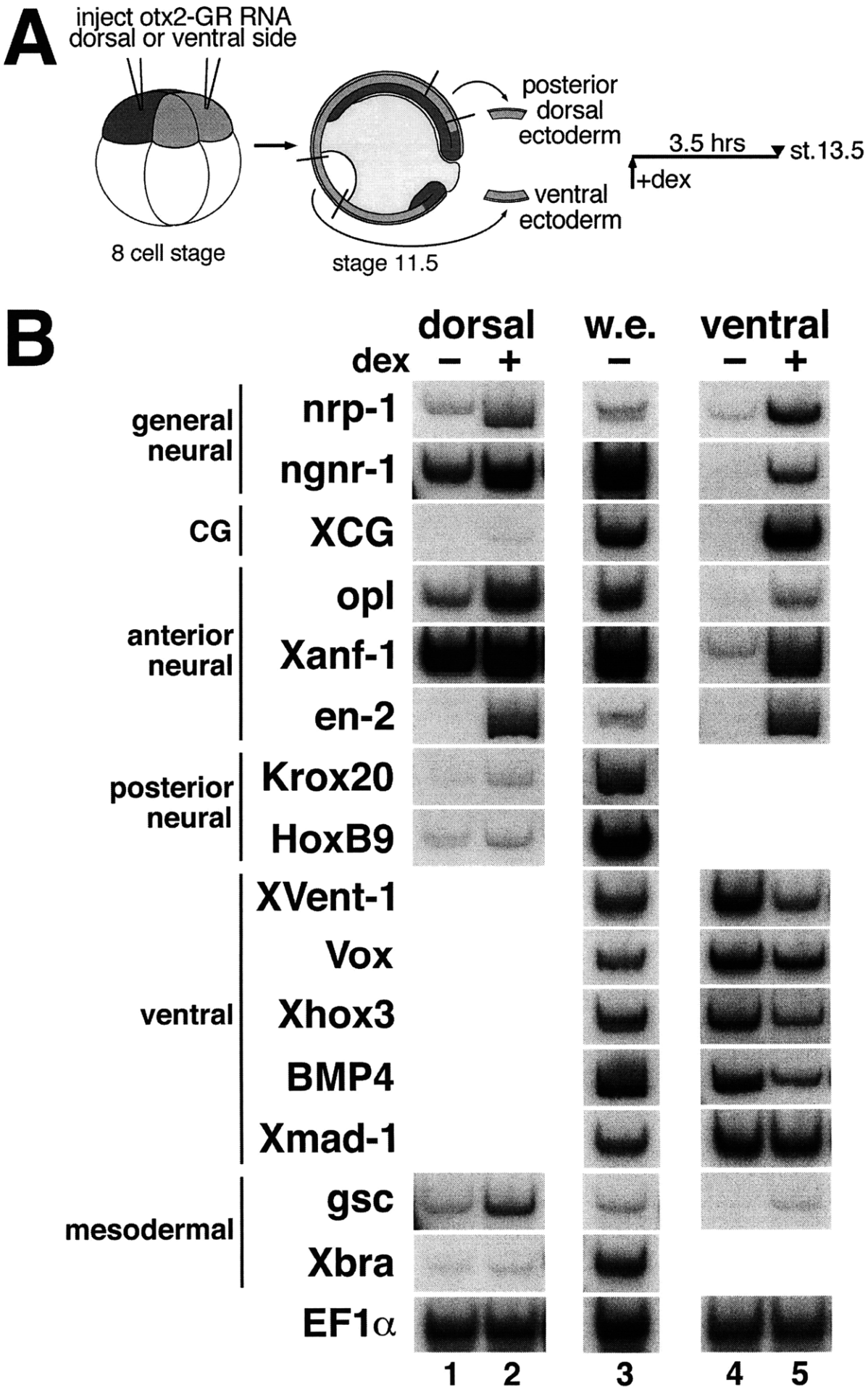
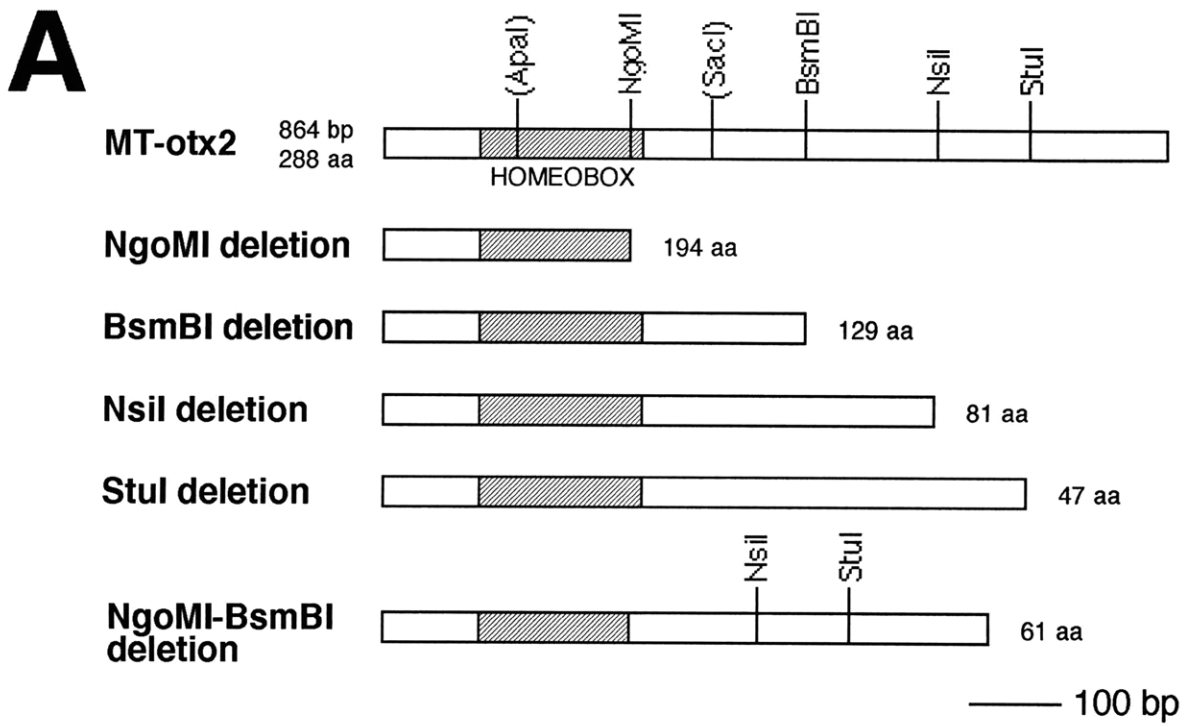
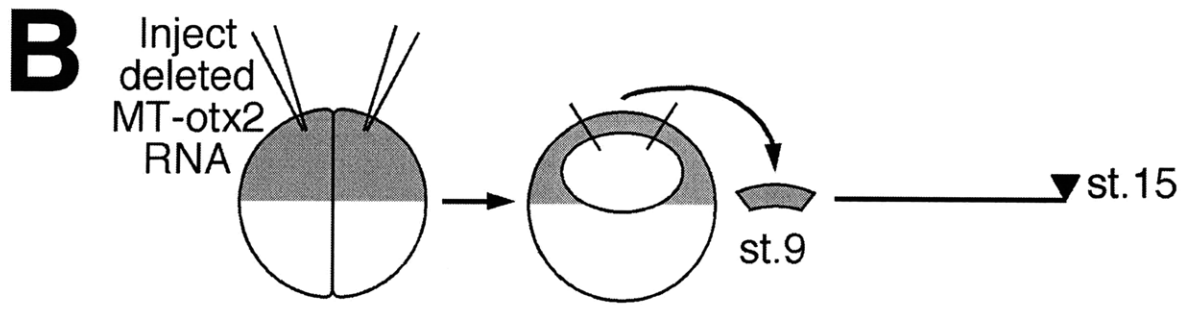

A
inject otx2-GR RNA
dorsal or ventral side
8 cell stage
stage 11.5
posterior
dorsal
ectoderm
ventral
ectoderm
3.5 hrs
st.13.5
+dex
B
dorsal
w.e.
ventral
dex
–
+
–
–
+
general
neural
nrp-1
ngnr-1
CG
XCG
anterior
neural
opl
Xanf-1
en-2
posterior
neural
Krox20
HoxB9
ventral
XVent-1
Vox
Xhox3
BMP4
Xmad-1
mesodermal
gsc
Xbra
EF1α
1
2
3
4
5

Figure 4.3 The transcriptional activation domain of *otx2* resides in the carboxyl terminus.

(A) Deletions were created in MT-*otx2* by restriction endonuclease digestion at sites indicated in *otx2*. MT-*otx2* is 864 nucleotides and 288 amino acids in length. 194 amino acids were deleted by *NgoMI* digestion, 129 amino acids were deleted by *BsmBI* digestion, 81 amino acids were deleted by *NsiI* digestion, 47 amino acids were deleted by *StuI* digestion, and internal amino acids were deleted by with *NgoMI* and *BsmBI* digestion. **(B)** Embryos were injected at the two-cell stage with 100 pg of each deleted MT-*otx2* RNA. Animal caps were dissected at late blastula stage 9 and cultured until mid neurula stage 15. **(C)** Animal caps from deleted MT-*otx2* injected embryos were assayed by RT-PCR for the cement gland markers *XCG* and *XAG* using *EF-1α* as a loading control. Lane 1, uninjected; lane 2, full-length MT-*otx2*; lane 3, *NgoMI* deleted MT-*otx2*; lane 4, *BsmBI* deleted MT-*otx2*; lane 5, *NsiI* deleted MT-*otx2*; lane 6, *StuI* deleted MT-*otx2*; lane 7, *NgoMI* to *BsmBI* internally deleted MT-*otx2*.


A
(ApaI)
NgoMI
(SacI)
BsmBI
NsiI
StuI
MT-otx2
864 bp
288 aa
HOMEOBOX
NgoMI deletion
194 aa
BsmBI deletion
129 aa
NsiI deletion
81 aa
StuI deletion
47 aa
NsiI
StuI
NgoMI-BsmBI deletion
61 aa
100 bp


B
Inject deleted MT-otx2 RNA
st.9
st.15

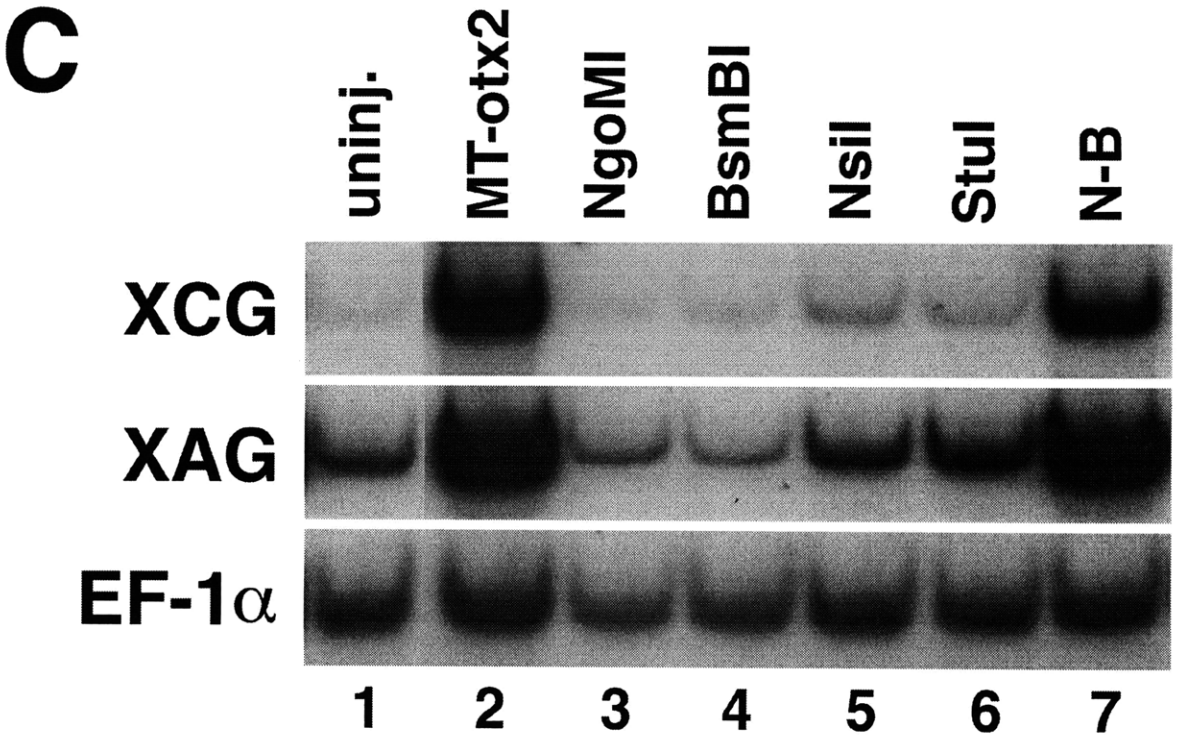

C
uninj.
MT-otx2
NgoMI
BsmBI
NsiI
StuI
N-B
XCG
XAG
EF-1α
1
2
3
4
5
6
7

Figure 4.4 *otx2*-En interferes with *otx2* activity.
(A) 162 amino acids including the activation domain (A) were deleted from the carboxyl terminus of *otx2* (top) and fused to the engrailed minimal repressor domain to create *otx2*-En (bottom). **(B)** Embryos were injected with *otx2*-En and/or *otx2* at the two-cell stage. Animal caps were dissected at late blastula stage 9 and harvested at hatching stage 30. **(C)** Animal caps from embryos injected with 500 pg *otx2*-En, 50 pg *otx2*, or both were analyzed by RT-PCR for expression of the cement gland markers *XCG* and *XAG* and the neural marker *NCAM*, using *EF-1α* as a loading control. Lane 1, 500 pg *otx2*-En; lane 2, 50 pg *otx2*; lane 3, 500 pg *otx2*-En and 50 pg *otx2*; lane 4, whole embryo.

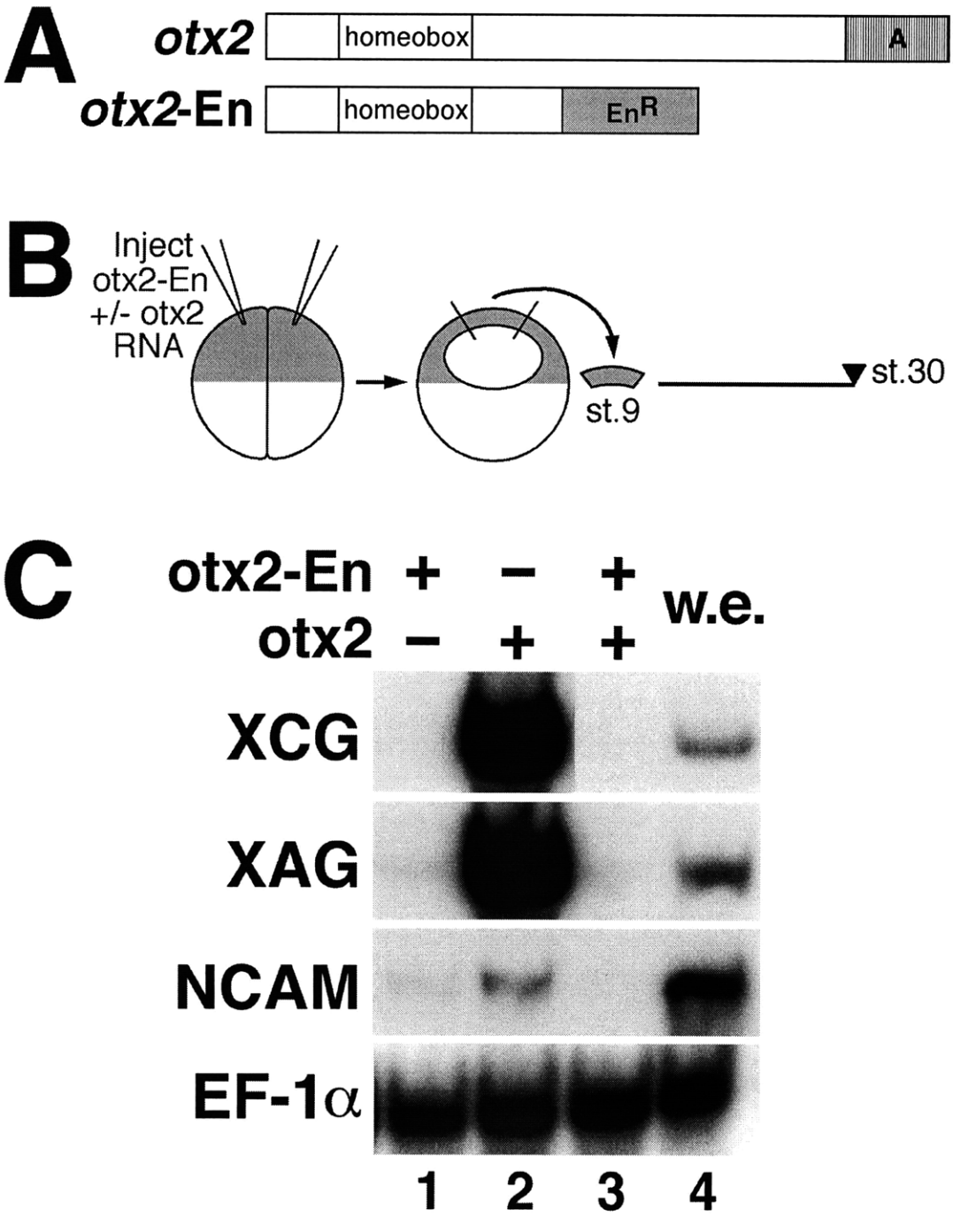

A
otx2
homeobox
A
otx2-En
homeobox
EnR
B
Inject
otx2-En
+/- otx2
RNA
st.9
st.30
C
otx2-En + − +
otx2 − + +
w.e.
XCG
XAG
NCAM
EF-1α
1 2 3 4

Figure 4.5 *otx2*-En does not affect activity of the paired class homeobox gene, *siamois*.

(A) Embryos were injected at the two-cell stage with *otx2*-En and/or *siamois* RNA. Animal caps were dissected at late blastula stage 9 and cultured until early gastrula stage 10.5. **(B)** Animal caps from embryos injected with 100 pg *otx2*-En, 50 pg *siamois*, or both were assayed by RT-PCR for expression of the organizer specific markers *goosecoid*, *chordin*, and *noggin*, using *EF-1α* as a loading control. Lane 1, *siamois* injected; lane 2, *otx2*-En and *siamois* injected; lane 3, *otx2*-En injected; lane 4, uninjected; lane 5, stage 10.5 whole embryo.

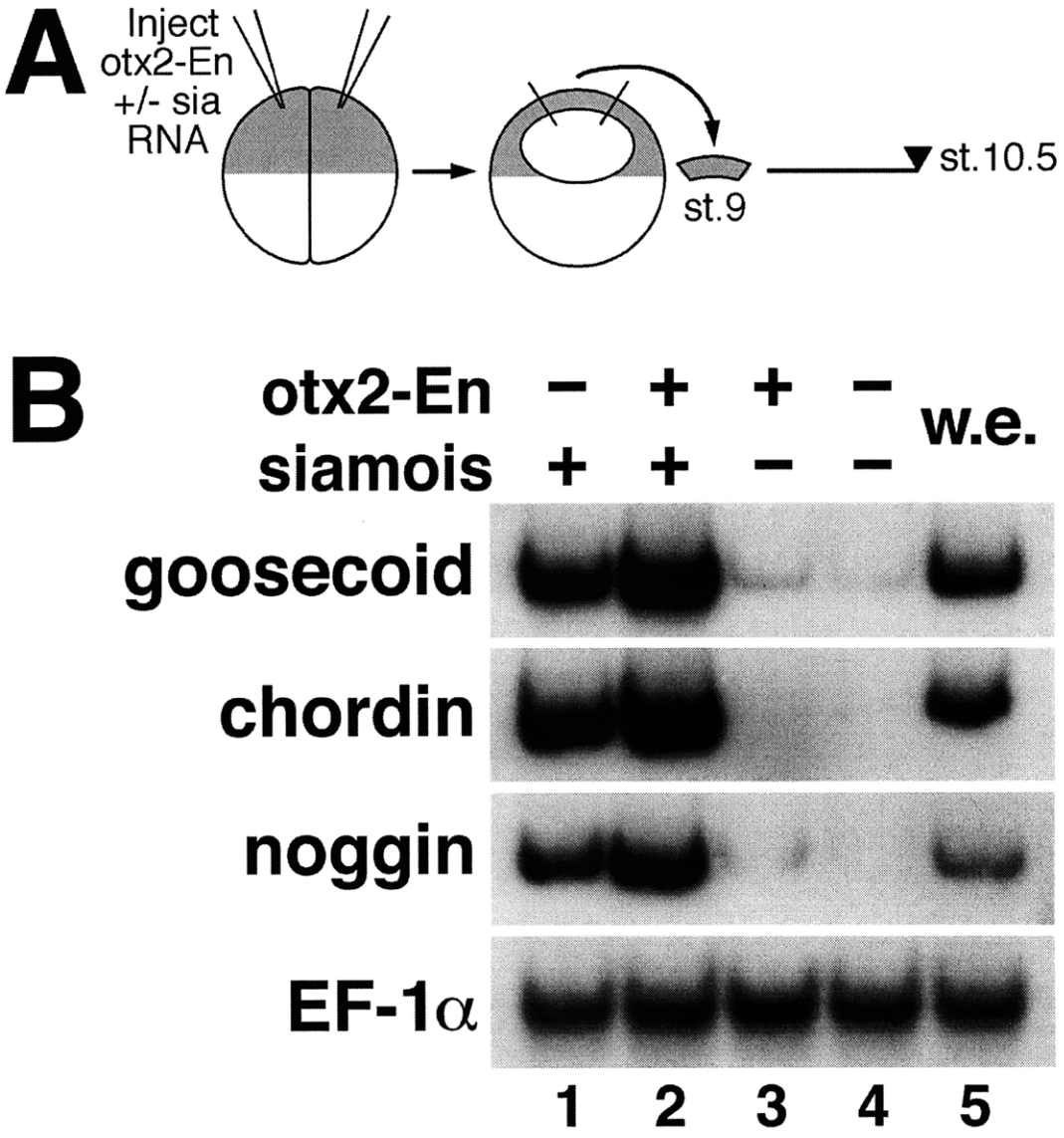

A
Inject
otx2-En
+/- sia
RNA
st.9
st.10.5
B
otx2-En
siamois
w.e.
goosecoid
chordin
noggin
EF-1α
1
2
3
4
5

Figure 4.6 *otx2*-En dominant negatively interferes with cement gland formation. **(A)** Embryos were injected at the two-cell stage with *otx2*-En RNA without or with *otx2* RNA, targeting the future anterior/ventral ectoderm by injecting superficially in the animal pole. *lacZ* RNA was included as a lineage tracer. Embryos were allowed to develop until hatching stage 30. **(B)** Embryos were injected with 100 pg *otx2*-En (a), 100 pg *otx2*-En plus 100 pg *otx2* (b), 100 pg *otx2*-En plus 200 pg *otx2* (c), or 100 pg *globin* (d) RNA mixed with 120 pg *lacZ* RNA. Injected embryos were stained for β-galactosidase activity (blue) and scored for cement gland formation (white arrowheads). **(C)** Embryos from (B) were scored for the absence of cement gland formation ("no CG", dotted bars), minimal cement gland formation (defined as a few pigmented cells; "v.sm. CG", hashed bars), small cement glands (a round dot of pigmented cells; "sm. CG", grey bars), reduced cement glands (typical oblong cement gland, but reduced in size; "reduc. CG", checkered bars), or normal cement glands (relative to size of average control cement gland; "normal", black bars). The data represent four separate rescue experiments. Rescued cement gland often included some ectopic cement gland formation due to the presence of *otx2* RNA.

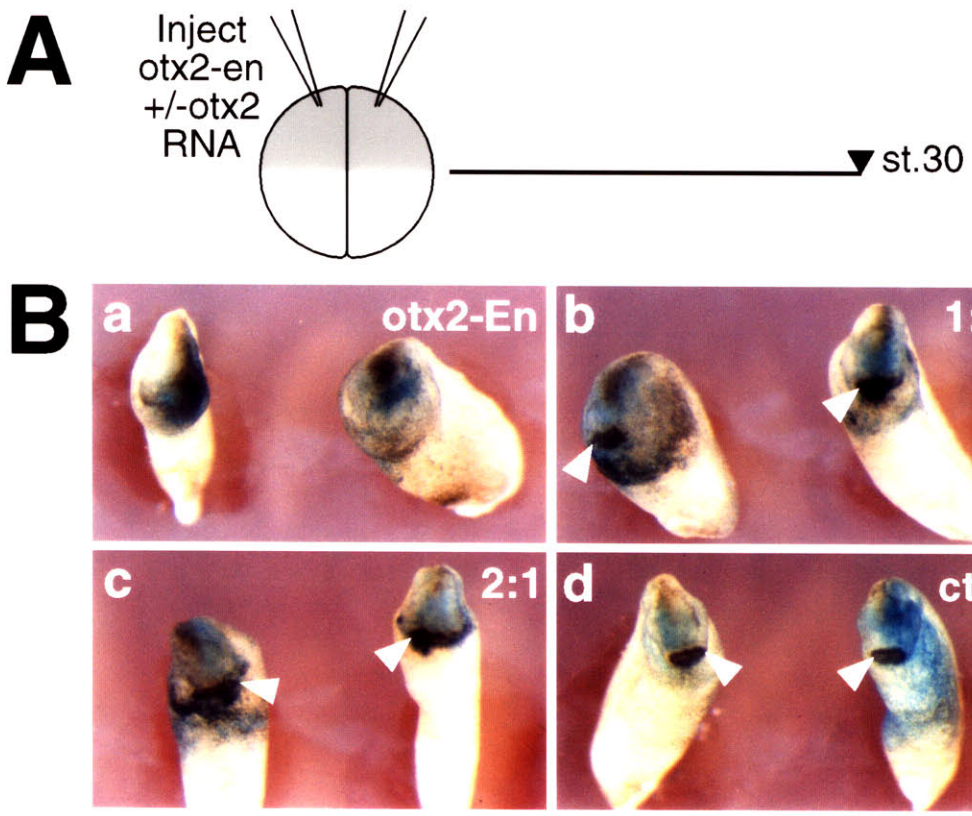
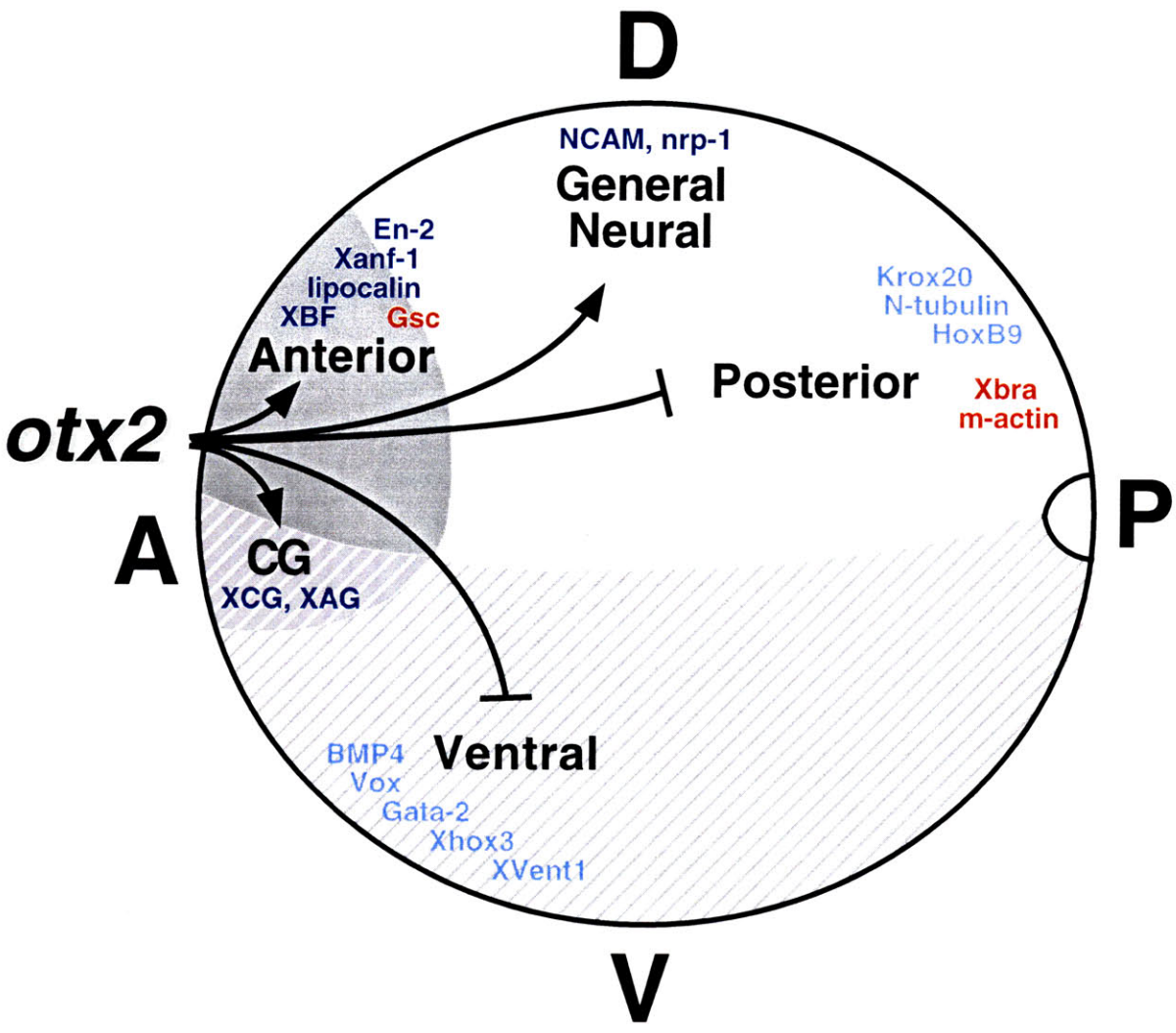

A
Inject
otx2-en
+/-otx2
RNA
st.30
B
a
otx2-En
b
1:1
c
2:1
d
ctrl
C
n=149
n=125
n=69
n=104
% rescue
100 otx2-en
100 otx2:
100 otx2-en
200 otx2:
100 otx2-en
globin
a
b
c
d
no CG
v.sm. CG
sm.
CG
reduc. CG
normal

Figure 4.7 *otx2* activates anterior and represses posterior and ventral gene expression.

otx2 (grey shading) stimulates expression (→) of ectodermal (blue) and mesodermal (red) markers normally expressed within the *otx2* domain. Cement gland (CG) markers can only be induced in the ventrolateral permissive area (hatching; Gammill and Sive, 1997), while general neural and, to some extent, anterior neural markers can be induced dorsally and ventrally in the ectoderm. Ectopic expression of the anterior mesodermal marker goosecoid (gsc) is limited to the mesoderm. Meanwhile, *otx2* represses (⊣) expression of posterior and ventral markers which do not coexist with *otx2* in the embryo. A is anterior, P is posterior, D is dorsal, V is ventral.



BLUE = neural induced

Lt. BLUE = neural repressed

RED = mesoderm

Chapter 5. Summary and future directions.

5.1 Summary

The induction, patterning, and subsequent regionalization of the dorsal ectoderm is a complicated process that begins during gastrulation. Although molecules that induce the neurectoderm and a number of genes that are expressed in response to induction have been identified in recent years, very little is known of how these factors are coordinated to yield complex pattern and region-specific differentiation within the neural plate. The anterior ectoderm gives rise to anterior neural derivatives and, in *Xenopus*, a non-neural organ, the cement gland. All of these structures express the evolutionarily conserved homeobox gene *otx2* almost as soon as anterior neural induction takes place (Blitz and Cho, 1995; Pannese, et al., 1995). Mouse mutants have demonstrated that *otx2* plays a critical, early role in the formation of anterior structures (Acampora, et al., 1995; Ang, et al., 1996; Matsuo, et al., 1995), however the function and requirement of *otx2* during ectodermal patterning have not been determined. Does *otx2* regulate the formation of anterior ectodermal structures? If so, what modulates *otx2* activity to give rise to multiple fates?

In this thesis, I have examined the activity of *otx2* specifically in the ectoderm in order to understand how positions are defined in the anterior neural plate. The results presented here indicate that *otx2* directs anterior fate, while *otx2* activity is both temporally and spatially modulated by interaction with other factors (Fig. 5.1). Specifically, these experiments demonstrate that *otx2* directly regulates expression of the cement gland marker *XCG*, indicating that *otx2* controls region-specific differentiation in the ectoderm. The cement gland inducing ability of *otx2* is regulated by other factors, since *otx2* can activate *XCG* expression only after mid-gastrula and only in the ventrolateral ectoderm. This cement gland permissive region overlaps with endogenous *otx2* expression exclusively in the cement gland primordium, and could normally restrict cement gland formation to this area. Ventrolateral permissiveness is molecularly defined in part by expression of BMP4, a factor that appears to modulate the ability of *otx2* to induce cement gland since cement gland formation correlates with coexpression of *otx2* and *BMP4*. The overlap of *otx2* and a BMP4 gradient may position the formation of anterior ectodermal structures. In the BMP4-free dorsal aspect of the *otx2* expression domain, *otx2* activates general neural and anterior neural markers while repressing posterior and ventral fates. This effect demonstrates that *otx2* actively signals anterior position in the ectoderm, and

indicates that maintenance and elaboration of anteroposterior ectodermal pattern is a dynamic process of mutual anterior and posterior repression.

5.2 Questions that remain

The findings presented in this thesis add to our understanding of how regionalization of the anterior ectoderm takes place. However, they also raise several questions that need to be addressed in future research.

5.2A Is *otx2* required for anterior ectodermal patterning?

Overexpression analysis indicates that *otx2* is sufficient to induce cement gland (Chapter 2) and anterior neural fates (Chapter 4). But is *otx2* activity necessary in the ectoderm for pattern formation and development of anterior ectodermal structures? Use of the *otx2* dominant negative described in Chapter 4 will allow this issue to be resolved. Mouse tissue explant recombinants of *otx2* mutant ectoderm and wildtype mesoderm, which normally induces *engrailed-2* expression in the ectoderm (Ang and Rossant, 1993), have demonstrated that *otx2* function is required in the ectoderm for induction of *engrailed-2* expression and thus formation of the midbrain/hindbrain boundary (Rhinn, et al., 1998). However, the extreme difficulty of obtaining mouse explants prevents a thorough analysis of anterior markers in this assay. Therefore, use of similar and more readily obtained recombinants between *Xenopus otx2*-En expressing ectoderm and wildtype mesoderm will be extremely informative for assessing the absolute requirement of *otx2* for the induction of anterior fates. Complementary experiments can also be performed by in situ hybridization in whole embryos to address the overall effect of *otx2*-En on ectodermal patterning. Are posterior markers expanded at the expense of anterior in the absence of *otx2*, reciprocal to the effect of overexpressing *otx2*? Analysis of gene expression in mouse chimeras suggests that this may be the case (Rhinn, et al., 1998). Direct comparison of decreased versus increased levels of *otx2* activity in *Xenopus* embryos will be informative for our understanding of *otx2* function and will indicate whether mutual anterior-posterior inhibitory interactions are relevant to ectodermal patterning in the embryo. These important experiments are currently underway.

5.2B What are the targets of *otx2* in addition to cement gland?

otx2 activates *XCG* expression in the absence of protein synthesis (Chapter 2; Gammill and Sive, 1997)). Thus, *XCG* is a direct target of *otx2*, indicating that

otx2 regulates regional identity and differentiation in the ectoderm. However, *otx2* is expressed in a broad region of the anterior neurectoderm. Does *otx2* serve a similar role elsewhere in its expression domain? What are other targets of *otx2* in the anterior ectoderm? We can begin to address these questions by performing subtractions between *otx2*-GR injected caps that are left untreated or treated with dexamethasone. If the treatment period is limited, early targets of *otx2* should be identified with this technique. Isolation of additional *otx2* targets and characterization of their regulation (see below) will allow us to better understand the role of *otx2* during patterning of the dorsal ectoderm and will potentially create a connection between patterned gene expression and later region-specific differentiation. Furthermore, *otx2* targets will serve as markers of the anterior ectoderm, providing new tools for assessing pattern formation.

5.2C How is *otx2* activity modulated in the neurectoderm?

Another aspect of *otx2* function that needs to be analyzed in greater detail is the spatial modulation of *otx2* activity. As it becomes clear whether *otx2* is required to instruct identities throughout its expression domain, it will be important to determine how the readout of *otx2* activity is determined. This addresses the downstream question of pattern formation: how do complex patterns of gene expression in the neural plate regulate morphogenesis? In the case of *otx2*, we have begun to address this issue with respect to cement gland formation (Chapter 3). But how is *otx2* activity modulated within the dorsal aspect of its expression domain to give rise to diverse structures in the forebrain and midbrain? Using available markers, the most likely candidates to interact with otx2 in the neural plate are other paired class homeodomains (Wilson, 1993). As indicated in the introduction, several members of the paired class in addition to *otx2* are expressed in the anterior ectoderm and could combinatorally regulate the formation of different anterior structures. However, modulation of *otx2* activity can be addressed more broadly by two-hybrid analysis to identify proteins, known and unknown, that interact with otx2. The ability of proteins to modulate *otx2* activity can be determined by coexpression in animal caps, using expression of *otx2* targets as markers of different anterior positions. Furthermore, promoter analysis of select targets of *otx2* would determine the requirements for region-specific gene expression in the neural plate. New protocols for creating transgenic frogs make these types of analyses accessible (Kroll, et al., 1994).

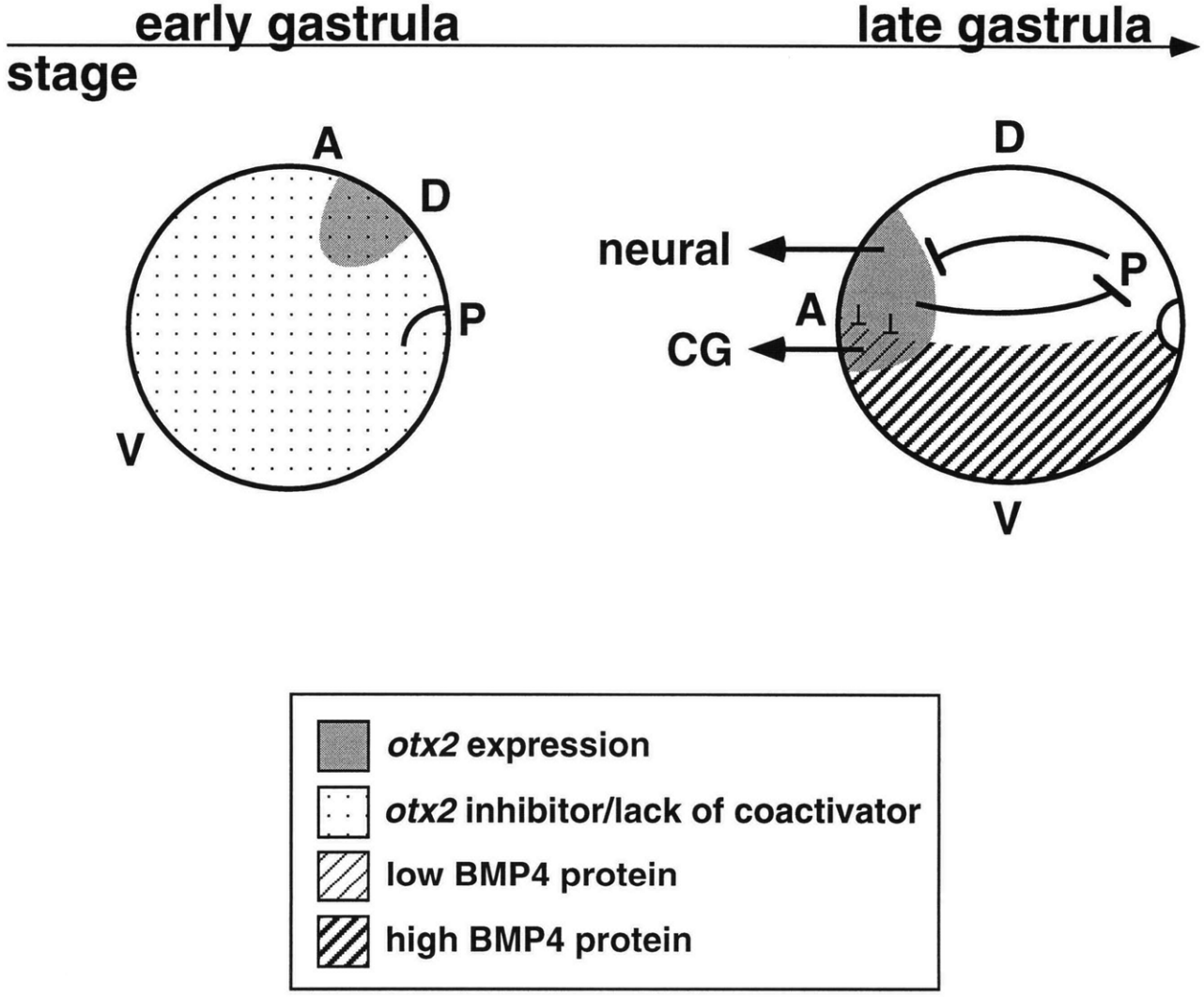
5.2D How is *otx2* expression regulated?

In addition to issues regarding regulation of *otx2* function, many questions also remain concerning the establishment of *otx2* expression. How do BMP4 inhibitors induce expression of genes such as *otx2*? What is the role of wnt signaling and the anterior inducers cerberus and dickkopf during induction of the anterior ectoderm? What are the cross-regulatory interactions between transcription factors expressed in the neurectoderm? These questions can be addressed by examining the *otx2* promoter. Identification of binding sites and the factors that recognize them will be very important for a general understanding of how the myriad signals during gastrulation are synthesized to achieve a patterned dorsal ectoderm. As other early, more restricted markers of the anterior ectoderm such as opl/Zic-1 (Kuo et al., in press; Mizuseki, et al., 1998), and fkh5 (Gamse and Sive, in preparation) are analyzed in a similar fashion, a picture should emerge of how diverse patterns of gene expression are generated in the dorsal ectoderm.

5.2E What defines temporal competence?

One last problem raised by these experiments is the temporal regulation of *otx2* activity in the dorsal ectoderm. Why does *otx2* activate cement gland marker expression only after mid-gastrula stages? This observation could prove to be a general mechanism to regulate the timing of differentiation within the neural plate, and has many different possible explanations to consider. One of the most obvious is that a necessary cofactor required for *otx2* to activate target genes, perhaps something that would be identified in the experiments described above, is not expressed until gastrula stages. Alternatively, a factor with a function analogous to *Drosophila yan*, which inhibits neuronal determination and keeps cells in an undifferentiated state (Rebay, et al., 1995), could be active in the dorsal ectoderm during early gastrula stages. It is also plausible that chromatin structure may regulate the accessibility of target gene promoters. Addressing these types of issues represents the next stage in the investigation of anteroposterior patterning, determining what controls the timing of the events that are being defined today.

Figure 5.1 Summary of *otx2* regulation and activity.
otx2 (dark grey) is expressed in the dorsal ectoderm beginning at early gastrula, but does not activate target gene expression at this stage, perhaps due to the presence of an inhibitor or the absence of a co-activator in the ectoderm (stippling). At late gastrula *otx2* expression defines an anterior domain in the embryo. *otx2* directly activates cement gland formation only in the region of *otx2* expression that overlaps with a gradient of BMP4 activity (light to dark hatching), which defines a ventrolateral permissive area. In the absence of BMP4, following neural induction or *otx2* overexpression (which inhibits BMP4 expression; ⊥), *otx2* induces anterior neural tissue. *otx2* actively maintains anterior position in the neurectoderm by inhibiting posterior gene expression, contrary to the theory of posterior dominance. Posterior genes also inhibit anterior (Epstein, et al., 1997; Pownall, et al., 1996), creating a dynamic circuit of mutual anterior and posterior repression during ectodermal patterning. A, anterior; P, posterior; D, dorsal; V, ventral.


early gastrula
late gastrula
stage
A
D
P
V
D
neural
P
A
CG
V
otx2 expression
otx2 inhibitor/lack of coactivator
low BMP4 protein
high BMP4 protein

Appendix A. The role of retinoids during anteroposterior patterning.

A.1 Introduction to Retinoid Signaling

Retinoic acid (RA) is a vitamin A derivative required for normal development. When present in excess, however, RA causes severe developmental defects. As first demonstrated in *Xenopus* (Durston, et al., 1989; Ruiz i Altaba and Jessell, 1991a; Sive, et al., 1990) and later in zebrafish and mouse (Holder, et al., 1991; Morriss-Kay, et al., 1991). A/P axis formation is sensitive to RA. In *Xenopus*, for example, incubation of gastrula stage embryos in 1 μM RA for two hours results in both anterior and posterior truncations of the body axis, with anterior structures being most sensitive (Durston, et al., 1989; Ruiz i Altaba and Jessell, 1991a; Sive, et al., 1990). In the most severe phenotype, the cement gland (an anterior ectodermal organ), eyes, and heart are absent, the forebrain, midbrain, and anterior hindbrain are reduced in size, and there is a loss of overt segmentation of the hindbrain (Durston, et al., 1989; Papalopulu, et al., 1991a; Ruiz i Altaba and Jessell, 1991a; Ruiz i Altaba and Jessell, 1991b; Sive, et al., 1990). Morphological alterations are underscored by an inhibition of anterior gene expression and an increase in the expression of some posterior genes (see below; (Sive, et al., 1990). These effects seem to be the result of RA acting to directly alter the fate of both the ectoderm and the mesoderm (Ruiz i Altaba and Jessell, 1991a; Sive and Cheng, 1991; Sive, et al., 1990). It is notable that the sensitivity of the A/P axis to RA is temporally limited (Avantaggiato, et al., 1996; Morriss-Kay, et al., 1991; Ruiz i Altaba and Jessell, 1991a; Simeone, et al., 1995; Sive, et al., 1990). By neurula stages, development of structures such as cement gland, eyes, and heart have become resistant to RA treatment, indicating the competence to respond to RA has been altered in the tissues which go on to form these organs.

A1.1 Retinoic acid is present in the developing embryo.

The fact that RA can so profoundly alter axial patterning suggests that it may be present in embryos and play an endogenous role during development. HPLC analysis indicates that *Xenopus* embryos do contain RA at a mean concentration of 1.5×10^{-7} M (Durston, et al., 1989). In chick embryos, Hensen's node and the floorplate of the neural tube were shown to be sites of retinoid synthesis using HPLC and reporter cell assays (Chen, et al., 1992; Hogan, et al., 1992; Wagner, et al., 1990). Transgenic indicator mice carrying a retinoic acid response element (which confers RA inducible gene expression; see below) upstream of a *lacZ* reporter show β-galactosidase activity starting at the headfold stage in the neural plate, with a sharp anterior boundary of expression in the

posterior presumptive hindbrain following neural tube closure (Balkan, et al., 1992; Rossant, et al., 1991). After treatment with RA, transgene activity is detectable in all germ layers throughout the embryo, suggesting that the teratogenicity of RA is due to elevated levels of retinoids in affected areas such as the anterior hindbrain where retinoids are not normally present (Balkan, et al., 1992; Rossant, et al., 1991).

A1.2 The complex retinoid signaling cascade.

Although the synthesis of RA itself represents one level of control, the retinoid signaling pathway is also regulated during development by the limited expression of a complex system of binding proteins, receptors, and response elements. The retinoid binding proteins CRBP I and II (cellular retinol binding proteins) and CRABP I and II (cellular retinoic acid binding proteins) serve to modulate the levels of free RA in the cell by facilitating RA metabolism (CRBP I and II) and catabolism (CRABP I) and by shuttling RA to the nucleus (CRABP II; for a review, see Morriss-Kay, et al., 1996). Retinoic acid receptors (RARs), which bind all-trans RA and 9-cis RA, and retinoid X receptors (RXRs), which bind only 9-cis RA, are ligand-dependent transcription factors and members of the steroid-thyroid hormone superfamily of nuclear receptors (Allenby, et al., 1994; Heyman, et al., 1992; Mangelsdorf, et al., 1992). There are three different types of RARs and RXRs, α, β, and γ, with RAR isoforms of each type being generated through differential promoter usage and alternate splicing (for a review, see Leid, et al., 1992). RARs bind to response elements as heterodimers with RXRs, preferentially recognizing elements composed of direct repeats of the half site (G/A)G(T/G)TCA spaced by 2 or 5 nucleotides (Kliewer, et al., 1992; Leid, et al., 1992; Yu, et al., 1991). "Consensus" retinoic acid response elements (RAREs) have been identified flanking many genes in the mouse, indicating that RA signaling is important for the developmental regulation of gene expression (for examples, see Dupé, et al., 1997; Langston, et al., 1997; Marshall, et al., 1994; Ogura, et al., 1995a).

A1.3 *Hox* genes are inducible by retinoic acid.

It has been proposed that retinoic acid may act during development to regulate *Hox* gene expression. The mouse and human *Hox* genes consist of four homologous clusters (A - D) believed to have evolved from duplications of an ancestral cluster related to the *Drosophila ANT-C* and *BX-C* complexes (for

reviews see Akam, 1989; McGinnis, et al., 1992). Studies in mouse and *Xenopus* have shown that *Hox* genes are expressed in overlapping domains in the embryo such that the genes located 3' in each cluster generally have the most anterior boundaries of expression, are turned on the earliest in development, and are the most highly responsive to RA (Conlon, et al., 1992; Dekker, et al., 1992b; Krumlauf, 1993a). The 3'-most genes in clusters A, B, and D are the *Drosophila labial* homologues *A1*, *B1*, and *D1*. Genes of the *labial* family are inducible by RA in mouse and human tissue culture cells (LaRosa, et al., 1988a; Simeone, et al., 1991) and in *Xenopus* ectoderm and mesoderm (Kolm and Sive, 1994) in the absence of protein synthesis. Importantly, *HoxA1* and *HoxB1* have been shown to contain RAREs in mouse (Langston, et al., 1992; Marshall, et al., 1994; Ogura and Evans, 1995a).

The combination of *Hox* genes expressed in a particular region of the embryo is thought to confer positional information and therefore axial identity (Hunt, et al., 1992). Treatment with RA causes an anterior shift in *Hox* expression domains, and in mouse and *Xenopus* embryos the expansion of *Hox* gene expression occurs long before any morphological alterations are evident (Conlon and Rossant, 1992; Kessel, 1993; Papalopulu, et al., 1991b; Sive and Cheng, 1991). This suggests that the teratogenic effects of RA could be mediated by changes in *Hox* gene expression due to increased levels of RA and leads to the intriguing possibility that RA is an endogenous signal involved in regulating *Hox* gene expression and therefore contributing to the specification of positional identities along the anteroposterior axis during development. Recently the use of dominant negative RARs has demonstrated that retinoid signaling is required for activation of *Hox* genes and appropriate patterning of the neurectoderm (Blumberg, et al., 1997; Kolm, et al., 1997).

A.2 Results and Discussion

A2.1 What is the endogenous role of retinoids?

The goal of this study was to better understand the in vivo role of RA by studying the regulation of *HoxD1*, a direct target of RA signaling (Kolm and Sive, 1995a). As indicated above, there was evidence to suggest that RA was present during early development, that RA was able to transduce a signal via the RARs, and that RA caused severe A/P defects, most likely by altering positional information through changes in *Hox* gene expression. *HoxD1* was an attractive model for studying the endogenous role of retinoids during A/P patterning

because it is induced as an immediate early response to RA and because it is first expressed at early gastrula stages and maximally expressed during early neurula stages, encompassing the period when axial patterning occurs (Kolm and Sive, 1994).

I chose to study regulation of *HoxD1* by isolating genomic clones (Fig. A.1) and characterizing promoter elements responsive to RA. *HoxD1* 5' and 3' promoter fragments were inserted upstream of a minimal promoter linked to chloramphenicol acetyl transferase (CAT) as a reporter. Following microinjection of these construct, embryos were cultured and treated with 1 μM RA at early gastrula (stage 10-10.5) for three to four hours. After treatment, embryos were lysed and reporter gene activity was assayed. The feasibility of this approach is demonstrated using control elements in Table A.1. However, due to our inability to recover sufficient 3' promoter sequence and identify an RA-responsive fragment, this project was not pursued any further.

Since this project was terminated, progress has been made to decipher the endogenous role of retinoids. RAREs 3' of *HoxA1* and *HoxB1* in mouse have been shown to be critical to the correct expression of these genes (Dupé, et al., 1997; Langston, et al., 1997; Marshall, et al., 1994; Ogura, et al., 1995b) . Furthermore, dominant negative receptors have been used to demonstrate that RA-signaling is required to activate expression of the *Hox* genes in *Xenopus* (Blumberg, et al., 1997; Kolm, et al., 1997), and *HoxD1* function in *Xenopus* has been studied in detail (Kolm and Sive, in preparation).

A2.2 Characterizing the competence of the ectoderm to respond to retinoic acid.

The competence of the ectoderm to respond to RA changes dramatically during gastrulation in both *Xenopus* and mouse embryos (Avantaggiato, et al., 1996; Conlon and Rossant, 1992; Morriss-Kay, et al., 1991; Ruiz i Altaba and Jessell, 1991a; Simeone, et al., 1995; Sive, et al., 1990; Zimmer, et al., 1992). While treatment of embryos with RA during early gastrula stages results in animals with anterior and posterior defects, by late gastrula stages, axial structures form even in the presence of RA (Morriss-Kay, et al., 1991; Ruiz i Altaba and Jessell, 1991a; Sive, et al., 1990). One explanation for this observation is that the RA sensitive anterior region of the embryo becomes protected from the posteriorizing influence of RA during anteroposterior patterning. We wanted to characterize the stage-dependent effects of RA on the anterior regions of the embryo in order to learn more about what defines the ability of a particular tissue

to respond to RA and how these changes in competence are important during the patterning of the embryo.

To address this, we exploited the ability of retinoic acid (RA) to inhibit cement gland formation (Sive et al., 1990) and precisely defined the period when cement gland development was sensitive to RA. Embryos were treated at various stages with 1 μM RA and *XCG* or *XAG* expression was later analyzed by in situ hybridization (Fig. A.2). Early to mid-gastrula treatment (stages 10, 10.5 or 11) resulted in severely abnormal embryos with no *XAG* (a) or *XCG* (b) expressed. However, embryos treated with RA at mid-gastrula (stage 11.5) went on to express small patches of *XCG* and *XAG* (white arrowheads). Increasing amounts of *XCG* and *XAG* were present after late gastrula treatment (stage 12 and 12.5), and after RA addition at early neurula (stage 13), an essentially normal sized cement gland formed (white arrowheads). These experiments indicated that ectoderm of the cement gland primordium became resistant to RA at mid-gastrula stages, suggesting that the anterior ectoderm loses competence to respond to RA at this stage.

In order to measure changes in RA sensitivity more generally, we also monitored expression of *HoxD1*, an immediate early target of RA (Kolm and Sive, 1994). Embryos were treated with RA at gastrula stages and gene expression was assayed at mid-neurula (stage 15; Fig. A.3). *HoxD1* is normally expressed in a posterior domain (a; white arrowhead), while RA treatment early during gastrulation induced *HoxD1* expression anteriorly (b). However, when RA was added later during gastrulation, the anterior limit of *HoxD1* expression receded (c and d). This indicated that a change in sensitivity to RA during gastrulation was not specific to cement gland formation.

We also examined whether RA resistance was acquired in isolated dorsal ectoderm. Anterodorsal ectoderm was dissected from mid-gastrula (stage 11.5) embryos and cultured until control embryos reached hatching stage (stage 32; Fig. A.4, top schematic). These explants went on to express the cement gland marker *XCG* as assayed by whole mount in situ hybridization (Fig. A.4; panel a). However, when anterodorsal ectoderm was transferred to RA when control embryos reached stage 12 (late gastrula), cement gland failed to form (b). If RA was added when control embryos reached stage 13.5 (early neurula), *XCG*-positive cells were observed in 29% of the explants (white arrowheads; 3 independent experiments). Following later RA treatments, a greater proportion of the explants expressed *XCG*, and the size of the patches increased (panels d-f). Ectodermal

explants treated with RA when control embryos reached early tailbud (stage 19; panel f) often went on to express *XCG* in a manner indistinguishable from control explants. However, even stage 19 RA-treated explants did not generally form cement glands that were as large as untreated controls and not all explants were RA resistant (25% not resistant in 3 experiments). These data show that the loss of competence to respond to RA was at least in part acquired autonomously by the dorsal ectoderm. However, RA resistance was delayed in explants, suggesting that either the tissue is being removed from some signal which confers resistance, or that the explants have slowed down. Preliminary experiments were not able to distinguish between these possibilities (data not shown).

These data described the change in competence to respond to RA that occurs during gastrulation. Expression of *XCG*, *XAG*, and *HoxD1* together defined an anterior domain which became RA-insensitive as gastrulation progressed, and suggested that other tissues might regulate the mid-gastrula change in the competence of the ectoderm. This work served as a prelude to the experiments described in Fig. 2.2, Fig. 2.3, and section 2.2C, and as a beginning to the study of *otx2* contained in this thesis.

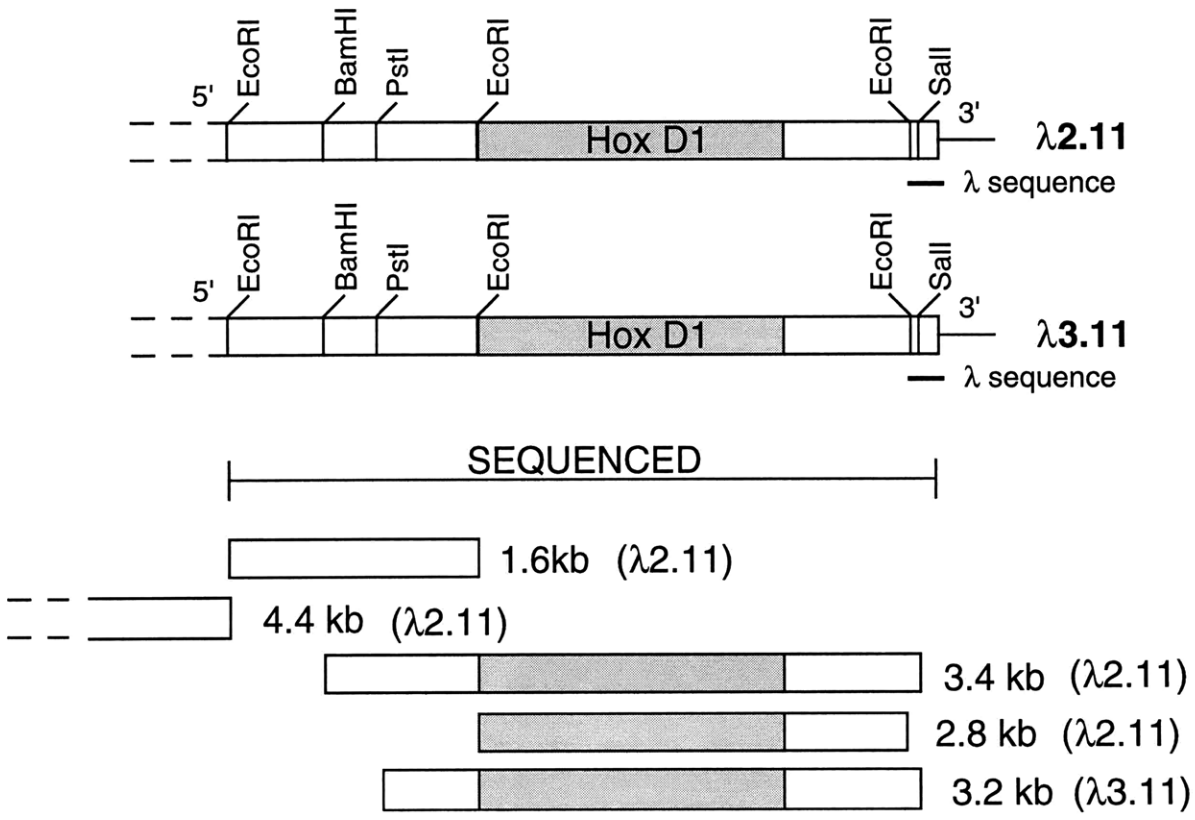


Figure A.1 *HoxD1* genomic clones.
Map of *Xenopus* HoxD1 genomic clones λ2.11 and λ3.11 and resulting subcloned fragments. Approximately 4 kb, including all available 3′ regions, were sequenced. The size and origin of the subcloned fragments is indicated to the right.

Exp.	Uninjected		PA-PTCAT		MLP		TK		RARE3-MLP		Induction
	- RA	+ RA	- RA	+ RA	- RA	+ RA	- RA	+ RA	- RA	+ RA	
1	868		830				11420				
2	762	768	654	742	1456	994	21850	21488			
3									5358 6534	11148	1.9
4	702								6544	13642 34656	4.0
5	856								2472	6302	3.4
6	782	892	828	850	928				3406 3468	4704 5562	1.6
7	738 858	794 802							2690 2730	5752 5784	2.6

Table A.1 Preliminary results of reporter construct microinjection experiments. *Xenopus* embryos were microinjected with approximately 50 pg of plasmid DNA at the 2 cell stage and incubated in RA (where indicated) for 3 to 4 hours during gastrula stages. At late gastrula/early neurula, embryos were harvested in pools of 5 in CAT assay lysis buffer. 2 1/2 embryo equivalents were used in each CAT assay. The fold induction was calculated for the $RARE_3$-MLP construct by dividing the average +RA (minus uninjected background, where possible) by the average -RA (minus uninjected background, where possible). Units represent ^3H counts incorporated.

Figure A.2 Cement gland formation loses sensitivity to retinoic acid at mid-gastrula.

Albino embryos were treated with retinoic acid (RA) at the stage indicated and cultured until stage 32 when they were analyzed for *XAG* (a) or *XCG* (b) expression (white arrowheads) by in situ hybridization. Each embryo pictured is representative of a pool of at least six embryos analyzed simultaneously. Lateral view, anterior to the right (a) or left (b).

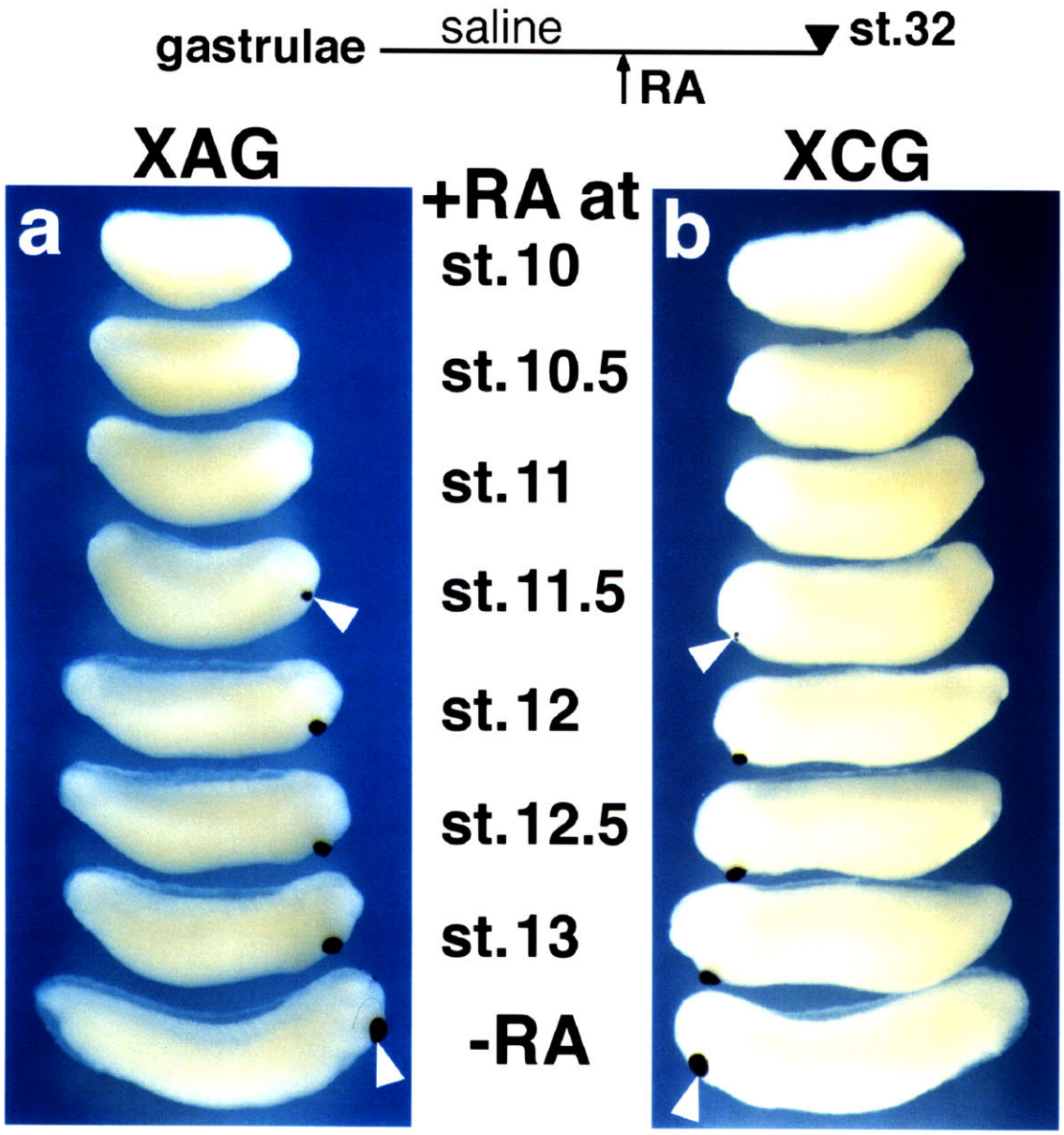
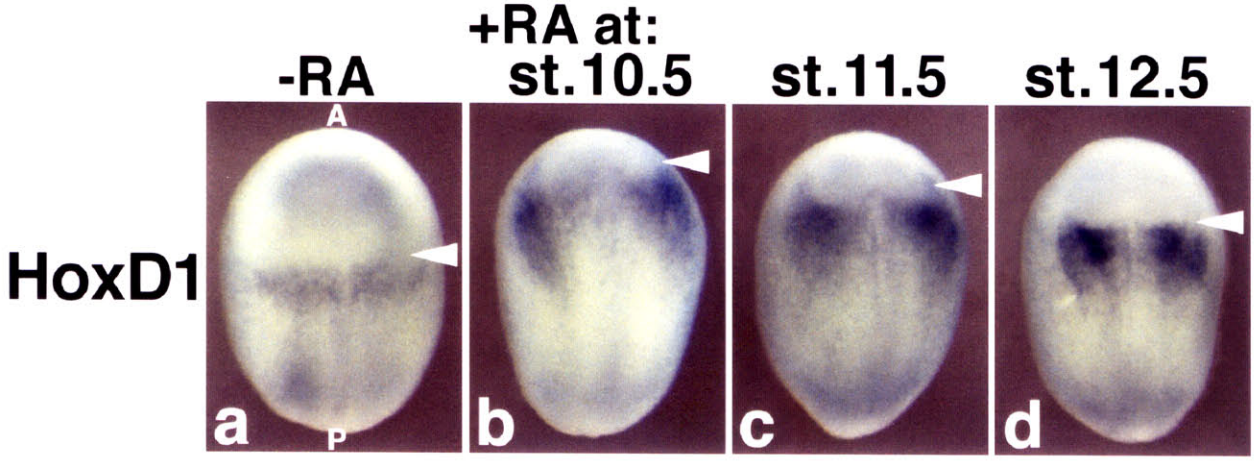
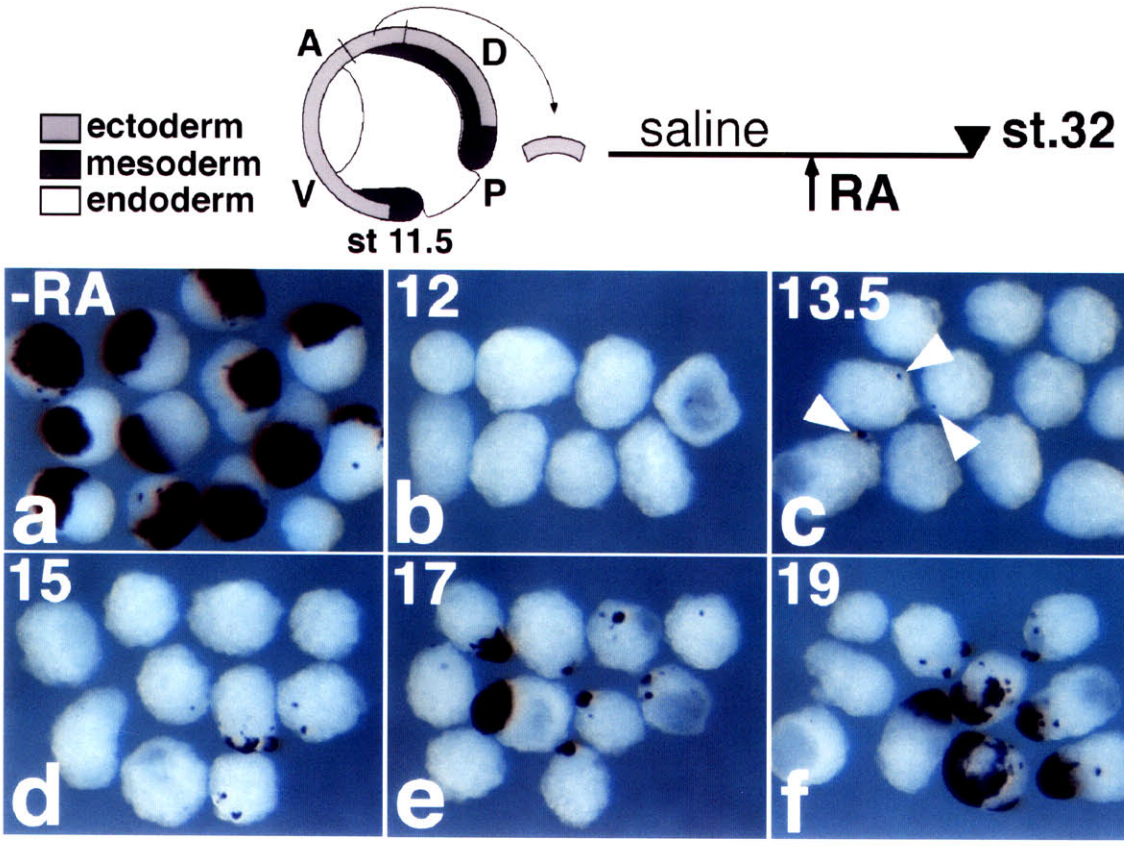

gastrulae
saline
st.32
RA
XAG
XCG
+RA at
a
b
st.10
st.10.5
st.11
st.11.5
st.12
st.12.5
st.13
-RA

Figure A.3 *HoxD1* expression defines an RA-sensitive anterior domain.
Albino embryos were left untreated (a) or treated with retinoic acid (RA) at stage 10.5 (b), stage 11.5 (c), or stage 12.5 (d) and harvested at stage 15 for analysis of *HoxD1* expression by in situ hybridization. White arrowheads indicate the anterior limit of *HoxD1* expression. Dorsal view, anterior up.

Figure A.4 RA-resistance is acquired later in explants of the cement gland primordium.
Anterior dorsal ectoderm, including specified cement gland, was dissected from mid-gastrula embryos (schematic; A=anterior, P= posterior, D=dorsal, V=ventral) and left untreated or placed in RA until control embryos reached stage 32. *XCG* expression was visualized by in situ hybridization. White arrowheads mark the first RA-resistant *XCG* expression at stage 13.5. Panel a, untreated; panel b, +RA st. 12; panel c, +RA st. 13.5; panel d, +RA st. 15; panel e, +RA st. 17; panel f, +RA st. 19.


+RA at:
-RA
st.10.5
st.11.5
st.12.5
HoxD1
A
P
a
b
c
d


ectoderm
mesoderm
endoderm
A
D
V
P
st 11.5
saline
st.32
RA
-RA
12
13.5
15
17
19
a
b
c
d
e
f

Appendix B. Determination of the enveloping layer and lack of autoneuralization in zebrafish embryos.

B.1 Background

This work represents a collaboration with Charles Sagerström in which we compared epidermal specification in the zebrafish, *Danio*, and in *Xenopus*. I contributed data examining expression and regulation of the *Xenopus* epidermal cytokeratin *XK81*. I also addressed the function of the *Xenopus* outer layer in the neural plate. These data are currently being prepared for publication as Sagerström, Gammill, and Sive.

B.2 Abstract

The zebrafish early gastrula ectoderm comprises two layers, a deep loosely adherent layer (DEL) and a superficial, tightly adherent monolayer, the enveloping layer (EVL). We have analyzed the developmental potential of these two cell layers using in vitro explant assays and a novel EVL-specific cytokeratin gene (*cyt1*), that is similar to epidermal-specific cytokeratins from *Xenopus*. As in frogs, the zebrafish EVL forms an epithelial barrier characterized by tight junctions. Based on these similarities, we consider the zebrafish EVL directly analogous to the *Xenopus* outer ectodermal layer. In *Xenopus*, ventral, *XK81* positive outer layer forms the larval epidermis, while dorsal, *XK81* negative cells become folded into the neural tube. In contrast, zebrafish cytokeratin expression is not restricted to the ventral side of the embryo, but is expressed throughout the EVL. This correlates with the failure of the dorsal EVL to become incorporated into the neural keel. We find that explants containing superficial cells from mid-blastula stage embryos are already committed to an EVL fate. This commitment requires cell contact since explants that are dissociated and cultured as single cells or reaggregated before culture do not activate *cyt1*. In *Xenopus*, epidermal determination requires both cell contact and BMP signaling. In contrast, we have shown that several members of the BMP family (BMP-2, 4, 7, 2/6 dimers) are unable to restore *cyt1* expression in dissociated/reaggregated zebrafish explants. We suggest that epidermal determination in fish requires only signals dependent on cell contact, whereas in *Xenopus*, signals involving both cell contact and formation of ventral fates are required. It is only the ventral restriction of cytokeratin expression that is dependent on BMP signals, and this level of control is absent in fish. Ectodermal explants from blastula and gastrula stages go on to express dorsal specific (neural marker) genes (including *otx2* and *opl*) and express a ventral specific gene (*gta3*). Dissociating and reaggregating these explants prevents *otx2* and *opl* expression, but does not suppress *gta3* expression,

indicating that dorsal specific gene expression in fish also requires cell contact. We find no evidence for autoneuralization in zebrafish ectodermal explants, in contrast to *Xenopus*, where dissociation of ectodermal explants activates dorsal ectodermal gene expression and autoneuralization. Our results highlight differences between ectodermal determination between vertebrate embryos.

Figure B.1 The zebrafish *cyt1* gene and the *Xenopus XK81* gene are expressed in analogous outer epithelial layers.

(A) Northern blot analysis of *cyt1* expression. A *cyt1* probe was hybridized to Northern blots containing 1 μg of total RNA from various stages of zebrafish development. Hybridization to a probe for 18S rRNA (B. Sun and H. Sive unpublished) was used as a loading control. **(B)** Whole mount in situ hybridization analysis of *cyt1* expression. A *cyt1* probe was hybridized to zebrafish embryos from various stages: a, late blastula, sphere stage, 4 hpf; b, early gastrula, shield stage, 6 hpf; c, late gastrula, 85% epiboly, 9 hpf; d, mid somitogenesis, 14 somite stage, 16 hpf; e, end of somitogenesis, prim 5, 24 hpf. Some embryos were sectioned following in situ hybridization to further define the expression of *cyt1*: f, section through embryo in panel b; g, section through embryo in panel d; h, section through embryo in panel e; i, enlargement of boxed region in panel h. The level of each section is indicated by a black line. Anterior is up in panels a-d and to the right in panel e. Dorsal is to the right in panels b-d and up in panel e. Dorsal can not be assigned at the stage in panel a (sphere stage, 4 hpf). Dorsal is up in panels f-i. **(C)** *XK81* expression by wholemount in situ hybridization. An *XK81* probe (see methods) was hybridized to *Xenopus* embryos from various stages; a and b, late gastrula, st. 12.5; c and d, mid-neurula, st. 15; e and f, tailbud, st. 20. Embryos were sectioned to further define *XK81* expression: g, section through an early neurula stage embryo (st. 14) at the level indicated in panel c; h, enlargement of area boxed in panel g; i, section through a late neurula stage embryo (st. 18) at the level indicated in panel e; j, enlargement of boxed area in i. Anterior is up in panels a-f. a, c and e are dorsal views. b, d and f are lateral views. Dorsal is up in g-j.

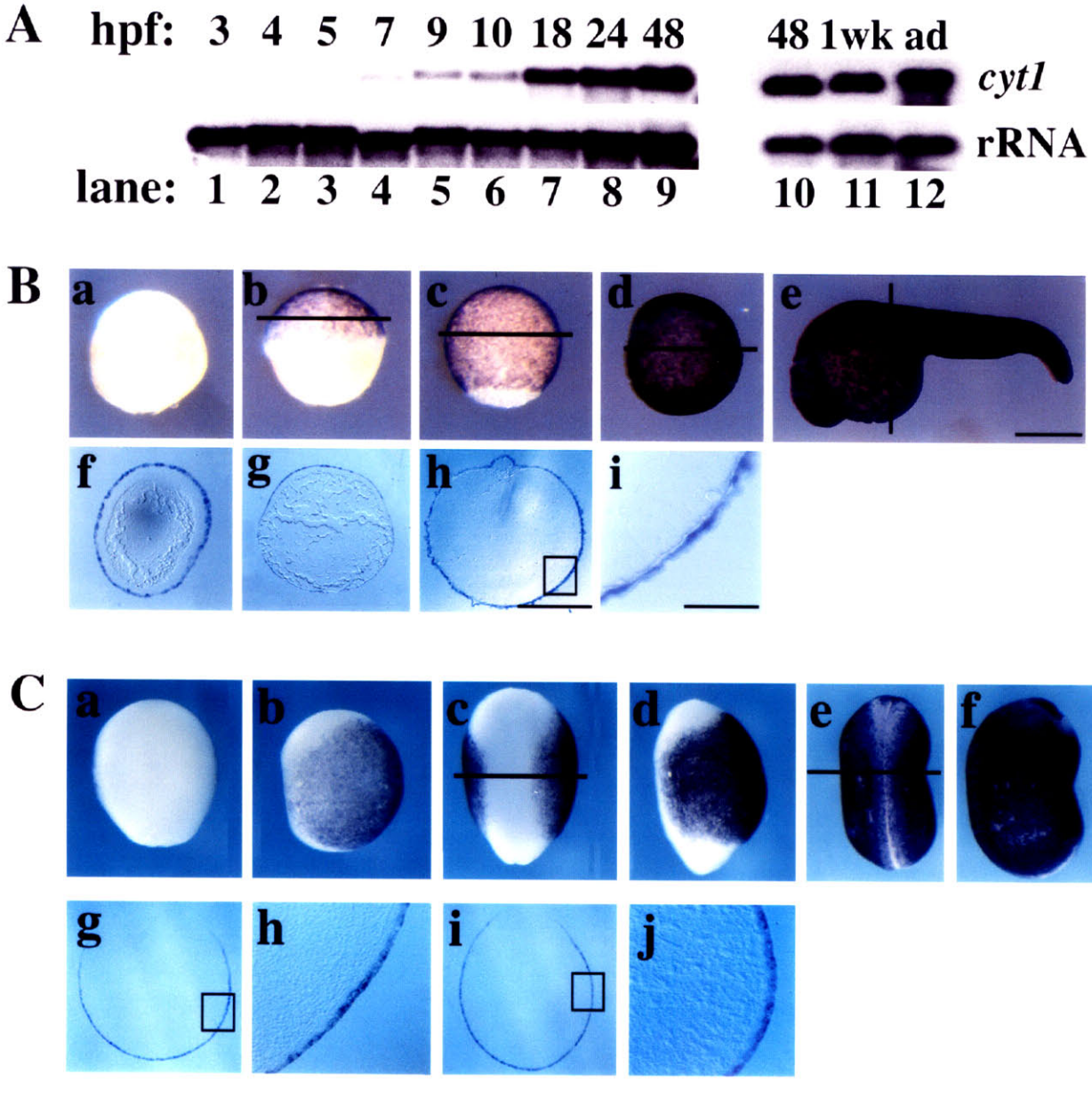
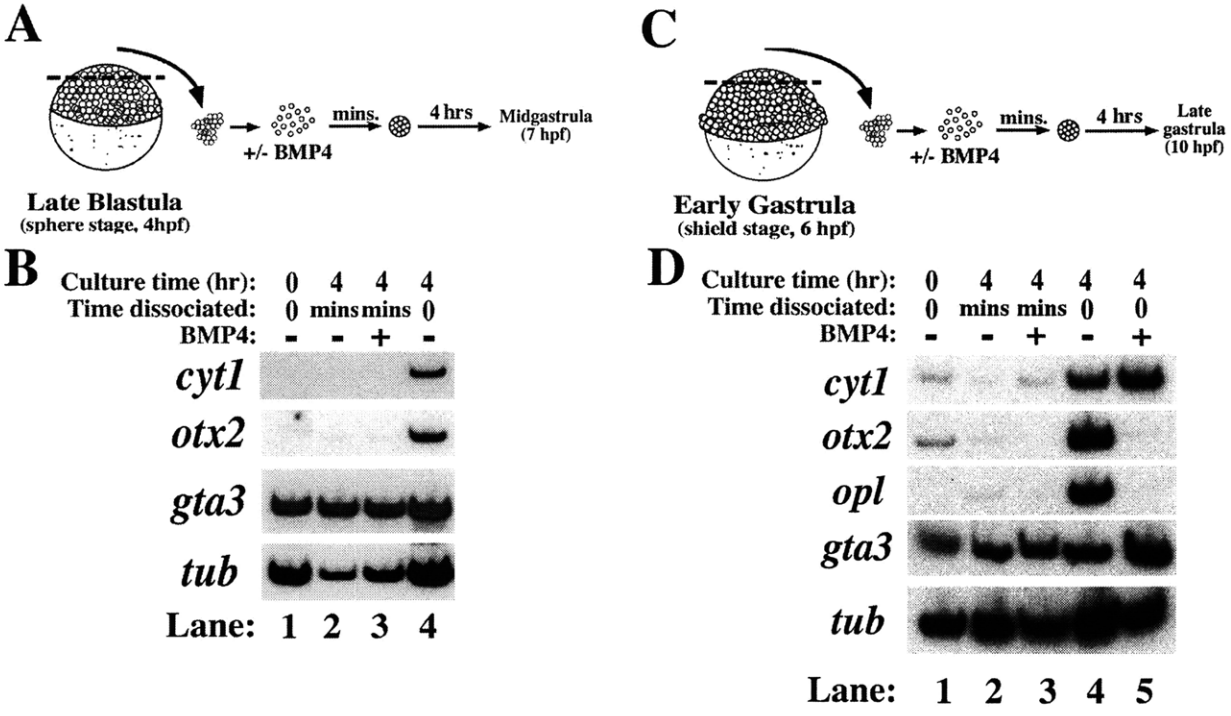
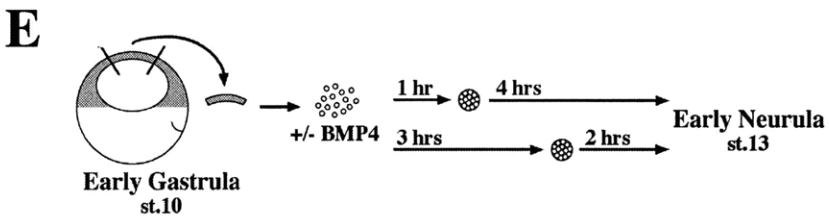

A
hpf: 3 4 5 7 9 10 18 24 48
48 1wk ad
cyt1
rRNA
lane: 1 2 3 4 5 6 7 8 9
10 11 12
B
a
b
c
d
e
f
g
h
i
C
a
b
c
d
e
f
g
h
i
j

Figure B.2 Dissociated zebrafish explants do not autoneuralize, revealing a role for cell contact during epidermis vs. neural fate determination.
(A) Dissociation and BMP4 treatment of zebrafish blastula stage animal caps. Animal caps were dissected at late blastula (4 hpf, sphere stage), aggregated into groups of ten, and either cultured intact or dissociated and reassociated (see methods). Explants were then incubated for four hours (until 8 hpf, equivalent to mid-gastrula) in the presence or absence of BMP4, followed by analysis by RT-PCR for expression of *cyt1*(expressed in the zebrafish EVL), *otx2*, (expressed dorsally), *gta3* (expressed ventrally), and *tubulin* (as a loading control). **(B)** Results of experiment in A. Animal caps were harvested immediately after dissection (lane 1) or after 4 hours in culture (lanes 2-4). Explants cultured for 4 hours were either dissociated into single cells and immediately reassociated (lanes 2 and 3) or left intact (lane 4). Explants were cultured in the presence (lane 3) or absence (lanes 2 and 4) of 50 ng/ml of BMP4. **(C)** Dissociation and BMP4 treatment of zebrafish gastrula stage animal caps. Animal caps were dissected at early gastrula (6 hpf, shield stage), aggregated into groups of ten, and either cultured intact or dissociated and reassociated (see methods). Explants were then cultured for four hours (until 10 hpf, equivalent to end of gastrulation) in the presence or absence of BMP4, followed by analysis by RT-PCR for expression of *cyt1, otx2, opl* (expressed dorsally), *gta3* and *tubulin*. **(D)** Results of experiment in C. Animal caps were harvested immediately after dissection (lane 1) or after 4 hours in culture (lanes 2-5). Explants cultured for 4 hours were either dissociated into single cells and immediately reassociated (lanes 2 and 3) or left intact (lanes 4 and 5). Explants were cultured in the presence (lane 3 and 5) or absence (lanes 2 and 4) of 50 ng/ml of BMP4. **(E)** Dissociation and BMP4 treatment of *Xenopus* gastrula stage animal caps. Animal caps were dissected at early gastrula (st. 10) and either left intact, or dissociated and reassociated after 1 or 3 hours (see methods). Explants were then cultured for a total of 5 hours (until st. 13, equivalent to early neurula) in the presence or absence of BMP4 followed by analysis by RT-PCR for expression of *XK81* (expressed in the outer layer), *otx2, opl* (both dorsal, neural markers), *XVent-1* (expressed ventrally), and *EF1a* (as a loading control). **(F)** Results of experiment in E. Animal caps were harvested immediately after dissection (lane 1) or after 5 hours in culture (lanes 2-6). Explants cultured for 5 hours were either dissociated into single cells and reassociated after 1 (lanes 2 and 3) or 3 (lanes 4 and 5) hours, or left intact (lane 6). Explants were cultured in the presence (lane 3 and 5) or absence (lanes 2, 4 and 6) of 500 ng/ml of BMP4.


A
mins.
4 hrs
Midgastrula
(7 hpf)
+/- BMP4
Late Blastula
(sphere stage, 4hpf)
C
mins.
4 hrs
Late
gastrula
(10 hpf)
+/- BMP4
Early Gastrula
(shield stage, 6 hpf)
B
Culture time (hr): 0 4 4 4
Time dissociated: 0 mins mins 0
BMP4: - - + -
cyt1
otx2
gta3
tub
Lane: 1 2 3 4
D
Culture time (hr): 0 4 4 4 4
Time dissociated: 0 mins mins 0 0
BMP4: - - + - +
cyt1
otx2
opl
gta3
tub
Lane: 1 2 3 4 5


E
1 hr
4 hrs
Early Neurula
st.13
+/- BMP4
3 hrs
2 hrs
Early Gastrula
st.10

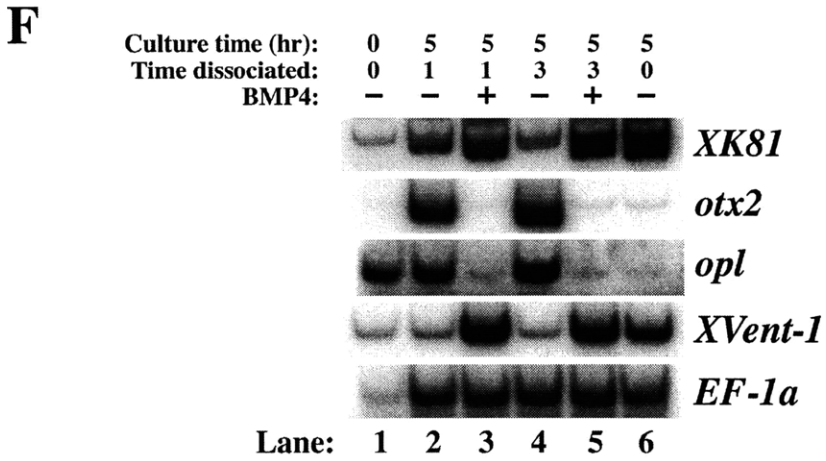
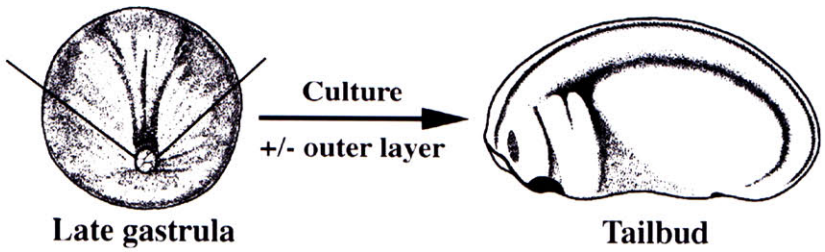

F
Culture time (hr): 0 5 5 5 5 5
Time dissociated: 0 1 1 3 3 0
BMP4: – – + – + –
XK81
otx2
opl
XVent-1
EF-1a
Lane: 1 2 3 4 5 6

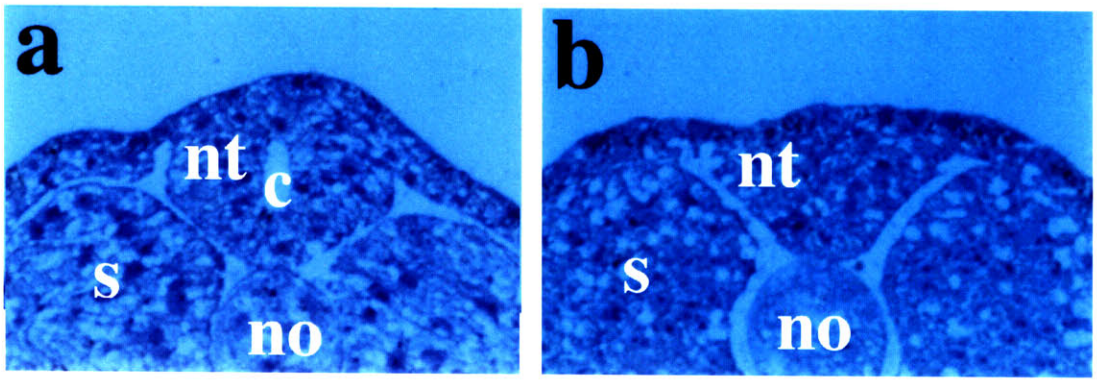
Figure B.3 The *Xenopus* outer layer is required for formation of a cavity in the neural tube.

(A) The dorsal portion of the *Xenopus* outer layer was removed at mid-gastrula (st. 12.5) as indicated by the black lines, and the resulting embryos cultured until tailbud stage. After culture, embryos were fixed, sectioned, and stained with Toluidine Blue as described in the methods. **(B)** Results of experiment outlined in A. Embryos with intact outer layers developed normally and showed a central cavity in the neural tube (a), while embryos without the dorsal outer layer lacked a central cavity (b). no=notochord, s=somite, nt=neural tube, c=cavity. Sections were taken through the mid-trunk level.

A



B



Appendix C. Materials and Methods.

Growth and culture of embryos

Wildtype and albino embryos were collected and cultured as described in Sive et al. (1989). Stages were assigned according to Nieuwkoop and Faber, 1994. Embryos were cultured in 0.1x Modified Barth's Saline (0.1x MBS).

Dissection of embryos

Micro-dissection was performed in 1x MBS, with subsequent culture of explants in 0.5x MBS. Animal caps were isolated using a pair of forceps as scissors, removing a small circle of animal-most tissue about 1/3 the diameter of the embryo.

Anterior dorsal ectoderm was dissected using an eyebrow knife to make parallel incisions along the dorsal side, including about 80° of the area above the mid-gastrula lip. The ectoderm was then carefully teased back by making a cut in the ectoderm over the blastocoel and peeling the ectoderm down. A rectangle was cut from this flap of ectoderm starting at the limit of involution (marked when peeling back) and continuing posteriorly about 1/2 the width of the piece.

The epithelial layer was removed from stage 12.5 embryos in 1X MBS using an eyebrow knife to carefully tease off the outer layer over the dorsal half of the embryo. Resulting embryos were cultured in 1X MBS.

Factor treatments

All factors were freshly diluted in 0.1x or 0.5x MBS as required.

1 μM all-*trans* retinoic acid (Sigma) was diluted from a 0.1M stock prepared in DMSO and stored in the dark at -20°C. Diluted RA was kept dark and remixed thoroughly before pouring into dishes for embryo incubations.

10 μM dexamethasone (Sigma) was diluted from a $5x10^{-3}$M stock prepared in 100% ethanol and stored at -20°C.

10 μg/ml cycloheximide (Sigma) was diluted from a 10 mg/ml stock prepared in water. All cycloheximide incubations included a 30 minute pre-treatment. Caps were sliced open before cycloheximide treatment to ensure that interior cells were exposed to the inhibitor. Translational inhibition was assayed by parallel incubations in dexamethasone/cycloheximide solutions containing 1 mCi/ml of [^{35}S]methionine (NEN). Counts incorporated were determined as described in Cascio, et al. (1987). In all cases, translation was inhibited >90% (data not shown) with remaining activity most likely due to mitochondrial translation (Schatz, et al., 1974).

500 ng/ml BMP4 (Genetics Institute) was diluted from a 69 µg/ml stock stored at -80°C. Pipette tips were precoated with BSA before aliquoting to prevent the protein from sticking to the plastic. MBS solutions also included 0.5 µg/ml BSA.

Microinjection

Embryos were dejellied 15 to 20 minutes after fertilization and placed in 4% Ficoll (Sigma) in 1x MBS. Embryos were injected at the two-cell stage with 5 to 10 nl of RNA per injection at sites indicated for each experiment (for animal cap experiments, embryos were injected with 5 nl in each blastomeres at the animal pole). Injected embryos were allowed to recover for one or more hours in 4% Ficoll in 1x MBS, then were transferred to 1% Ficoll in 0.1x MBS.

In vitro transcription

Capped mRNA was transcribed according to Krieg, et al. (1987b). Templates are indicated in Table C1. Anti-sense in situ hybridization probes were labeled with digoxygenin-11-UTP (Harland, 1991). Templates are indicated in Table C2.

Table C.1 Templates for in vitro transcription of sense RNA.

gene	plasmid	enzyme[a]	pol.[b]	reference
otx2	CS2+*otx2*	*NotI*	SP6	Gammill, unpublished
otx2-GR	T7TS*otx2*-GR	*BamHI*	T7	Gammill and Sive, 1997
otx2-GR	CS2+*otx2*-GR	*NotI*	SP6	Gammill and Sive, 1997
MT-*otx2*-GR	CS2+MT-*otx2*-GR	*NotI*	SP6	Gammill and Sive, 1997
lacZ	pSP6nucßgal	*XhoI*	SP6	Smith and Harland, 1991
noggin	CS2+noggin	*NotI*	SP6	Smith et al., 1993
BMP4	p64T-BMP4	*EcoRI*	SP6	Schmidt et al., 1995
otx2-En	CS2+otx2-En	*NotI*	SP6	Gammill, unpublished
GFP	pβGFP/RN3P	*SfiI*	T3	(Zernicka-Goetz, et al., 1996)
Siamois	pBSRN3XSia	*SfiI*	T3	Carnac et al., 1996

a. Restriction endonuclease used to linearize the template.
b. RNA polymerase used to synthesize RNA.

Table C.2 Templates for in vitro transcription of anti-sense RNA for in situ hybridization probes.

gene	plasmid	enzyme[a]	pol.[b]	reference
XCG	H8-1	*NotI*	T3	Sive et al., 1989
XAG	pXAG-1 (1.12)	*NotI*	T7	Sive et al., 1989
otx2	p*otx2*-BS	*XhoI*	T3	Pannese et al., 1995
HoxD1	pXhox.lab1.ATG	*HindIII*	T7	Kolm and Sive, 1995a
NCAM	pSP71-NI	*BglI*	SP6	Kintner and Melton, 1987
BMP4	BMP4-pBS	*EcoRI*	T7	Schmidt et al., 1995
N-tubulin	p24-10	*BamHI*	T3	Richter et al., 1988
lipocalin	p13-8	*BamHI*	T3	Richter et al., 1988
XBF-1	XBF-1	*XbaI*	T3	Pappalopulu and Kintner, 1996
Gbx2	xGbx-62	*EcoRI*	T7	von Bubnoff et al., 1995
engrailed 2	pSP6-En2-3.0-AS	*XbaI*	SP6	Hemmati-Brivanlou, 1991
Krox20	pKrox-20	*EcoRI*	T7	Bradley et al., 1993
HoxB9	pGIS	*SmaI*	T7	Sharpe et al., 1987
GATA-2	XGATA-2/pGEM	*XhoI*	SP6	Kelley et al., 1994
XVent-1	pXVent-1 (2.15)	*SalI*	T7	Gawantka et al., 1995
Vox	pXVent-2	*SalI*	T7	Onichtchouk et al., 1996
goosecoid	pAB-Goosecoid	*XbaI*	SP6	Cho et al., 1991
brachyury	pXT1	*EcoRV*	T7	Smith et al., 1991
muscle actin	pBS-AC100	*EcoRI*	T3	Gurdon et al., 1985
XK81	pGEM81B	*EcoRI*	SP6	(Jonas, et al., 1985)

a. Restriction endonuclease used to linearize the template.
b. RNA polymerase used to synthesize RNA.

In situ hybridization

Whole mount in situ hybridization was performed as described in Harland (1991), with modifications described in Sagerström, et al. (1996). Double in situs were performed by mixing digoxygenin and fluorescein labeled probes during the hybridization step. The digoxygenin labeled probe was detected first by incubating with anti-digoxygenin and staining with BCIP alone (175 µg/ml; blue color) or with BCIP (250 µg/ml)/INT (250 µg/ml; orange color). Alkaline phosphatase activity was subsequently heat inactivated in MAB containing 10 mM EDTA at 65°C for 10 minutes followed by dehydration in methanol for 10 minutes. Tissue was rehydrated by washing 5 minutes each in 75% MeOH/25% H_20, 50% MeOH/50% H_20, 25% MeOH/75% MAB, and 4 times in MAB. Embryos were

then reblocked in 2% BMB diluted in MAB, incubated in anti-fluorescein and stained with NBT/BCIP to visualize the fluorescein probe.

Sectioning

Embryos were embedded in JB-4 media according to the manufacturers protocol. 1 μm sections were cut with a microtome and stained with Toluidine Blue prior to photography.

β-galactosidase staining

Embryos microinjected with β-galactosidase mRNA were fixed in MEMPFA (0.1M MOPS pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, and 4% paraformaldehyde) for one hour. Fixed embryos were then washed three times for 10-30 minutes in PBS containing 1 mM $MgCl_2$ and once for 10 minutes in Xgal buffer (10 mM $K_3Fe(CN)_6$, 10 mM $K_4Fe(CN)_6$, 1 mM $MgCl_2$, in PBS). Embryos were stained in Xgal buffer containing 1.5 mg/ml freshly added 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (XGAL) for 30 minutes to several hours at 30°C. After staining, embryos were washed two to three times in PBS, refixed in MEMPFA for 30 minutes, and stored in 100% ethanol at -20°C until needed for in situ hybridization.

Western Analysis

Pools of 10 animal caps or 5 embryos were homogenized in lysis buffer containing 50mM Tris, pH 7.5, 150 mM NaCl, 0.5% NP-40, 2 mM DTT, 20% glycerol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2.5mM Na_2β-glycerophosphate, 1mM NaF, 0.1mM sodium orthophosphate. Lysates were centrifuged for 10 minutes at 4° C before loading onto a gel. Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and immunoblotted with the c-myc 9E10 monoclonal antibody (Santa Cruz Biotechnology) diluted 1:1000 (0.1 μg/ml). Binding was visualized by a secondary antibody linked to horseradish peroxidase and chemiluminescence (ECL, Amersham).

Isolation of RNA and Northern Analysis

RNA was prepared by Proteinase K treatment and phenol extraction and analyzed by Northern analysis as described in Kolm and Sive (1995a). Antisense probe was prepared by asymmetric PCR amplification (Sive and Cheng, 1991) of *otx2*-BS linearized with *Xho*I using a T3 primer.

Relative Quantitative RT-PCR

RNA from pools of ten explants was treated with 100 units/ml DNAse at 37°C for 1 hour. First strand cDNA and PCR reactions were prepared and analyzed according to Sagerström et al. (1996). With this protocol, changes in expression of a particular gene can be analyzed, but expression levels of different genes cannot be compared to one another since amplification efficiencies vary between primer sets. Expression levels were quantitated using a phosphorimager (Fuji) and normalized to *EF-1α* (Krieg, et al., 1989). The linear range for each primer pair was determined using stage 12 whole embryo cDNA, or when treatments extended beyond neurula stages, using explants expected to give the maximal signal. Due to repeated sequences in *XCG*, primer pairs for this gene give a ladder of products. Since all bands amplified linearly at the cycle number selected, only the expected product is shown.

Table C.3 RT-PCR primer sequences.

gene	primer	F/R	sequence
XCG	XCG.C	F	5'-GGTTGATGTTACTTCCCCAGAGCAG-3'
	XCG.D	R	5'-GAGTTGCTTCTCTGGCAT-3'
XAG	XAG1.1	F	5'-CTGACTGTCCGATCAGAC-3'
	XAG1.2	R	5'-GAGTTGCTTCTCTGGCAT-3'
otx2	otx2-3'.1	F	5'-AACTTTGCTGAGACATTCCTA-3'
3'UTR	otx2-3'.2	R	5'-TGTTTTGCTTATTGCTTCTTCC-3'
EF-1α	EF1a.1	F	5'-TCTTGACTGCATTCTGCCAC-3'
	EF1a.2	R	5'-GACCAACTGGTACAGTACCAATACC-3'
NCAM	NCAM.1	F	5'-GGAATCAAGCGGTACAGA-3'
	NCAM.2	R	5'-CACAGTTCCACCAAATGC-3'
BMP4	XBMP4.A	F	5'-GCATGTAAGGATAAGTCGATC-3'
	XBMP4.B	R	5'-GATCTCAGACTCAACGGCAC-3'
XVent-1	XVent1.A	F	5'-GCATCTCCTTGGCATATTTGG-3'
	XVent1.B	R	5'-TTCCCTTCAGCATGGTTCAAC-3'
nrp-1	nrp-1.A	F	5'-TACCAACGGCTACCACTGAAAC-3'
	nrp-1.B	R	5'-CTCCGCATCTTACCAAAACAAA-3'
ngnr-1	Ngn-1A.1	F	5'-CAGCCCGGGACAGCACCAG-3'
	Ngn-1A.2	R	5'-GCCGGGGAGAAGGAGCAACA-3'
en-2	XEn-1.F	F	5'-GCACAAGAAAGACTCTGTGG-3'
	XEn-1.R	R	5'-CAGTCACTTGGCAGAGTTTC-3'
opl	Xopl3A.1	F	5'-AGGGGTAACAGGGAGGACGACA-3'
	Xopl3B.2	R	5'-GGTGGACGCTCAGACTGGAATAAT-3'

gene	primer	F/R	sequence
XANF-1	XANF.1	F	5'-AGCCTCACCATGTCTCCTG-3'
	XANF.2	R	5'-ACGGCGTGAACTTGTATGG-3'
gsc	Gsc.F	F	5'-GGATTTTATAACCGGACTGTGG-3'
	Gsc.R	R	5'-TGTAAGGGAGCATCTGGTGAG-3'
HoxB9	HLS51	F	5'-GCCCCTGCGCAATCTGAAC-3'
	HLS52	R	5'-CAGCAGCGGCTCAGACTTGAG-3'
Krox20	LCB9	F	5'-AACCGCCCCAGTAAGACC-3'
	LCB10	R	5'-GTGTCAGCCTGTCCTGTTAG-3'
Xbra	Xbra.1	F	5'-TTCTGAAGGTGAGCATGTCG-3'
	Xbra.2	R	5'-GTTTGACTTTGCTAAAAGAGACAGG-3'
Vox	Vox.A	F	5'-CTCAAAGCAGCCGCAGAT-3'
	Vox.B	R	5'-CCAGGGGATGATGATGTATTA-3'
Xhox3	HLS75	F	5'-ATATGATGAGCCACGCAGCAG-3'
	HLS76	R	5'-CAGATGCTGCAGCTCTTTGGC-3'
Xmad-1	XMad1.A	F	5'-TGCCGCCTGTCCTTGTTCC-3'
	XMad1.B	R	5'-TAGCTGCTGTTGGGCGAGTGA-3'
noggin	Noggin.A	F	5'-AGTTTGCAGATGTGGCTCT-3'
	Noggin.B	R	5'-AGTCCAAGAGTCTCAGCA-3'
chordin	CHD.3	F	5'-TTTCGCAACAGGAGCACAGACAC-3'
	CHD.4	R	5'-TACCGCACCCACTCAAAATACCAT-3'
otx2	Xotx2.A	F	5'-GAGGCCAAAACAAAGTGAGA-3'
	Xotx2.B	R	5'-ACAGTCCATACCCCCAAAG-3'
XK81	XK81.C	F	5'-TCATTCCGTTCCAGCTCTTCTTAC-3'
	XK81.D	R	5'-TCCAGGGCTCTTACTTTCTCCAG-3'

F = forward primer R = reverse primer

Construction of inducible *otx2* proteins

The ligand binding domain of the human glucocorticoid receptor was amplified from the plasmid MyoD-GR (Hollenberg, et al., 1993) by PCR with the primers 5'-GGCGCCGAGAGCTCCTCTGAAAAT-3' and 5'-GCGGCGGGCGAGCTCCACTTTTGA-3' for insert 1 or 5'-GGCGCCGAGGCCTCCTCTGAAAAT-3' or 5'-GGCGGGCAAGGCCTACTTTTGATG-3' for insert 2. Products were digested with *Sac*I (underlined, insert 1) or *Stu*I (underlined, insert 2) and inserted in frame at the *Sac*I and *Stu*I sites in *otx2* plasmid T7TSMC19/1 (Pannese et al., 1995). Both fusions gave equally efficient, hormone-dependent ectopic cement gland formation and were used interchangeably, although a stop codon within the antisense primer for the *Stu*I construct results in the deletion of 48 C-terminal

amino acids. CS2+ and myc-tagged *otx2*-GR (MT-*otx2*-GR) were created by cloning the *Bcl*I-*Spe*I (endfilled) fragment of the *otx2*-GR insert into the *Xba*I site (endfilled) of CS2+ or CS2+MT (Rupp, et al., 1994; Turner, et al., 1994). RNA transcribed from CS2+ constructs is very efficiently transcribed in *Xenopus* embryos. CS2+MT contains six multimerized copies of the myc epitope upstream of the insert. *otx2*/3'Δ-GR, which lacks *otx2* 5' and 3' untranslated sequence, was constructed by inserting the *Bcl*I-*Msc*I (endfilled) fragment of the *otx2*-GR *SacI* construct into the *Xba*I site (endfilled) of CS2+ (Rupp, et al., 1994; Turner and Weintraub, 1994). CS2+*otx2* and MT-*otx2* constructs containing unmodified wildtype *otx2* were made by removing the GR insert from the CS2+*otx2*-GR *SacI* fusion and recircularizing the plasmid.

Construction of a dominant negative *otx2*

Deletions were created in MT-*otx2* by digestion with the restriction endonucleases *NgoMI*, *BsmBI*, *NsiI*, and *StuI* in *otx2* and *SnaBI* at the 3' end of the upstream CS2+ polylinker. Plasmid fragment ends were endfilled with Klenow when necessary and ligated to the *StuI-SnaBI* fragment of CS2+. This fragment provides in-frame stop codons in all three frames to complete the deleted sequences. An internal *NgoMI-BsmBI* deletion was created by removing this fragment of *otx2*, endfilling with Klenow, and recircularizing the remaining plasmid.

The minimal engrailed repressor domain (amino acids 228 to 282 of *Drosophila* engrailed; Han and Manley, 1993) was amplified from the plasmid pTβ-en using the upstream primers 5'-GCTGGAGGCCTGCCGGCAACAGCAAGC -3' (primer 1) or 5'-GTGGATCTGCTGAGCTCTAGCCGGCAACAG-3' (primer 2) and the downstream primer 5'-GGATAGGCCTAGCTCATCTTGGAGCTC-3'. The downstream primer inserts an in-frame stop codon at the end of the repressor sequence. Products were digested with *StuI* (primer 1) or *SacI* and *StuI* (primer 2; restriction sites underlined in primer sequences). CS2+*otx2* or MT-*otx2* were digested with *StuI* (for primer 1; deletes only the minimal cement gland inducing region) or *SacI* (for primer 2; deletes most *otx2* sequence C-terminal to the homeodomain) in *otx2*, and *SnaBI* in CS2+ (at the 3' end of the upstream polylinker) to excise the desired region of *otx2*. The *otx2* and engrailed repressor fragments were ligated to achieve in-frame fusion of the deleted *otx2* and the engrailed repressor. Amplified sequence was verified by PCR. The *StuI*-deleted *otx2*-En was extremely toxic and was therefore not pursued.

Luciferase assay

Luciferase assays were performed according to the manufacturers instructions (Promega). Briefly, 15 animal caps were rinsed with 1x PBS and lysed in 100µl 1X lysis buffer, incubated at room temperature for 10 minutes, frozen at -20°C, and then thawed. Lysates were microfuged briefly at top speed, and 10 µl of extract or extract diluted in lysis buffer was assayed in a luminomiter after addition of 100 µl of assay reagent.

Chloramphenicol Acetyl Transferase (CAT) assay

CAT activity was assayed essentially as described in Kuo et al. (1996). Pools of 5 embryos injected with 50 pg of the appropriate DNA construct were lysed in PBS + 0.5% NP-40 (10 µl lysis buffer/embryo). Extracts were heated for 10 minutes at 65°C, then microcentrifuged for 10 minutes at 4°C. 25 µl of this extract (2.5 embryo equivalents) was combined with CAT assay solution (40 mM Tris pH7.6, 1.6 mM chloramphenicol, 50 µM acetyl CoA) and 0.25 µCi ^{3}H-acetyl CoA (0.5 µl; 200 mCi/mmol; NEN) in a total reaction volume of 100 µl. Reactions were incubated at 37°C for 2 hours, then extracted with 400 µl ice-cold ethyl acetate. 300 µl of this extract was mixed with 2.5 ml of scintilation fluid, and counted for 1 minute. The cpm from uninjected embryo lysates was defined as background and subtracted from experimental values to determine fold induction.

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Appendix D. References

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